Textbook of Vascular Medicine

Rhian M. Touyz
Christian Delles
Editors

Springer

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ISBN 978-3-030-16480-5 ISBN 978-3-030-16481-2 (eBook)
https://doi.org/10.1007/978-3-030-16481-2

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Foreword I

In 2014, the British Heart Foundation (BHF) was pleased to award the Institute of Cardiovascular and Medical Sciences a prestigious Research Excellence award to advance their science in vascular biology and medicine. Not only has the award supported new discoveries in vascular biomedicine, but it has provided outstanding training opportunities for the next generation of cardiovascular researchers and clinician scientists. While not our expectation, I am delighted that the award has been instrumental in the birth of this excellent *Textbook on Vascular Medicine*. The editors are to be applauded for bringing together a group of experts from the Glasgow BHF Centre of Research Excellence, as well as international authorities, on vascular biology and medicine that has resulted in this comprehensive, informative, and up-to-date book. The vast range of topics covering not only the anatomy, cell biology, physiology, and pharmacology of the vessel wall but also some of the key regulators, all written in succinct and well-organized chapters, makes the book user-friendly and a pleasure to read. Given the central importance of vascular dysfunction to many heart and circulatory diseases, this book is a must-read for any student, researcher, or clinician who has an interest in vascular biology and/or vascular medicine. I am particularly proud that this book which is a product of BHF support will benefit many across the world.

Nilesh Samani
London, UK
July 2019

Foreword II

As the Chair of the Scientific Advisory Board of the Institute of Cardiovascular and Medical Sciences, University of Glasgow, I am very familiar with the outstanding contributions made by Glasgow researchers in vascular biology and cardiovascular biomedicine. It is therefore not surprising that they would produce a state-of-the art textbook of the highest quality that provides up-to-date knowledge on how the vascular system is regulated and how it functions in health and disease. The growing field of vascular biology has provided important insights into fundamental mechanisms that underlie cardiovascular and cerebrovascular disease. With the advancement of vascular biology, it is essential that clinicians are kept abreast of relevant information and that scientists are familiar with the clinical relevance. I believe that this easy-to-read book fulfils these needs. The reader will learn about the anatomy, physiology, and molecular biology of the vasculature and will gain knowledge on vascular disorders and associated cardiovascular diseases. This textbook on vascular medicine is a valuable resource for both clinicians and researchers interested in the vascular system from basic science to clinical practice.

Victor J. Dzau
Washington, USA
July 2019

Preface

It is with great pleasure that we present our *Textbook of Vascular Medicine* to you. In 43 chapters, this book aims to cover all areas of vascular biology, physiology, and pathology, with a focus on mechanisms of disease that translate into clinical practice.

Many cardiovascular conditions including hypertension, ischemic heart disease, dementia, and stroke are in fact vascular conditions. Other diseases such as chronic kidney disease and diabetes in turn affect the vasculature and are thereby associated with increased cardiovascular risk. In addition, many new anticancer drugs have unwanted vascular effects, predisposing cancer patients to increased risk of cardiovascular disease. Clinically, specialists across multiple disciplines are therefore involved in the management of patients with vascular disorders, including cardiologists, nephrologists, endocrinologists, neurologists, oncologists, and vascular surgeons.

With this book, we aim to provide an introduction to vascular physiology and pathobiology to clinicians but particularly to graduate students and early career researchers. We sincerely hope that it is an easy and exciting read that will provide a broad overview of this fascinating and important topic.

This book would never have been possible without the enthusiastic support and commitment by colleagues and collaborators who contributed to this textbook; they provided authoritative, concise, and up-to-date chapters that are easy to read yet scientifically accurate. We would also like to thank our colleagues who helped us with the peer-review process and of course the Springer team, namely Donatella Rizza, Hemalatha Gunasekaran, and Jeyaraj Allwyn Kingsly, for all their expert help. We also express our sincere thanks to the British Heart Foundation for funding our Centre of Research Excellence, from which this work derived, and the Canadian Vascular Network, whose members contributed significantly to this project.

We hope you will enjoy reading this textbook and that it supports you in your studies, research, and clinical practice.

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July 2019

Acknowledgments

The editors would like to thank the following colleagues for critically reviewing the chapters of this book:

- Dr. Angela Bradshaw, Glasgow, UK
- Dr. David Carty, Glasgow, UK
- Prof. Fadi Charchar, Ballarat, Australia
- Dr. Gemma Currie, Glasgow, UK
- Prof. Elanor Davies, Glasgow, UK
- Dr. Adam Greenstein, Manchester, UK
- Prof. Michael Hecht Olsen, Odense, Denmark
- Dr. Simon Kennedy, Glasgow, UK
- Mr. David Kingsmore, Glasgow, UK
- Dr. Ninian Lang, Glasgow, UK
- Prof. Mandy MacLean, Glasgow, UK
- Dr. Pasquale Maffia, Glasgow, UK
- Prof. Patrick Mark, Glasgow, UK
- Prof. Una Martin, Birmingham, UK
- Dr. Carmel McEniery, Cambridge, UK
- Prof. Markus Mohaupt, Bern, Switzerland
- Dr. Augusto Montezano, Glasgow, UK
- Prof. Stuart Nicklin, Glasgow, UK
- Dr. Terry Quinn, Glasgow, UK
- Dr. Susana Ravassa, Pamplona, Spain
- Prof. Carmine Savoia, Rome, Italy
- P.D. Dr. Bernhard Schmidt, Hannover, Germany
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Anatomy and Physiology

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Anatomy and Pharmacology of Vessels

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_1

1

Key Concepts

- Arteries are composed of an intima, media and adventitia.
- The endothelium influences artery diameter and prevents adhesion of platelets.
- The media contains concentric layers of smooth muscle and elastic tissue.
- The adventitia contains nerve endings which can modulate vascular diameter.
- Adipose tissue surrounding the vessel releases paracrine mediators.
- Artery structure can be modified in some diseases such as hypertension.

1.1 Artery Structure

Arteries are composed of three major components: endothelial cells, smooth muscle cells and extracellular matrix (ECM). In recent years, the perivascular adipose tissue which surrounds all blood vessels with the exception of cerebral vessels has received increasing attention, and this will be discussed later in the chapter. The ECM is composed of collagen, elastin and glycosaminoglycans (GAGs) and supports the mechanical load of the vessel. The ECM is of great interest in that it can communicate with vascular smooth muscle cells in the vascular media, and changes in the ECM can also contribute to vascular stiffening or arteriosclerosis, a hallmark of ageing and many cardiovascular (CV) diseases (reviewed in Lacolley et al. [1]).

A typical blood vessel is arranged in three distinct layers forming concentric circles. The innermost layer adjacent to the vessel lumen and in contact with the bloodstream consists of a monolayer of endothelial cells on a basement membrane. Collectively this is termed the vessel intima (tunica intima). The basement membrane contains fenestrations through which the endothelial cells can communicate with the underlying layers of the vessel, and there is evidence that the communication and orientation of the intimal cells become altered in some disease such as hypertension [2] and pulmonary hypertension [3]. Myoendothelial gap junctions (MEGJ) are points of very close contact between endothelial cells and vascular smooth muscle cells, and such sites are thought to facilitate the local action of signalling molecules and/or the passage of current between the two cell types. Since these sites have the potential for bidirectional communication between the endothelium and smooth muscle, they are seen as a key pathway for coordinating vascular function and also as a target for therapeutic agents in vascular diseases [4]. Recent work in this area has focussed not only on the integration between various cell types in the artery wall but also on the importance of integration of consecutive segments within the microvasculature. Here, connexins appear to be central, not only acting as gap junctions but also linking with proteins to form signalling complexes (a recent review on the subject was published by Pogoda et al. [5]). A simple representation of vascular structure is shown in Fig. 1.1.

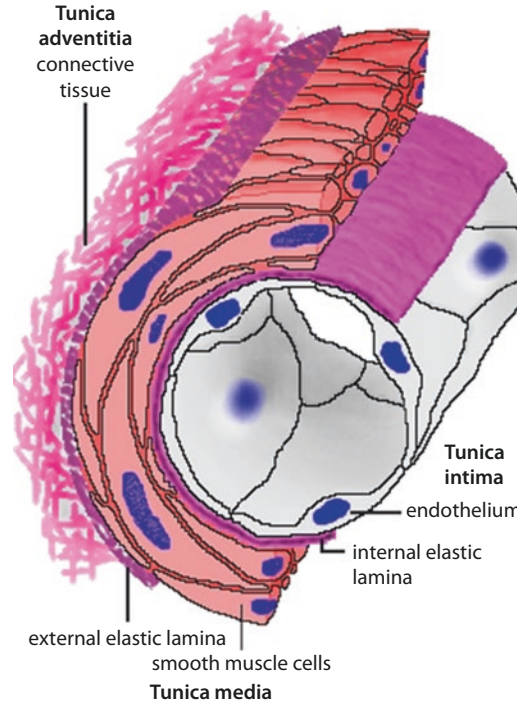

Fig. 1.1 Diagrammatic representation of an artery showing the three main components: endothelium, medial smooth muscle cells separated by the two laminae – the internal and external elastic laminae. (Courtesy of Dr. Craig Daly, University of Glasgow)

1.2 The Endothelium

Previously viewed as an inert barrier between the blood and the artery wall, the pioneering work of Robert Furchgott and other pharmacologists demonstrated the complexity of the endothelium and its ability to modulate the function of the blood vessel wall and also components of the blood (for a review of the discovery of gasotransmitters and the paracrine function of the endothelium, see Nava and Llorens [6]). Intact endothelium provides an anticoagulant and antithrombotic luminal surface rich in heparin-like glycosaminoglycans. Prostacyclin and nitric oxide (NO) are the most important factors produced and released by endothelial cells that inhibit platelet aggregation and coagulation. The production and release of these molecules from endothelial cells can be activated by agonists involved in platelet aggregation and coagulation, such as thrombin. Thrombomodulin is another important anticoagulant factor expressed by endothelial cells. When binding to thrombin, thrombomodulin reduces thrombin's activity in converting fibrinogen to fibrin and activates the anticoagulant protein C pathway. Endothelial cells are also the source of circulating vWF, which binds and stabilises circulating factor VIII. Endothelial cells also control the fibrinolysis balance by secreting tissue-type plasminogen activator (t-PA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1). For a recent review of the thrombomodulatory role of endothelial cells, see Urano et al. [7].

1.2.1 Endothelium-Derived Vasoconstrictors

Endothelial cells also regulate vascular tone and blood pressure by synthesising and secreting a spectrum of vasoactive substances (Table 1.1). Endothelin-1 (ET-1) is one of the most potent vasoconstrictors released by endothelial cells, which contributes to the maintenance of endogenous vascular tone. ET-1 also induces tonic NO production in the endothelium to oppose vasoconstriction. In addition to its constrictor effects, ET-1 has mitogenic effects on smooth muscle cells, influences homeostasis of salt and water and stimulates the renin-angiotensin-aldosterone (RAAS) system. ET-1 is synthesised as a precursor peptide which is then enzymatically converted to an inactive intermediate called big ET-1. Big ET-1 is then cleaved by another enzyme known as endothelin converting enzyme (ECE) as well as another route involving a two-step process. ET-1 induces its effects via activation of two G protein-coupled receptors: ETA and ETB. The former mediates vasoconstriction, while activation of ETB on the endothelium relaxes arteries, and activation of ETB on smooth muscle induce constriction. Recent evidence suggests that regional differences in ET receptor expression may determine the response and that ETA and ETB receptors may interact. This is an active area of research, and the system is seen as an attractive target for novel pharmacological agents (reviewed in Houde et al. [8]). However, currently available endothelin receptor antagonists, although effective in reducing systemic blood pressure, are often associated with peripheral oedema and liver toxicity (reviewed in Burnier [9]), and the future of this class of drugs to treat hypertension is uncertain.

Angiotensin II (Ang II) is another important vasoconstrictor released by endothelial cells. Although well known as a circulating hormone regulating the systemic blood pressure through the renin-angiotensin-aldosterone system, the major site for Ang II production is the endothelium [10]. In addition to its vasoconstrictor and proliferative effects on smooth muscle cells, Ang II promotes local production of prostacyclin and ET-1. Interestingly, ACE, the major enzyme for

Table 1.1 Endothelial cell-derived vasoactive substances

Vasoconstrictors	Vasodilators
Endothelin-1	Nitric oxide
Angiotensin II	Prostacyclin
Thromboxane A_2	Endothelium-derived hyperpolarizing factor
Platelet-activating factor	Adenosine
Leukotrienes	C-natriuretic peptide
Superoxide	Hydrogen peroxide

Ang II production in endothelial cells, is the same enzyme responsible for bradykinin degradation to inactive fragments. Bradykinin stimulates NO production and accordingly is a potent vasodilator. Hence activation of ACE leads to vasoconstriction through increased Ang II production as well as through reduced bradykinin-mediated vasodilation. The RAAS has been very successfully targeted to treat hypertension, and agents which either block Ang II receptors (the AT-1 receptor subtype) or prevent its formation by blocking the synthesising enzyme (ACE) are first-line drugs in many patients with hypertension. Recent research has highlighted the importance of aldosterone in blood pressure and that the deleterious effects of aldosterone may not be limited to reabsorption of sodium and water by the kidney (reviewed in Te Riet et al. [11]). The RAAS system is complex, comprising the classical system where ACE cleaves Ang I to produce Ang II and the counter-regulatory system composed of angiotensin converting enzyme 2 (ACE2), angiotensin (1–7), and Mas receptor. In contrast to the classical RAAS, this system generally opposes the actions of Ang II-AT1R. Growing evidence suggests that the counter-regulatory system may be an attractive target in diseases that involve adverse inflammation in the blood vessels and structural changes [12].

1.2.2 Endothelium-Derived Vasodilators

In contrast to vasoconstrictors, endothelial cells also release a number of vasodilators, such as NO and prostacyclin. Nitric oxide is a small gaseous molecule synthesised by nitric oxide synthase (NOS) that induces vascular relaxation by activation of guanylyl cyclase and cGMP accumulation. Nitric oxide was initially discovered as endothelium-derived relaxing factor (EDRF) in 1980 and is released by the endothelium under the challenge of acetylcholine and relaxes isolated vascular beds. EDRF was then proposed to be NO on the basis of pioneering work by Palmer, Ferrige and Moncada using a chemiluminescence assay to detect the EDRF being released in a bioassay set-up. Nitric contains an unpaired electron and thus regarded as a free radical species. Although relatively stable compared to other free radical species such as hydroxyl radicals, nitric oxide is still reactive and readily combines with a number of substances including other radicals, transition metal ions and nucleophiles such as thiols and amines. Nitric oxide is highly hydrophobic and has a very low solubility in water. This characteristic enables nitric oxide to diffuse through cell membranes. Nitric oxide is produced from L-arginine which is oxidised into L-citrulline in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADP) by a family of enzymes called NOS. There are three isoforms of NOS: the neuronal NOS (nNOS), the inducible NOS (iNOS) and the endothelial NOS (eNOS). eNOS is constitutively expressed in vascular endothelial cells and maintains tonic release of nitric oxide, which plays a critical role in vascular homeostasis. The activity of eNOS and nNOS requires reversible calcium-dependent calmodulin binding and is influenced by intracellular calcium concentration, while physiological factors such as shear stress and changes in oxygen tension also affect the activity and expression of eNOS. iNOS is not constitutively expressed in most cardiovascular cells. The expression of iNOS is normally induced by pro-inflammatory stimuli. Almost all types of cells including endothelial cells, SMC, fibroblast and leukocytes can express iNOS when activated. Unlike eNOS, iNOS binds calmodulin irreversibly, and the activity of iNOS is not sensitive to the intracellular calcium concentration. iNOS is regarded as a high-output isoform of NOS, and it is of importance in a number of blood vessel pathologies. NO is sometimes referred to as a "gasotransmitter" and also within this category is hydrogen sulphide (H_2S). H_2S is also produced by the vasculature (both endothelium and vascular smooth muscle), and, in contrast to NO which signals through the sGC/cGMP pathway, H_2S produces vasodilation via activation of potassium channels and muscle membrane hyperpolarisation (for a recent review of gasotransmitters, see Gheibi et al. [13]). Therapeutically, many agents in current use to treat conditions such as angina and heart failure do so by liberating NO to promote primarily venous dilatation, and future efforts may be directed towards targeting other gasotransmitters such as H_2S.

Another endothelium-derived vasodilator, prostacyclin, is produced from membrane-bound phospholipid by a series of enzymes including phospholipase A_2, cyclooxygenase and prostacyclin synthase when endothelial cells are activated.

Prostacyclin activates G protein-mediated activation of adenylate cyclase through IP receptors and leads to cAMP formation and then vasorelaxation [14]. In addition, NO and prostacyclin are reported to inhibit vascular SMC proliferation, maintaining the vessel media in a state of quiescence with a very low cell turnover. They are likely to do this by antagonising the cellular effects of growth factors and vasoactive peptides including Ang II and ET-1 (reviewed by Kapakos et al. [15]). The importance of this process in diseases where structural changes occur cannot be overstated. Perturbations in the endothelial layer caused by high blood pressure, atherosclerosis, aneurysms or mechanical injury during percutaneous interventions can result in excessive proliferation and development of a neointima, and this may be a key step in pathologies such as vein graft hyperplasia and in-stent restenosis.

Inflammation in the vascular tree is characteristic of many cardiovascular diseases, and mediators produced by the endothelium may also play a role. Endogenous NO inhibits the expression of adhesion molecules and pro-inflammatory cytokines. Inhibition of basal nitric oxide production results in increased leukocyte adhesion in the post-capillary venules, while addition of exogenous nitric oxide reduces leukocyte infiltration in acute inflammation. Induction of iNOS in inflammation might contribute to limit the extent of the inflammatory response. However, a high concentration of NO is also well known to react with ROS to produce cytotoxic peroxynitrite and cause tissue damage (for a recent review on the biochemistry of peroxynitrite and how it damages biological membranes, see Bartesaghi and Radi [16]). Paradoxically, acute exposure of isolated segments of artery to peroxynitrite actually induces vasodilation which may have a physiological role. Interestingly, in atherosclerotic vessels from experimental animals, the response to some vasodilators is attenuated although the response to peroxynitrite is maintained [17].

1.3 Vascular Smooth Muscle Cells

Immediately underlying the basement membrane is a fenestrated layer of ECM called the IEL. This represents the innermost layer of the vessel media (tunica media). All blood vessels, with the exception of the smallest capillaries, contain a muscular media. Arteries have a degree of muscularisation which is related to function. Resistance arterioles are particularly well muscularised relative to their size in order to dissipate arterial blood pressure prior to blood entering the capillaries, while conduit vessels such as the aorta and carotid arteries have a comparatively less muscular media with more connective tissue, making for a more elastic vessel. Indeed, a term sometimes coined to describe conduit vessels is "windkessel" which literally means "air-chamber". When blood pressure rises during ejection of blood from the heart (systole), windkessel vessels distend and then recoil as blood moves out and blood pressure falls during diastole. There is a net storage of blood during systole, and, by their distensibility, these vessels act like capacitors. Vascular stiffness increases with age, likely due to increased calcification and loss of elastin, and so the ability of the elastic vessels to dampen the fluctuations in blood pressure over the cardiac cycle diminishes. This can give rise to isolated systolic hypertension and an increased pulse pressure.

The medial smooth muscle cells are arranged concentrically, and their coordinated contraction and relaxation are responsible for changes in blood vessel diameter. Vascular smooth muscle (VSM) is continuously active with the concentration of cytosolic calcium (Ca^{2+}) being the major determinant of vasoconstriction or vasodilation (for a recent review of calcium and contractile processes in VSM, see Liu and Khalil [18], Touyz et al. [19]). The movement of Ca^{2+} into the cytosol results from a change in membrane potential (and the opening of voltage-operated calcium channels) or the activation of receptors by contractile agents. This results in the production of inositol 1, 4,5-trisphosphate (IP_3) and diacylglycerol from the hydrolysis of phosphatidylinositol-bisphosphate by phospholipase C. Following activation, IP_3 causes Ca^{2+} release from its internal store, the sarcoplasmic reticulum (SR) via the IP_3 receptor. The calcium-binding protein calmodulin forms a complex with Ca^{2+} which in turn activates myosin light-chain kinase. The activated enzyme phosphorylates smooth muscle myosin light chain (SM MLC) with this process driven by adenosine triphosphate (ATP). This results in the interaction of SM MLC with alpha smooth muscle actin (αSMA) filaments, initiating crossbridge cycling and the generation of force which in turn produces contraction. For relaxation to occur, the level of free

cytosolic Ca^{2+} must decrease which occurs by the movement of Ca^{2+} back into the lumen of the SR via the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump with the hydrolysis of ATP. Other pumps may extrude calcium across the plasma membrane to reduce intracellular calcium such as the plasma membrane calcium ATPase (PMCA) or sodium-calcium exchanger (NCX). Many of these processes are regulated by reactive oxygen species in vascular cells. Expression of these calcium extrusion pumps is altered in atherosclerotic mice [20], and there may be compensatory changes that may maintain vasodilator function of the vessel wall as atherosclerotic plaques develop. Thus, VSM regulates the luminal diameter of the vessel and therefore is fundamental in vascular tone as well as arterial blood flow and pressure. In addition, vascular dysfunction which is characteristic of many CV diseases appears to be associated with abnormalities in PKC and ROCK activity, and active efforts are underway to develop modulators of these signalling proteins which could be future therapeutic agents.

1.4 The Adventitia

The adventitial layer (tunica adventitia) surrounds the media and is comprised of connective tissue within which nerve fibres and a network of small blood vessels called the vasa vasorum are located. The vasa vasorum supplies oxygen and other nutrients to the adventitia and outer portion of the media, while the inner portion of the media and intima receive nutrients from the luminal blood by the process of diffusion. The innervation of the adventitia consists of varicosities of the sympathetic branch of the autonomic nervous system. Varicosities, which are small enlargements along the nerve fibres, are the point at which noradrenaline is released. By activating postsynaptic alpha adrenoceptors, these nerves induce contraction of the medial smooth muscle cells and a reduction in vessel diameter. It has been known for some time that varicosities release more than one neurotransmitter, a process known as cotransmission. There can be interaction between the transmitters released by the nerves (e.g. noradrenaline and adenosine 5′-triphosphate (ATP)) as well as interactions between nerve-derived transmitters and locally derived mediators released by the blood vessel (e.g. prostanoids). For an authoritative review, the reader is directed towards Burnstock [21].

Parasympathetic fibres are found to be associated with blood vessels in certain organs such as salivary glands and gastrointestinal glands and in genital erectile tissue. These specialised nerves release acetylcholine (ACh) which binds to muscarinic ACh receptors to induce vasodilation through NO formation and guanylyl cyclase activation. The tone of vessels is thus an amalgamation of the influence of the autonomic nervous system coupled with factors released by the endothelium (and also other parts of the vessel which will be discussed below). Many currently used pharmacological agents reduce blood pressure by altering this balance – for example, by blocking the alpha adrenoceptors or blocking the production, metabolism or action of endothelium-derived vasoconstrictors. The outer regions of blood vessels may also contain sensory neurones which release vasoactive substances such as calcitonin gene-related peptide (CGRP) which can dilate blood vessels and may have a role in diseases such as migraine [22].

1.5 Perivascular Adipose Tissue

Surrounding the adventitia of the vast majority of blood vessels (capillaries and cerebral blood vessels being the exception) is a cushion of perivascular adipose (PVAT) tissue (Fig. 1.2), and it is becoming increasingly apparent that it too can influence blood vessel function [23]. PVAT has a mixed composition of both white adipose (WAT) and brown adipose tissue (BAT), with varied WAT/BAT ratio according to its anatomical location. The PVAT around abdominal and mesenteric vessels is mainly WAT, while the thoracic aorta appears to have more BAT. Besides adipocytes, PVAT contains other cells grouped together as the stromal vascular fraction (SVF), including fibroblasts, mesenchymal stem cells, lymphocytes, macrophages and endothelial cells derived from structures such as the vasa vasorum (reviewed in Almabrouk et al. [24]). In addition to the release of free fatty acids/non-esterified fatty acids (NEFAs) by lipolysis, adipose tissue secretes bioactive proteins. These are collectively termed as adipokines and have endocrine, autocrine and paracrine actions. PVAT-

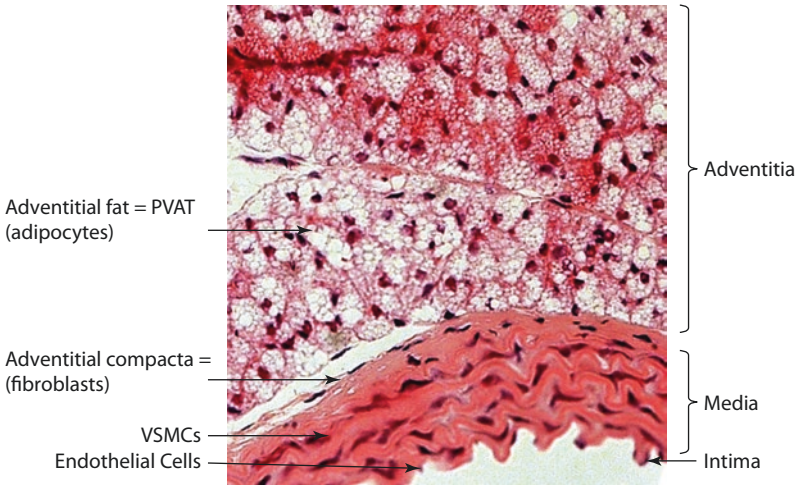

Fig. 1.2 Haematoxylin- and eosin-stained mouse aorta showing the structure of the blood vessel and the surrounding perivascular adipose tissue (PVAT). The blood vessel wall is composed of three layers: tunica intima (endothelium), tunica media (VSMCs layer) and adventitia which includes the PVAT. PVAT contains primarily adipocytes but also vasa vasorum and other cells (macrophages, adipocyte stem/progenitor cells, lymphocytes, fibroblasts, etc.). (Photograph courtesy of Dr. T.A.M. Almabrouk, University of Zawia. Libya)

derived adipokines such as adiponectin, leptin, omentin and resistin participate in the regulation of vascular function (reviewed in Mattu and Randeva [25]). Many of the substances released by PVAT from healthy blood vessels are beneficial, and in vitro studies have shown that adiponectin can augment the ability of vessels to relax [26]. PVAT can also release a whole host of other substances with direct or indirect effects on the underlying blood vessel such as angiotensin, reactive oxygen species (ROS) including superoxide, hydrogen peroxide (H_2O_2) and gaseous molecules such as H_2S and NO. Recent work has demonstrated that an enzyme involved in controlling cellular metabolism known as AMPK and which is expressed in PVAT may be pivotal in release of some adipokines [26].

PVAT also produces classical chemokines (or cytokines) including IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and PAI-1 (plasminogen activator inhibitor-1). Interestingly, inflammatory cells such as macrophages and T-lymphocytes, fibroblasts and capillary endothelial cells have also been demonstrated to contribute to the chemokine and cytokine profile of adipose tissue, and in disease states or in animal models of disease, inflammation within the PVAT increases [27]. In addition, dysfunctional fat can affect the activity of other tissues within the vessel wall such as the endothelium, and adiponectin has been proposed to mediate crosstalk between PVAT and vascular endothelium [28]. Cytokines generated from within the PVAT may also induce changes in vascular reactivity indirectly through upregulation of iNOS. Currently, there is a large research effort underway to understand the function of PVAT better and in particular to appreciate whether changes in PVAT underlie the vascular dysfunction often seen in obese, insulin-resistant patients.

Conclusions and Clinical Perspectives

The basic structure of a blood vessel consists of three concentric layers: endothelium, vascular smooth muscle cells, adventitia and, in most vessels, perivascular adipose tissue associated with the adventitia. The thickness of the medial smooth muscle and the amount of elastic fibres within the medial layer depend on the position of the vessel within the vascular tree and the pressure of the blood within the vessel. Veins are adapted to their function of moving low pressure blood back to the heart and storing the majority of blood by virtue of having valves and distensible, thin walls. The function of blood vessels is to conduct blood ejected by the heart around the body. The diameter of a blood vessel affects the flow of blood within that vessel, and diameter can be varied by a huge number of factors including release of mediators by the blood vessel itself, circulating vasoactive factors, neural

control within the adventitia, release of adipocytokines by the perivascular adipose tissue and metabolic activity of the tissue served by the blood vessel. With increased understanding of the physiological regulation of the structure and function of blood vessels, novel therapeutic targets for vascular disorders may become apparent.

Gaps in Knowledge

- How cells within the artery wall communicate with each other is not fully understood.
- The processes of remodelling in response to diseases such as high blood pressure are incompletely understood.
- It is unclear whether vascular remodelling is a cause or effect of increased blood pressure.
- The perivascular adipose tissue, which produces adipokines and other vasoactive factors, modulates vascular function, although exact mechanisms and the clinical relevance still remain unclear.

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Vascular Physiology

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_2

2

Key Concepts

- The continuous circulatory system consists of distribution, resistance, exchange, and capacitance vessels.
- Blood pressure is the force exerted on the wall of blood vessels by its contained blood.
- Blood pressure is controlled by multiple systemic (neural, hormonal) and local (myogenic, endothelial) systems.
- Differences in blood pressure within the vascular system provide the driving force that keeps blood moving from higher to lower pressure areas.
- Certain tissues possess specialised mechanisms of blood flow regulation which are critical for the proper functioning of the organs.

2.1 Introduction

The vascular system is a complete circuit of blood vessels which distributes blood via the arterial system to the microcirculation of organs and tissues where the exchange of materials between the blood and the interstitial space takes place and is returned from the capillary region via the venous system. The complete circuit of blood was first described by William Harvey (1578–1657) in his work entitled *Exercitatio Anatomica de Motu Cordis et Sanguinis* (Frankfurt, 1628) [1]. The circulatory system is divided into two main sections: the pulmonary circulation which supplies the lungs for the exchange of oxygen and carbon dioxide between the internal environment and atmospheric air and the systemic circulation which supplies the tissues of all other organs in the organism with oxygen and nutrients. The main function of the circulation is to maintain homeostatic conditions in the tissue for optimal function and survival of the cells [2, 3]. These needs are served by transporting different nutrient molecules (e.g. amino acids, fatty acids, glucose, vitamins, minerals, oxygen, etc.) to the tissues, transporting away waste products, carrying chemical information molecules (e.g. hormones and vitamins) from one part of the organism to another, and distributing heat energy in the body. The blood is propelled from the heart by the pulsatile pumping activity of the left and right heart chambers, and an appropriate pressure is maintained in both the systemic and pulmonary circulation through the specific properties of the blood vessels.

Healthy functioning of the circulatory system is determined by several physiological factors including normal arterial blood pressure, blood flow, blood viscosity, vascular elasticity, capillary permeability, and local as well as systemic control. The haemodynamic performance of the cardiovascular system is normally extremely high. The resting value of cardiac output in adults is 5–5.5 l/min, but this can rise to 25–35 l/min during heavy exercise, depending on the trained state of the body. Over an average 60-year life span, approximately 200,000 m^3 blood is pumped by the heart into the circulation, 5000–6000 m^3 fluid is filtered across the capillary wall, and 8,000,000 l of oxygen is supplied for metabolic processes in the body. The average capillary density in the body is 600 vessels/mm^3 tissue with around 1000 m^2 surface area available for exchange of materials [4], which is equivalent to the surface area of almost four tennis courts.

The circulatory system is provided with multiple systemic (neural, hormonal) and local (myogenic, endothelial, etc.) control systems [2, 3]. Blood pressure (BP) control is essentially the sum of the regulation of blood flow to a given tissue in proportion to its metabolic need. The local mechanisms that control blood flow include acute and chronic vasoconstriction and dilatation and a change in the number and calibre of the blood vessels supplying a tissue. Endothelial autocrine secretions play an important role in these vasoconstriction and vasodilatation mechanisms [5]. The global control of blood flow involves changes in cardiac output, vascular resistance, and control of arterial BP mediated by the autonomic nervous system.

2.1.1 Functional Units of Systemic Blood Vessels

The systemic circulation can be divided into units according to their major haemodynamic functions (see Fig. 2.1) [6, 7]. These include conducting (elastic) vessels, precapillary resistance vessels, precapillary sphincters, exchange vessels, postcapillary resistance vessels, capacity or

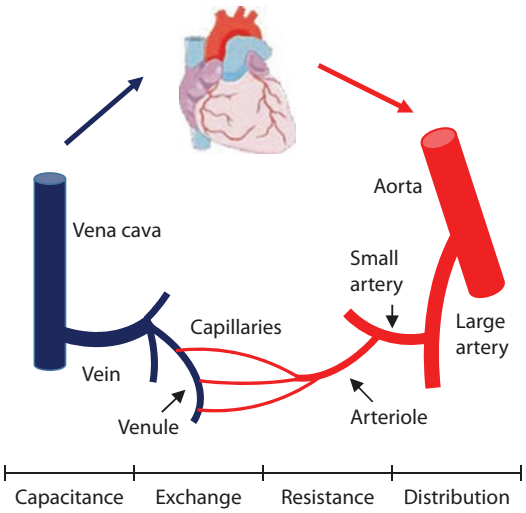

Fig. 2.1 The systemic circulation can be divided into major haemodynamic functional units

Table 2.1 Function and diameter of the various vessels that form the vascular system

Vessel type	Diameter (mm)	Function
Aorta	25	Pulse dampening and distribution
Large Arteries	1.0–4.0	Distribution of arterial blood
Small Arteries	0.2–1.0	Distribution and resistance
Arterioles	0.01–0.20	Resistance (pressure and flow regulation)
Capillaries	0.006–0.010	Exchange
Venules	0.01–0.20	Exchange, collection, and capacitance
Veins	0.2–5.0	Capacitance function (blood volume)
Vena Cava	35	Collection of venous blood

volume vessels, and shunt vessels. Table 2.1 summarises the function and diameter range of the various vascular units.

Elastic (conducting) arteries are the *largest arteries* and include the *aorta* and other nearby branches. The tunica media of elastic arteries contains a large amount of elastic connective tissue, which enables the artery to expand as blood enters the lumen from the contracting heart. During relaxation of the heart, the elastic wall of the artery recoils to its original position, forcing blood forward and smoothing the pulsatile discharge of blood from the heart. These vessels, also known as Windkessel vessels (which is a term generally taken to imply an elastic reservoir), provide relatively little resistance to blood flow and are much more distensible than the peripheral arteries; consequently they dampen the pulsatile pressure generated by the left ventricle. These large arteries, although capable of constricting and dilating, serve virtually no role in the regulation of pressure and blood flow under normal physiological conditions.

Precapillary resistance vessels (muscular arteries) are represented by the *small arteries* and the *arterioles*. They provide most of the peripheral resistance against blood flow. Abundant smooth muscle in the thick tunica media allows these arteries to regulate blood flow by vasoconstriction (narrowing of the lumen) or vasodilation (widening of the lumen). These vessels usually exhibit a substantial intrinsic myogenic tone and are highly innervated by autonomic nerves (particularly sympathetic adrenergic). They respond to changes in nerve activity, circulating hormones and endothelial substances. Together, the small arteries and arterioles represent the primary vessels that are involved in the regulation of arterial blood pressure as well as blood flow within the organ.

The precapillary sphincter is the terminal component of the precapillary resistance vessels. This is a very short section at the entrance of each systemic capillary which is composed of a few circular smooth muscle cells. These elements control the number of open capillaries at any given time and thereby the size of the capillary surface area available for exchange of materials across the capillary wall. The spontaneous cyclic closure and opening activity of these elements is called vasomotion. Local vasodilator metabolites play a major role in controlling the open phase of these vessel sections and thus the level of local blood supply.

Exchange vessels (*capillaries*) promote the exchange of materials between the external and internal environment of the living organism. Capillaries are made up of a single layer of endothelial cells and a basement membrane, without the presence of smooth muscle cells. The mechanisms of transmural capillary transport involve

ultrafiltration governed by the Starling forces, diffusion, pinocytosis, and occasionally bulk flow. Exchange across the capillary endothelium of oxygen, carbon dioxide, water, electrolytes, proteins, metabolic substrates and by-products (e.g. glucose, amino acids, lactic acid), and circulating hormones takes place between the plasma and the tissue interstitium surrounding the capillary.

Venules and *small veins* (postcapillary resistance vessels) provide only a small contribution to the total peripheral blood flow resistance; however they are important in order to maintain the ratio of pre- to postcapillary resistance. This ratio determines capillary hydrostatic pressure, which is a major component of the filtration forces driving fluid across the capillary wall. In addition to the postcapillary resistance, this vascular section also contributes to the exchange function (resorption of fluid) and participates significantly in the capacitance function of the venous system. These venous vessels, like the resistance vessels, are capable of dilating and constricting and serve an important role in regulating capillary pressure.

Capacity (or volume) vessels make up a major part of the venous system. The vascular capacity of the venous system is substantially larger than that of the arterial system, thus the distribution of blood volume between them is asymmetrical. Capacitance (or compliance) of veins is greater than that of arteries, which means that small changes in venous blood pressure is accompanied by large change in intravascular volume (and vice versa). The final venous vessels are the inferior and superior *vena cava*, which carry the blood back to the right atrium of the heart.

Shunt vessels can be found in certain regions of the body (e.g. ears, fingers, etc.) as direct vascular connections between small arteries and veins, bypassing the capillary bed. Their function is closely related to thermoregulation.

The microcirculation is a collective name for the smallest vessels in the circulatory system, i.e. arterioles, venules, shunts, capillaries, and collecting ducts. Microcirculation exists in all tissues and organs except the cornea.

2.1.2 Blood Pressure (BP)

Blood pressure (expressed as mmHg) is defined as the force per unit area exerted on the wall of a blood vessel by its contained blood [8]. It usually refers to the pressure in the large arteries of the systemic circulation. Pressure of arterial blood (mean arterial pressure, MAP) is regulated primarily by total peripheral resistance (TPR) and cardiac output (CO): MAP=CO × TPR.

Cardiac output is dependent on the filling of the heart (preload) and the strength of contractility, which is regulated by fibre length (the Frank-Starling law of the heart), autonomic (sympathetic) nervous system activity, circulating factors such as catecholamines, and disease [9]. *Peripheral resistance* is determined by vasoconstrictors (locally released and circulating) including catecholamines, angiotensin II, and endothelin and by vasodilators such as nitric oxide, prostaglandins, histamine, and atrial natriuretic peptide (ANP) [6].

Blood pressure is expressed as two values (systolic pressure and diastolic pressure). Systolic pressure represents the amount of pressure exerted on the blood vessels when the ventricle of the heart contracts (systole). Diastolic pressure represents the amount of pressure on the blood vessels when the heart is at rest between contractions (diastole). Normal ranges of blood pressure are less than 120 mmHg for systolic and less than 80 mmHg for diastolic according to the World Health Organization [10].

Pulse pressure (mmHg) is the difference between the systolic and diastolic pressure readings. It represents the pressure resulting from left ventricular ejection and is influenced by the stroke volume and the compliance or elasticity of the aorta.

Mean arterial pressure (MAP) is the pressure that propels the blood to the tissues. MAP = diastolic pressure + 1/3 pulse pressure.

2.1.3 Distribution of Pressures and Volumes

Differences in BP within the vascular system provide the driving force that keeps blood moving from higher to lower pressure areas [6]. The aorta and arteries have the highest pressure (see Fig. 2.2). The mean aortic pressure (solid red line) is approximately 95 mmHg in a normal individual. There is only a small fall in mean blood pressure as the blood flows down the aorta and through large distributing arteries. However, when reaching the small arteries and arterioles,

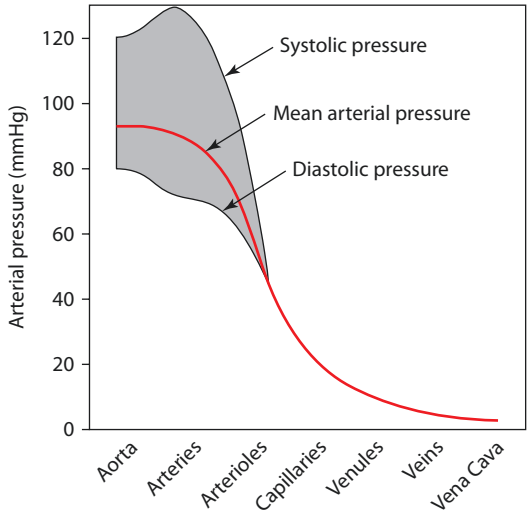

Fig. 2.2 Blood flows through the vasculature down a pressure gradient created by the contraction of the heart

there is a much larger fall in mean blood pressure with approximately 50–70% of the pressure drop along the vasculature occurring within this region of the circulatory system. By the time blood reaches the capillaries, the mean pressure may be 25–30 mmHg, depending upon the organ. The pressure falls further as blood travels into the veins and back to the heart. Pressure within the thoracic vena cava near the right atrium is very close to zero and fluctuates from a few mmHg negative to positive with respiration. Many veins, especially those in the limbs, have valves, which are needed to assist pumping of blood back towards the heart and prevent backflow due to gravity. Dynamically, active skeletal muscle contraction/relaxation helps in pumping of venous blood.

The arterial pulse pressure (shown as the grey band around the mean pressure in Fig. 2.2) increases as the blood flow travels down the aorta and into distributing arteries. The systolic pressure rises and the diastolic pressure falls, therefore the pulse pressure increases. This occurs because of reflective waves from vessel branching and from decreased arterial compliance (increased vessel stiffness) as the pressure pulse travels from the aorta into distributing arteries. As the blood flows into smaller arteries and arterioles, the pulsatility declines.

In terms of blood volume distribution within the circulation, the greatest volume resides in the venous vasculature, where 70–80% of the blood volume is found. For this reason, veins are referred to as capacitance vessels. The relative volume of blood between the arterial and venous sides of the circulation can vary considerably depending upon total blood volume, intravascular pressures, and vascular compliance.

2.1.4 Blood Flow

Blood flow is the movement of blood through the vessels, tissues, or organs and is denoted in terms of the volume of blood flowing in unit time. It is directly proportional to the difference in blood pressure (ΔP) between two points in the circulation and flows down the pressure gradient.

$$\text{Flow} = \Delta P / R$$

where flow = flow rate of blood through a vessel, ΔP = pressure gradient, and R = resistance of blood vessels (R is more important than ΔP in influencing local blood pressure).

Resistance is regulated by constant factors, i.e. blood viscosity (thickness or 'stickiness' of the blood), haematocrit (packed red cell volume), and blood vessel length (the longer the vessel the greater the resistance encountered), and by dynamic factors, i.e. blood vessel diameter.

Resistance of blood flow in a vessel is described by Poiseuille's law [6]. This law expresses the relationship between the rate of flow of a liquid in a tube and the pressure gradient in the tube, the radius of the tube, the length of the tube, and the viscosity of the liquid.

2.1.5 Compliance

The ability of a blood vessel wall to expand and contract passively with changes in pressure is an important function of large arteries and veins. This ability of a vessel to distend and increase volume with increasing transmural pressure (inside minus outside pressure) is quantified as vessel compliance (C), which is the change in volume (ΔV) divided by the change in pressure (ΔP).

$$C = \frac{\Delta V}{\Delta P}$$

The volume-pressure (compliance) relationship for an artery and vein is illustrated in Fig. 2.3a

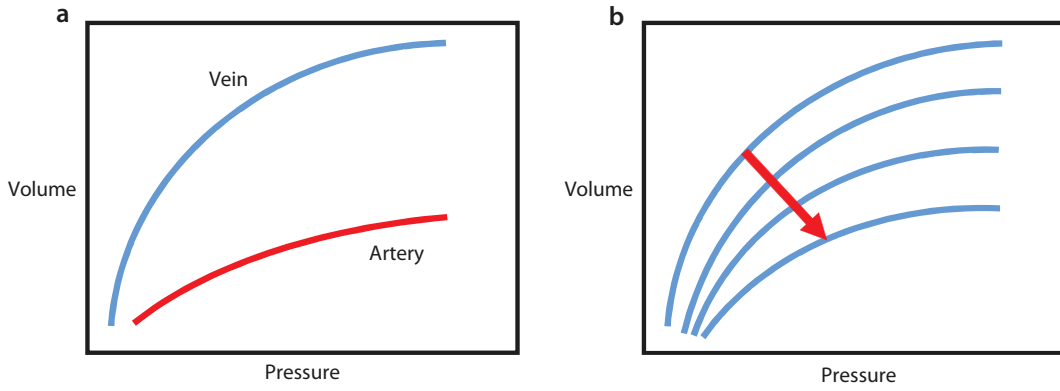

Fig. 2.3 **a** Compliance curve for an artery and vein. The slope of the curve is compliance. At low pressures venous compliance is 10 to 20 times greater than arterial compliance, but arterial and venous compliance are similar at high pressures. **b** Compliance curves for a vein showing how increasing smooth muscle contraction (arrow) decreases venous volume and increases venous pressure by decreasing compliance

[6]. Two important characteristics are demonstrated. Firstly, the slope is not linear because the blood vessel wall is a heterogeneous tissue. Therefore, compliance decreases at higher pressures and volumes (i.e. vessels become 'stiffer' at higher pressures and volumes). Secondly, at lower pressures, the compliance of a vein is about 10 to 20 times greater than an artery. Therefore, veins can accommodate large changes in blood volume with only a small change in pressure. However, at higher pressures and volumes, venous compliance (slope of compliance curve) becomes similar to arterial compliance. This makes veins suitable for use as arterial bypass grafts.

Vascular smooth muscle contraction, which increases vascular tone, reduces vascular compliance; conversely, smooth muscle relaxation increases compliance [6]. This is particularly important in the venous vasculature for the regulation of venous pressure and cardiac preload. Contraction of smooth muscle in arteries reduces their compliance, thereby decreasing arterial blood volume and increasing arterial blood pressure.

2.1.6 Myogenic Tone

Myogenic tone is an intrinsic property of vascular smooth muscle, which contracts in response to stretching, independent of any nerve or humoral regulation [11]. All arteries have myogenic tone and therefore contract in response to an increase in blood pressure. Myogenic tone gains in importance with decreasing vessel calibre, and only in small arteries and arterioles (diameter, 15–300 m, depending on species and organ) is it able to provoke substantial luminal narrowing in reaction to an increase in transmural pressure. The purpose of myogenic tone is to protect the distal capillaries against deleterious local hypertension. This short-term protection has an immediate impact whereby increased myogenic tone amplifies arteriolar resistance to blood flow, leading to a proximal increase in blood pressure [12].

2.1.7 Blood Pressure Regulation

Mean arterial pressure is constantly monitored by baroreceptors (pressure sensors) within the circulatory system [13]. When deviations from 'normal pressure' are detected multiple reflex responses are initiated in order to return mean arterial pressure to its normal value. *Short-term* (within seconds) adjustments are made by alterations in cardiac output (CO) and total peripheral resistance, mediated by means of autonomic nervous system influences on the heart, veins, and arterioles. *Long-term* (requiring minutes to days) control involves adjusting total blood volume by maintaining normal salt and water balance through mechanisms that regulate urine output and thirst. The size of total blood volume, in turn, has a profound effect on cardiac output and mean arterial pressure.

Arterial Baroreflex (Baroreceptor Regulation) The arterial baroreflex is a classic example of a negative feedback system and is designed to buffer

beat-to-beat fluctuations in arterial blood pressure from an internal set point or baseline. This sympathoinhibitory reflex is stimulated by acute changes in arterial blood pressure that are sensed by stretch receptors (baroreceptors) in the vessel wall of the carotid sinus and aortic arch. When blood pressure falls, reduced afferent activity from these receptors results in increased efferent activity from the nucleus tractus solitarii (NTS), which is the cardiovascular control centre of the brain. This causes vasoconstriction mediated by the sympathetic nervous system and circulating hormones and thus restores blood pressure. This system is dynamic enough to respond to a transient fall in blood pressure that occurs, for example, on standing. Increases in arterial pressure have the opposite effect, evoking reflex decreases in peripheral resistance, stroke volume, and heart rate to restore arterial pressure.

An important property of the arterial baroreflex is that it can be reset to operate around a new baseline blood pressure. This resetting can be acute or temporary, for example, during exercise, when the efferent baroreflex function curve shifts to the right and upward without a reduction in sensitivity. This adaptive response allows blood pressure, efferent sympathetic nerve activity, and heart rate to remain at higher levels during the period of exercise and then to return to baseline levels at the end of exercise. Baroreflex resetting can also be chronic, for example, during the development of hypertension as the baroreflex function curve gradually shifts to the right to operate around the new prevailing blood pressure. Over time, as arterial pressure remains elevated, the sensitivity of the baroreflex may also be reduced, rendering it less able to buffer acute pressure fluctuations.

Chemoreceptors The major function of peripheral chemoreceptors (carotid and aortic bodies) and central chemoreceptors (medullary neurons) is to regulate respiratory activity. This is an important mechanism for maintaining arterial blood PO_2, PCO_2, and pH within appropriate physiological ranges. However chemoreceptor activity also affects cardiovascular function either directly by interacting with medullary vasomotor centres or indirectly via altered pulmonary stretch receptor activity. Impaired gas exchange in the lungs decreases arterial PO_2 and pH and increases arterial PCO_2. These changes stimulate chemoreceptor activity leading to enhanced sympathetic outflow to the heart and vasculature via activation of the rostral ventrolateral medulla. Cerebral ischaemia activates central chemoreceptors which produces simultaneous activation of sympathetic and vagal nerves to the cardiovascular system.

2.1.8 Long-Term Regulation of Blood Pressure

The long-term control of arterial pressure is dependent on the relationship between arterial pressure and the urinary output of salt and water, which is affected by a number of factors including the integrated renal-endocrine systems (e.g. RAAS, ANP, and ADH) [14].

The Renin-Angiotensin-Aldosterone System The RAAS is a hormone system that regulates blood pressure and water (fluid) balance. When blood volume is low, it is detected by a decrease in blood flow and thus a decrease in glomerular filtration rate (GFR) in the kidneys. This decrease in GFR is sensed as a decrease in Na^+ levels by the macula densa. The macula densa cause an increase in Na^+ reabsorption, and thus water is reabsorbed via osmosis leading to an ultimate increase in plasma volume. The macula densa also releases adenosine which causes constriction of the afferent arterioles. In parallel, the juxtaglomerular cells sense the decrease in blood pressure and release renin into the circulation. Renin converts angiotensinogen (inactive form) to angiotensin I (active form). Angiotensin I is subsequently converted to angiotensin II by angiotensin-converting enzyme produced in the lungs. Angiotensin II is a potent vasoactive peptide that causes blood vessels to constrict, resulting in increased blood pressure. Angiotensin II also stimulates the secretion of the hormone aldosterone from the adrenal cortex. Aldosterone causes the distal convoluted tubules of the nephron to increase the reabsorption of sodium and water into the blood. This increases the volume of fluid in the body, which also increases blood pressure.

Antidiuretic Hormone (ADH) Baroreceptors in low-pressure zones (mainly in the venae cavae, pulmonary veins, and the atria) result in feedback by regulating the secretion of renin, aldosterone, and antidiuretic hormone (ADH). ADH, which is also known as arginine vasopressin (AVP), has two principle sites of action: the kidneys and blood

vessels. The primary function of AVP is to regulate extracellular fluid volume by affecting renal handling of water. AVP acts on renal collecting ducts via V_2 receptors to increase water permeability (via cAMP-dependent mechanism), which leads to decreased urine formation. This increases blood volume, cardiac output, and arterial pressure. A secondary function of AVP is vasoconstriction. AVP binds to V_1 receptors on vascular smooth muscle to cause vasoconstriction through the IP_3 signal transduction pathway and Rho-kinase pathway, which also increases arterial pressure.

Natriuretic Peptides Natriuretic peptides (NPs) are involved in the long-term regulation of sodium and water balance, blood volume, and arterial pressure. The main physiological action of natriuretic peptides is to reduce arterial pressure by decreasing blood volume and systemic vascular resistance.

NPs directly dilate veins (increase venous compliance) and thereby decrease central venous pressure, which reduces cardiac output by decreasing ventricular preload. NPs also dilate arteries, which decreases systemic vascular resistance and systemic arterial pressure. The mechanism of systemic vasodilation involves NP receptor-mediated elevations in vascular smooth muscle cGMP as well as attenuation of sympathetic vascular tone. This mechanism may involve NPs acting on sites within the central nervous system as well as through inhibition of norepinephrine release by sympathetic nerve terminals.

NPs affect the kidneys by increasing GFR and filtration fraction, which produces natriuresis (increased sodium excretion) and diuresis (increased fluid excretion), but are potassium sparing. A second renal action of NPs is that they decrease renin release, thereby decreasing circulating levels of angiotensin II and aldosterone. This leads to further natriuresis and diuresis. Decreased angiotensin II also contributes to systemic vasodilation and decreased systemic vascular resistance. Natriuretic peptides therefore serve as a counter-regulatory system for the renin-angiotensin-aldosterone system.

2.1.9 Intrinsic Regulation of Blood Flow (Autoregulation)

Intrinsic mechanisms of local blood flow regulation contribute to the precise matching of a tissue's metabolic needs to the quantity of blood flow delivered by the microcirculation [15]. These mechanisms operate completely within the tissue itself and are thus independent of outside physiological inputs.

Vasodilator Mechanisms Vasodilator mechanisms are activated when insufficient blood flow to a tissue is detected resulting in the build-up of vasodilatory substances that locally diffuse and induce vasodilation of adjacent arterioles and precapillary sphincters. Some vasodilatory substances are produced as a result of normal cellular metabolism, while others are produced in contexts of low oxygen tension. Lactic acid, adenosine, CO_2, hydrogen ions, and potassium ions all appear to display properties of vasodilatory substances. These substances are leaked in small amounts by cells, and their concentrations locally increase when they are not washed away by normal levels of blood flow. On the other hand when blood flow is excessive, the baseline concentrations of these substances decrease, resulting in vasoconstriction of arterioles and precapillary sphincters, thus allowing fine-tuning of blood flow levels to a tissue's metabolic demands.

Endothelium-Dependent Regulation of Blood Flow Endothelial cells play a pivotal role in the regulation of blood flow [5]. Endothelium-derived nitric oxide and prostacyclin are released in response to physical stimuli, hormones, and platelet-derived substances and induce vascular relaxation and inhibit platelet function. Certain endothelium-derived substances are also able to invoke hyperpolarisation of smooth muscle cells (EDHF). In addition, endothelial cells can release several vasoconstrictor agents (e.g. endothelin, thromboxane A2, angiotensin II, superoxide). Endothelial cells are also a source of growth inhibitors and promoters, such as heparin and heparin sulphates, platelet-derived growth factor, and thrombospondin. Several vasoactive substances produced by the endothelium, such as nitric oxide, endothelin, and angiotensin II, also play a role in the regulation of vascular growth. Thus, the endothelial layer can regulate vascular tone and growth.

Oxygen Deficiency Mechanisms Vascular smooth muscle cells require sufficient oxygen and nutrients to maintain tension. Consequently, in the absence of sufficient oxygen and nutrients, the 'oxygen

deficiency' mechanism is activated whereby vascular smooth muscle cells naturally relax, causing vasodilation of arterioles and precapillary sphincters. This simple mechanism accounts for some of the vasodilation observed when blood flow to a tissue is reduced. Arterioles can inherently react to sudden changes in blood pressure by undergoing vasoconstriction. While the precise mechanism of this reactive vasoconstriction is not currently understood, it is thought to play a role in the ability of arterioles to autoregulate local blood flow in spite of changes in systemic arterial pressure.

2.1.10 Organ-Specific Mechanisms of Blood Flow Regulation

While a combination of all of the above mechanisms likely contributes to local blood flow regulation in most tissues, certain tissues possess specialised mechanisms of blood flow regulation which are critical for the proper functioning of the organs. For example:

- *Kidneys:* tubuloglomerular feedback is an important regulator of blood flow to the kidneys and is important for guaranteeing a relatively constant level of glomerular filtration.
- *Lungs:* reductions in oxygen concentration tend to cause vasoconstriction of pulmonary arterioles rather than the vasodilation observed in other tissues.
- *Brain:* cerebral blood flow is much more sensitive to changes in CO_2 and hydrogen ions than other metabolites because neuronal function is highly sensitive to changes in pH which is determined by the balance between local CO_2 and hydrogen ion concentrations.
- *Heart:* vasodilator mechanisms appear to be the dominant process regulating blood flow in the coronary vasculature.

Conclusions and Clinical Perspectives

The vascular system maintains cellular homeostasis through a complex network of arteries, capillaries, and veins. It is necessary for vessels of the circulatory system to exhibit some homology of cell structure and composition, yet be different enough in their cellular properties to fulfil their physiological roles. In-depth understanding of vascular cell structure and organisation which underlie the specific roles of the functional units will lead to advances in drug development for vascular-related pathophysiological diseases.

Gaps in Knowledge

Vascular physiology has many facets ranging from molecular to the intact system. Through understanding the specialised mechanisms at the subcellular and cellular levels, using cutting-edge methodology and novel in vivo and in vitro models, we are discovering how the vasculature serves the unique needs and functions of every individual organ and tissue. Rapid advancement of our knowledge of the vasculature is critical to improve insight into and treatment of vascular disease and related disorders.

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Cell Biology of Vessels

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_3

3

Key Concepts

- Vessels are composed of endothelial cells, vascular smooth muscle cells (VSMCs) and adventitial cells, comprising fibroblasts and adipose tissue. Endothelial cells play an important regulatory role in vascular function via release of nitric oxide (NO), endothelium-derived hyperpolarizing factors (EDHF) and several pro-contractile factors.
- VSMCs are the major component of the vessel wall and together with endothelial cells are responsible for the maintenance of vascular tone.
- There are three major VSMC phenotypes, which are classified as contractile, synthetic and pro-inflammatory. Contractile VSMCs have low proliferative rate and express contractile proteins. Synthetic VSMCs are proliferative and express low levels of contractile proteins. Inflammatory VSMCs produce pro-inflammatory mediators.
- Perivascular adipose tissue (PVAT) surrounds blood vessels and secretes adipokines that regulate vascular function.
- Dysregulation of PVAT and associated alterations in adipokine secretion contribute to vascular dysfunction.

3.1 Introduction

The heart and blood vessel, which are responsible for the transport of nutrients, hormones, oxygen and other gases throughout the body, form the cardiovascular system. Blood vessels are composed of three layers: tunica intima of endothelial cells, tunica media composed of vascular smooth muscle and the adventitia or tunica externa. The vascular system includes arteries, arterioles, capillaries, venules and veins. Arteries play an important role in organ nutrition and include elastic arteries and muscular arteries. Arteries, such as the aorta and pulmonary vessels, contain more elastic tissue in the tunica media than smooth muscle cells (SMCs) and are considered elastic, whereas brachial, radial and femoral arteries contain more SMCs in the tunica media and are classified as muscular arteries. Arterioles are composed mainly of SMCs and an adventitia composed mostly of collagen, nerve endings and fibroblasts. Arterioles have an important role in resistance to blood flow and, consequently, are important regulators of mean arterial pressure and tissue perfusion. Capillaries are composed of a single endothelial layer. Veins comprise three layers, similar to the arterial system, but, unlike arteries, are thinner, and they can accommodate a large volume of blood at relatively low pressures. Surrounding the vascular media is the adventitia, which comprises fibroblasts as well as adipocytes, which together make up the perivascular adipose tissue (PVAT), an important source of adipokines that are functionally active and can influence vascular function (Fig. 3.1).

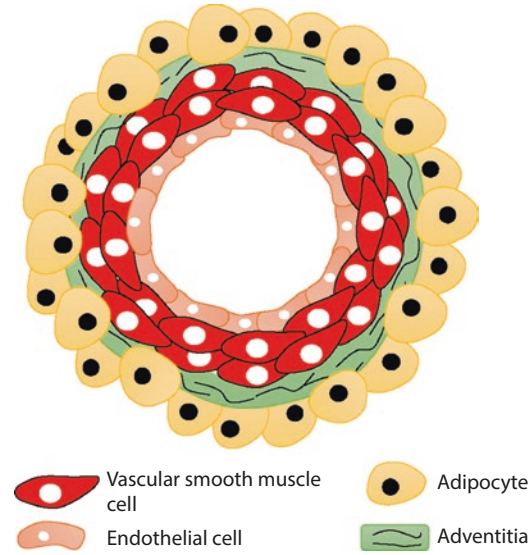

Fig. 3.1 Vessels are composed of endothelial cells, vascular smooth muscle cells and adventitia, mainly composed of collagen and fibroblasts. Surrounding the tunica externa, there is the adventitial fat layer, also known as perivascular adipose tissue (PVAT), which can influence vascular homeostasis by releasing numerous vasoactive factors, cytokines and adipokines

3.2 Endothelial Cells

The vascular system is essential for embryonic development, and therefore the cardiovascular system is the first organ system to develop in mammals; mesodermal precursors of haematopoietic and endothelial lineages differentiate into solid clumps known as blood islands. The inner

cells of these blood islands become haematopoietic cells, while outer cells differentiate into endothelial cells. Many transcription factors are known to play an important role in the activation and maintenance of endothelial gene expression which include vascular endothelial growth factor (VEGF), NOTCH4, ICAM-2, Ve-cadherin (Cdh5), FOXP1 and Gata2. Among these transcription factors, VEGF has a crucial role in endothelial cell generation. During embryonic development, VEGF receptor-2 (VEGFR-2) is restricted to endothelial cells, and deficiency of VEGF is lethal due to impaired vascular formation. Following the initial formation of endothelial cells and vasculature, the vascular system is rapidly expanded and remodelled. Next, the blood vasculature becomes further specialized into arteries, veins and capillaries [1].

Endothelial cells from different vessels exhibit regional characteristics, morphologically and functionally, due to the diversity of vascular channels and associated differences in haemodynamics, structure and embryonic origins. This phenomenon is observed in endothelial cells from large and resistance vessels, where disparities in mechanical and structural characteristics of these vessels and also different physiological roles in haemodynamics contribute to differences observed in these cells. In addition to the function, the shape of endothelial cells varies across the vascular tree. Endothelial cells are typically flat but can also be plump or cuboidal in venules. Their thickness varies from less than 0.1 μm in capillaries and veins to 1 μm in the aorta. Additionally in straight segments, they are aligned in the direction of blood flow of arteries but not at branch points [2].

The intimal layer in veins and arteries consists predominantly of endothelial cells, which have an important role in controlling vessel tone via the production and release of vasoactive factors that exert their action in SMC. Endothelial cells are also a barrier between blood components and extravascular tissues, and conditions such as inflammation promote alterations in the endothelial cytoskeleton that compromise microvascular permeability.

Endothelial cells play a key role in vascular relaxation, mainly through the production of NO, which control the degree of contraction of VSMCs. NO is a gas synthetized from L-arginine by the calcium-calmodulin-dependent enzyme nitric oxide synthase (NOS), as proposed by Furchgott and Ignarro in 1986. NO evokes vasodilatation through SMC relaxation, and abnormalities of endothelial regulation of vascular tone have been documented in hypertension.

L-arginine, which is synthesized from L-citrulline, is the best-characterized source of NO as a substrate for NOS. NO is produced by three NOS isoforms: neuronal NOS (nNOS or NOS-1), cytokine-inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). eNOS is the major isoform that regulates vascular function. The production of NO from L-arginine by NOS requires the presence of various cofactors including tetrahydrobiopterin (BH4). Reduced levels of BH_4, or its oxidation to the $BH_3\cdot$ radical by reactive oxygen species (ROS), are associated with NOS uncoupling, a condition where superoxide anion ($O_2^{\cdot -}$) is generated instead of NO from the oxygenase domain. NO has a high affinity for $O_2^{\cdot -}$, leading to peroxynitrite ($ONOO^-$) formation and reduction of NO bioavailability. Additionally, decreased availability of L-arginine and L-citrulline can contribute to decreased levels of NO production. eNOS activation is regulated by phosphorylation of Thr495 or Ser1177 residues, rather than eNOS expression. Rise in phosphorylated eNOS at Thr495 leads to a reduction in electron transfer in the enzyme, thus diminishing NO production, while phosphorylation of eNOS at Ser1177 enhances eNOS activity. Agonists such as acetylcholine enhance phosphorylation of eNOS at Ser1177 via activation of endothelial M3 receptors, which increases the influx of calcium, activation of calmodulin-binding domain of eNOS and synthesis of NO, which diffuses to adjacent VSMCs to induce vasorelaxation (Fig. 3.2). In VSMCs, NO increases intracellular levels of cGMP, inhibiting calcium entry into the cell and resulting in the activation of K+ channels and consequent membrane hyperpolarization, closure of calcium channels and vasorelaxation [3].

In addition to NO, endothelial cells modulate vascular function by releasing EDHFs and procontractile prostanoids and peptides including endothelin-1 (ET-1). EDHF is synthesized and released from the endothelium and can compensate the loss of NO bioavailability. EDHF acts by increasing potassium (K^+) conductance resulting in depolarization of VSMCs and consequent relaxation, an effect that can be mimicked by K^+ channel agonists and induced by acetylcholine in

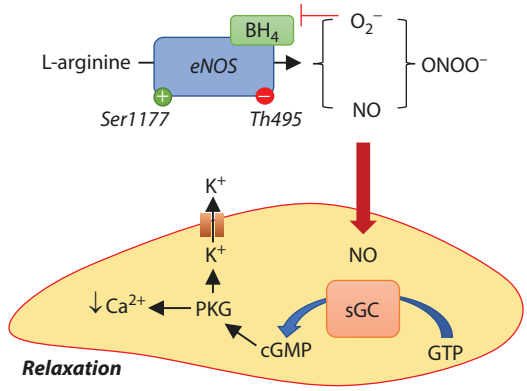

Fig. 3.2 In endothelial cells L-arginine is the best-characterized source of NO as substrate for eNOS. The production of NO requires the presence of BH_4. Oxidation of BH_4 leads to generation of O_2^{-} instead of NO. After released, NO diffuses to adjacent vascular smooth muscle cells to induce vasorelaxation via reduction of calcium entry into the cell and activation of K^+ channels

intact vessels, but not in endothelial-denuded vessels. Possible candidates for EDHF include hydrogen sulphide (H_2S) and hydrogen peroxide (H_2O_2). It has been demonstrated that H_2S can be generated in endothelial cells from cysteine by cystathionine γ-lyase (CSE) which is an event dependent on calcium-calmodulin. In VSMC H_2S causes hyperpolarization and vasorelaxation by activating the ATP-sensitive, intermediate conductance and small conductance potassium channels through cysteine S-sulfhydration. H_2O_2 induces VSMC hyperpolarization via arachidonic acid metabolism and activation of ATP-sensitive K^+ channels, leading to vascular relaxation. Experimental data indicate that, in cardiovascular diseases, there is a shift away from NO-mediated endothelium-dependent relaxation towards EDHF-dependent relaxation and impairment of EDHF-mediated responses has been reported in conditions such as, hypertension, atherosclerosis, diabetes, heart failure and ageing [4, 5].

ET-1 is a potent vasoconstrictor. Impaired balance between ET-1 and NO is implicated in the pathogenesis of many cardiovascular diseases. VSMCs express mRNA for ET-1, but its production is at least 100-fold less than endothelial cells. ET-1 effects are exerted via activation of two receptors, ET-A and ET-B, which are transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs) containing seven transmembrane domains with an extracellular N-terminal and intracellular C-terminal. ET-A receptor is highly expressed in VSMC, while ET-B is expressed in endothelial cells and VSMC. VSMC binding of ET-1 to ET-A and ET-B leads to vasoconstriction, cell adhesion and cell growth, while binding to ET-B in endothelial cells stimulates release of NO and prostacyclin.

ET-1 binding to receptors activates heterotrimeric guanine nucleotide (G) proteins including Gq, followed by activation of phosphoinositide-specific phospholipase Cβ (PLCβ) and hydrolysis of the membrane phospholipid to hydrophobic diacylglycerol (DAG) and soluble inositol 1′,4′,5′-trisphosphate [Ins(1,4,5)P_3]. These downstream molecules diffuse into the cytoplasm to activate calcium channels of the sarcoplasmic reticulum. DAG with Ca^{2+} activates the phosphatidylserine-dependent protein kinase, protein kinase C (PKC), which is involved in vascular dysfunction and observed in pathologies including diabetes and hypertension. ET-1 receptor activation can also lead to mitogen-activated protein kinase (MAPK) cascade activation, including ERK 1/2, p38MAPK, c-Jun N-terminal kinases (JNK), MEK 4/7 and MEK 3/6, which control cell differentiation, proliferation, migration and survival and activation of pro-inflammatory markers [6].

In addition to the control of vascular tone, endothelial cells are important regulators of the transport of ions, glucose and amino acids and have an important role in host defence and angiogenesis. GLUT-1 and GLUT-4 are expressed in endothelial cells and regulate glucose transport though these cells; dysregulation of these transporters is particularly important in conditions such as diabetes and hypoxaemia. Caveolae are also important vesicle carriers responsible for the transport of molecules through endothelial cells including albumin, which are primarily transported across the endothelium via this system. Transports through endothelial cells are also modulated by intercellular junctions. These junctions form a barrier to transport between endothelial cells and help to maintain cell polarity between the luminal and abluminal side of these cells. Endothelial cells maintain vascular homeostasis by regulating platelet aggregation, fibrin formation and thrombosis [7].

Due to its strategic position, endothelial cells play a role in host defence. Endothelial

cells produce and react to a variety of cytokines and other mediators of inflammation, including chemokines, colony-stimulating factors (CSF), growth factors and interferons (IFN). Endothelial cells also produce an important factor involved in angiogenesis, VEGF. Besides its contribution to the formation of new blood vessels, VEGF regulates inflammatory responses through stimulation of the release of metalloproteinases, molecules of adhesion and nitric oxide [8].

3.3 Vascular Smooth Muscle Cells

VSMCs found in the vascular wall contribute to the structural integrity of arteries. VSMCs regulate vessel diameter by relaxation or contraction in response to vasoactive agents. The property to adapt to constant cycles of contraction/relaxation is very important in terms of maintaining transport of oxygenated blood, nutrients, hormones and metabolites, immune cells and waste products to and from all tissues/organs in the body [9]. During vascular development or vasculogenesis, endothelial precursor cells initiate the formation of the dorsal artery and cardinal vein by mechanisms including selective sprouting, cell segregation and complex interactions between signalling pathways important to cell fate, such as the hedgehog family of morphogens (Shh), the vascular endothelial growth factor family (VEGF) and Notch receptors and ligands (Notch 1–4, Jagged 1–2). Further to the activation of these signalling complexes, new arteries and veins start to form with differential expression of VEGF receptors and ephrin (Eph) tyrosine kinase receptors, determining whether a vessel will become an artery or a vein. At this stage, endothelial cells mainly form these primordial arteries or veins and start the recruitment of mural cells, also known as nascent VSMCs [9, 10].

3.3.1 VSMC Phenotypes

Nascent VSMCs originate from multiple embryonic bodies and reflect the local characteristics of the specific embryonic origin. VSMC within the aorta, which is a large elastic artery, originates from ectodermal cardiac neural crest cells, while VSMCs from the mesenteric arterial bed are from the mesothelium. The origin of VSMCs is an important factor that will affect differential responses to diverse vasoactive stimuli leading to distinct phenotype of VSMCs and distinct cellular responses in different arterial beds. There are three distinct VSMC phenotypes: contractile (or differentiated), synthetic (or undifferentiated) and inflammatory [11].

Contractile VSMC is characterized by the expression of contractile genes and proteins important for contraction and maintenance of vascular tone, such as α-smooth actin (αSMA), smoothelin, SM22α, h1-calponin and h-caldesmon. They also express high levels of extracellular matrix components, collagen I and IV, followed by lower expression of matrix metalloproteinases (MMPs) and higher expression levels of tissue inhibitors of MMPs (TIMPs). In culture, contractile VSMCs have an elongated spindle-shaped morphology with low proliferation rates. It is also suggested that the expression of integrins differs between the different VSMC phenotypes, where in contractile VSMCs, α1β1 and α7β1 integrins are mostly expressed.

In *synthetic VSMCs*, the expression of contractile proteins decreases, followed by an increase in the following proteins: osteopontin, l-caldesmon, non-muscle myosin heavy chain B (NMB-MHC), vimentin and tropomyosin 4. VSMCs in the synthetic phenotype are more proliferative and more associated with vascular injury. In addition to high proliferation rates, synthetic VSMCs also have increased migration, extensive ECM production/degradation, increased cell size and decreased total number of actin filaments and predominant expression of α4β1 integrins.

The *inflammatory phenotype* occurs in response to inflammatory responses and stimuli usually initiated by endothelial cells. VSMCs express markers of inflammation, such as cytokines, vascular cell adhesion molecule 1 (VCAM-1) and NFκB. Pro-inflammatory factors and cytokines induce expression and activation of pro-inflammatory transcription factors and genes in VSMCs, leading to phenotypic switches from a contractile to an inflammatory phenotype in pathological conditions. In this state, VSMCs also produce cytokines and factors that stimulate monocytes and macrophage recruitment to the vascular wall, further contributing to vascular inflammation [12]. This is especially important in atherosclerosis.

3.3.2 Factors that Influence the VSMC Phenotype

Growth factors, such as epithelial growth factor (EGF), platelet-derived growth factor (PDGF) and VEGF, stimulate the switch to a synthetic phenotype in order to promote growth and cell survival, where activation of receptor tyrosine kinases and downstream signalling involving PI3K and MAPKs are required. On the other hand, TGFβ or bone morphogenetic proteins (BMPs) induce a contractile phenotype by increasing the expression of αSMA and calponin. Interestingly, Ang II, an important vasoactive peptide, acts as a dual factor and is able to induce many types of phenotypes, highlighting the plastic and dynamic nature of VSMCs. Calcium influx and signalling induced by Ang II is associated with contraction, whereas activation of growth signalling pathways by Ang II leads to a proliferative phenotype [13].

The *Notch receptors* and ligands are master regulators of the contractile phenotype in VSMCs. However, the degree of activation of the contractile phenotype will depend on interactions with Notch signalling repressors, such as HRT1, adding an extra level of modulation of VSMC phenotypes during development, maintenance of vascular tone/integrity and vascular injury/damage. A synergistic interaction between Notch signalling and TGFβ or PDGF and VEGF has been suggested, influencing switches between a contractile phenotype and contraction and a synthetic phenotype and proliferation [13].

VSMC function is regulated by the surrounding components of the ECM and plasma membrane adaptors connecting both, VSMCs and the ECM. These interactions between VSMCs and the ECM are extremely important in maintaining vascular cell function, structure and signalling. It also plays a role in the phenotype modulation, since while maintaining a contractile phenotype; it acts as a link between other factors (i.e. growth factors) or mechanical forces as an anchorage system to modulate proliferation and migration of VSMCs (phenotype switch) [14].

Integrins and syndecans are two essential components of the cell membrane responsible for cell-ECM anchorage. Integrins are transmembrane receptors composed of a diverse combination of α and β subunits, important in the activation of a complex signalling network, leading to the control of changes in VSMC function. Integrins are also important in linking ECM components to the actin cytoskeleton. Syndecans are transmembrane proteoglycans, formed by an extracellular domain, composed by glycosaminoglycans chains and an integrin association site, and an intracellular domain containing phosphorylation and binding sites for many other signalling proteins, such as actin, fascin, syntenin, synectin, FAK, Src, calmodulin and others. Syndecans are co-receptors for growth factors, important to VSMC signalling. ECM components that regulate VSMC phenotype and function are fibronectin, collagens, laminin, elastin, fibrillins, fibulins and emillins. Fibronectin interacts with integrins to activate cell signalling and actin/myosin interactions. Collagens have been demonstrated to influence growth factors signalling in VSMCs. VSMC responses depend on the form and subtype of collagen, where fibrillary collagens influence cell signalling differently than monomeric collagens and collagen type I seems to activate the synthetic phenotype, while collagen type IV promotes the expression of contractile proteins and the contractile phenotype. Elastin provides elasticity to vessels but also controls the contractile phenotype of VSMCs. Elastin regulates actin polymerization, RhoA/Rho-kinase signalling and contraction. Elastin can be degraded during vascular damage, generating soluble-derived peptides, which in turn will stimulate cell cycle and mitosis, leading to a switch to a synthetic phenotype and proliferation [14].

3.3.3 Role of VSMCs in Vascular Contraction

The physiological function of VSMCs is the regulation of vascular contraction. Changes in vessel diameter due to contraction depend on the phosphorylation (activation) or inactivation of contractile signalling proteins, which can be calcium-dependent or calcium-independent. Detailed mechanisms of contraction are discussed in more detail in other chapters in the book. Briefly, after stimulation by a neural, humoral or mechanical stimulus, calcium channels, pumps or exchangers in the plasma membrane are activated, inducing an increase in intracellular calcium levels. The increase in calcium influx activates calmodulin and myosin light chain kinase

(MLCK), which in turn will phosphorylate myosin light chain (MLC) and induce actin polymerization and VSMC contraction. Calcium-independent modulation of contraction is achieved by the activation of RhoA/Rho-kinase pathway, which will decrease the activity of myosin light chain phosphatase, facilitating activation of MLC and contraction. Hyperactivation of the contractile machinery and changes from a contractile to a synthetic phenotype are common features of cardiovascular diseases, including hypertension and associated cardiovascular complications, leading to vascular dysfunction and remodelling [15].

3.4 Perivascular Adipose Tissue

PVAT is the adipose component that is contiguous with the adventitial layer surrounding vessels. PVAT was previously thought to serve primarily as a scaffold for vessels. However, more recently it is being appreciated that PVAT is a metabolically active endocrine organ with important effects on vascular function. PVAT has local effects via release of a variety of adipokines and other factors that influence the function of conductance and resistance arteries, which are vital for the regulation of vascular tone and blood pressure.

The composition of PVAT varies by vessel type, but the amount increases with increasing adiposity of the subject. While small vessels contain predominantly white adipose tissue showing less differentiated adipocytes with low vascularization and metabolism, PVAT from conductance vessels resembles brown adipose tissue, with abundant number of mitochondria and multilocular lipid droplets. Bioactive substances (adipokines) released from PVAT include adiponectin, leptin, chemerin, resistin, visfatin, tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-18 (IL-8) and monocyte chemoattractant protein-1 (MCP-1). These agents have an important role in vascular function under normal conditions. In PVAT-denuded aorta, vasoconstrictor responses are increased when compared to intact vessels, suggesting an anticontractile effect of PVAT. Several PVAT-derived agents proposed to cause this anticontractile effect include adiponectin, NO and H_2O_2. Dysregulation of PVAT function is observed in pathological conditions such as obesity, diabetes and hypertension as well as in ageing [16].

PVAT-induced anticontractile effects are associated with release of PVAT-derived relaxation factors, which favour vascular relaxation through an endothelium-dependent mechanism involving NO production and H_2O_2-induced endothelium-independent relaxation. The modulation of vascular function by PVAT is not limited to the secretion of relaxing factor but also pro-contractile agents. Adipocytes exhibit AT_1R and AT_2R receptors, whereby Ang II mediates local effects, including increase in inflammatory markers and oxidative stress and adipocyte growth and differentiation. In PVAT, Ang II through AT_1R-mediated activation of calcineurin/NFAT stimulates aldosterone synthase and consequent production of pro-contractile agent aldosterone, effect potentiated in obese mice, which contributes to impairment of PVAT-induced anticontractile response, observed in this condition. In addition to aldosterone, PVAT enhances the contractile response of resistance arteries through superoxide anion production and consequent reduction of NO levels. Adipokine chemerin is also an endogenous pro-contractile agent released by PVAT, and incubation of isolated vessels with this adipokine increases arterial sensitivity to endothelin-1.

Obesity is associated with a pro-inflammatory adipose phenotype, where PVAT produces inflammatory adipokines, including cytokines and ROS, which promote vascular inflammation and dysregulation. PVAT from obese subjects secretes more MCP-1 than adipocytes from lean subjects. This chemokine is involved in macrophage M1 differentiation, which is classically activated and produces pro-inflammatory cytokines and initiates an immune response. On the other hand, lean subjects have predominantly M2 macrophages, which secrete anti-inflammatory cytokines. Many of the pro-inflammatory agents released by PVAT in obesity can influence vascular function, including cell migration, apoptosis, NO production and consequent endothelial function, vascular content and vascular remodelling [17].

Conclusions and Clinical Perspectives

Endothelial cells, VSMCs and PVAT play an important role in regulating vascular tone. These systems are tightly controlled and functionally interconnected. Vascular dysfunction and impairment of the endothelium are involved in many cardiovascular diseases including hypertension, atherosclerosis,

myocardial infarction and heart failure. Hence, approaches to maintain a healthy endothelium and vasculature are pivotal in cardiovascular therapies. Also, there is growing evidence that non-invasive assessment of endothelial and vascular function, for example, examination of endothelium-dependent flow-mediated dilation (FMD) and pulse wave velocity, may be useful strategies to predict cardiovascular risk and events. Hence a clearer understanding of the anatomy, physiology and biology of the vascular system has important predictive, diagnostic and therapeutic implications in cardiovascular health and disease.

Gaps in Knowledge

Even though the understanding of endothelial cells and VSMC biology has advanced in the past few decades, there is still a paucity of information on how these two cell types talk to each other and what the signalling pathways and mechanisms are that coordinate endothelial and VSMC function. While many factors have been identified as EDHF, it is still unclear exactly which factors are physiologically and pathologically important. Regarding VSMCs, the exact triggers and mechanisms that drive cells from one phenotype to another are still unclear, especially since most studies examining phenotypic switches are conducted in cultured cells that do not reflect the situation in vivo. In addition, the molecular and cellular processes that underlie vascular heterogeneity in different vascular beds remain unclear. Finally, the importance of PVAT in the regulation of vascular function and structure in health and disease still needs to be unravelled.

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Stem Cell Biology and the Cardiovascular System

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_4

4

Key Concepts

- Tissues and organs are maintained by endogenous stem cell populations that may become dysregulated during disease.
- There are two main types of therapeutically useful stem cells (a) adult (somatic) stem cells that can be isolated from tissue/organs and (b) pluripotent stem cells that are generated in the laboratory from early embryos (ESCs) or by genetic reprogramming of somatic cells (induced pluripotent stem cells – iPSCs).
- Somatic stem cells (SSCs) have limited capacity for ex vivo expansion and only make the tissue that they came from; pluripotent stem cells (PSC) can proliferate indefinitely under the correct conditions and have the capacity to make all cell types of the body.
- The exact identity and location of progenitor/stem cells in the cardiovascular system are still debated, although resident stem cell (SC) populations have been reported in both the vasculature and the myocardium.
- Cell therapies, derived from SSC or PSC, are of great interest for the treatment of heart attack, heart failure and peripheral ischaemia.

4.1 Introduction

During development of the mammalian embryo, a small number of pluripotent stem cells within the inner cell mass of the blastocyst proliferate and specialise to form all of the cells of the various organs and systems in the body. During later life tissues and organs are maintained by resident populations of tissue-specific somatic stem cells (SSCs). Over the last 50 years, we have sought to understand these different stem cell populations and how they might be exploited to repair damaged or diseased tissue.

Stem cells (SCs) are defined by their capacity to self-renew; this property ensures that when a stem cell undergoes cell division, at least one of the daughter cells will be a functionally identical copy of the parent cell. The other daughter cell normally loses the capacity to self-renew and becomes a more differentiated progenitor which will undergo extensive proliferation and generate the specialised cells required to maintain the tissue.

It is this capacity to self-renew over a prolonged period of time that ensures that stem cell populations last throughout the life of an organism and do not become exhausted. In the absence of self-renewing divisions, stem cells would all be fated to differentiate, and the pool would be rapidly depleted; to protect this essential function, many stem cells remain quiescent within tissues for long periods, dividing only rarely (Fig. 4.1).

A second basic property of stem cells is their lack of specialised function; however they have the capacity to differentiate into specialised cells, and the variety of cells produced defines their potency. For example, the cells of the inner cell mass of the blastocyst have the potential to generate all tissues of the body, but not extraembryonic tissues such as the placenta, and are therefore pluripotent; however, somatic stem cells are now widely accepted to generate only the tissue in which they reside and are therefore either multi- or unipotent depending on whether they can generate a few lineages or just one.

4.2 Pluripotent Stem Cells

In 1998 the group of James Thomson at the University of Wisconsin isolated cells from the inner cell mass of human blastocysts and, by doing so, established the first human embryonic stem cell (hESC) lines [1]. The blastocysts, at approximately 5 days post-fertilisation, were produced by in vitro fertilisation (IVF) and later donated specifically for research use. After the inner cell mass was removed, the cells were maintained in suitable culture conditions and were found to undergo prolonged, undifferentiated proliferation and to retain the capacity to differentiate into all cells of the three germ layers of the embryo – the endoderm, mesoderm and ectoderm [2]. The cells are therefore considered pluripotent and have great potential for studies of development, drug discovery and toxicology and for transplantation and tissue repair. If they are to be used clinically, hESCs are by definition allogeneic, that is, they can be isolated from one source (the embryo) and transplanted into a genetically different recipient. This would mean that if hESC-

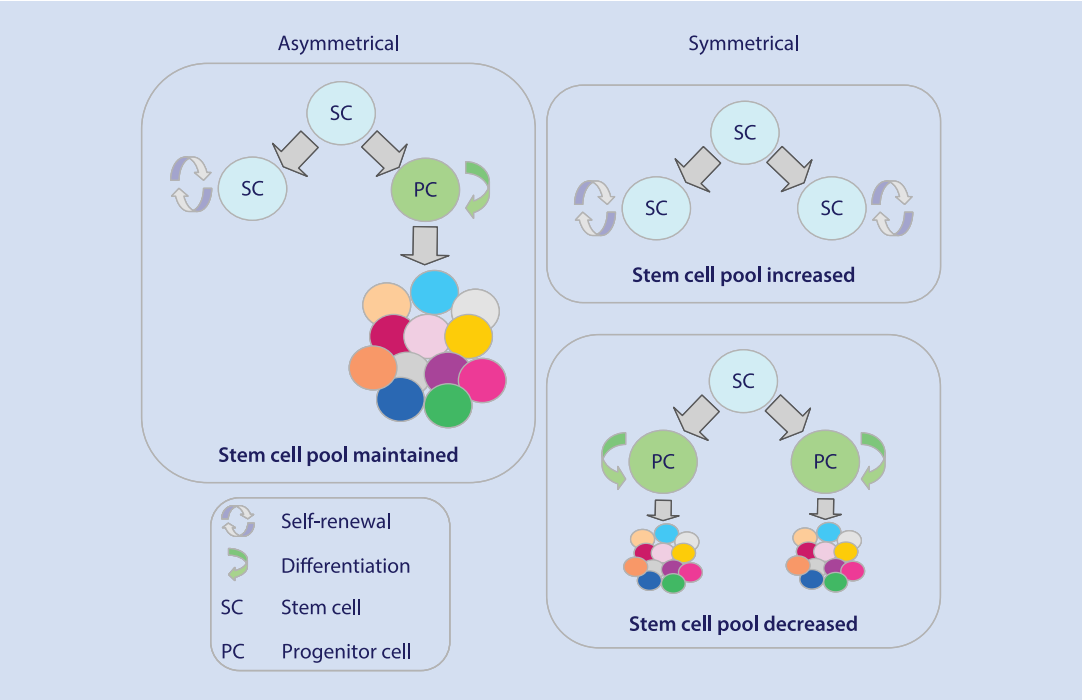

Fig. 4.1 Self-renewing stem cell division. Somatic stem cells normally undergo asymmetric, self-renewing division to maintain the number of stem cells present in an organ, thereby sustaining that organ throughout the life of an organism; however in times of stress, the system can respond by altering the kinetics of division to increase the number of progenitors, at the cost of SCs, and later to replace those lost SCs

derived cells and tissues are used as therapies, they would have to be tissue (HLA)-matched in the same way as solid organ transplants are currently and would be detected as 'foreign' and potentially rejected; therefore the recipient would need immune suppression to support retention of the graft [3]. The development of clinical therapies based on hESCs is also limited by ethical, moral and religious concerns around their embryonic origins. An individual cell line can be maintained and used by different laboratories around the world; indeed the most used hESC lines are still those first isolated by Jamie Thomson in 1998. In many countries, including the UK, new lines can only be derived under stringent licencing conditions by approved facilities; however, the fact that an embryo is destroyed in the derivation of the line poses significant concern to some. As such, varying levels of restriction on the use of hESC up to and including prohibition are present, which vary from country to country. European regulations are summarised at ► http://www.eurostemcell.org/stem-cell-regulations and those for the USA and Canada at ► https://stemcells.nih.gov/policy.htm and ► http://www.cihr-irsc.gc.ca/e/15255.html, respectively.

4.3 Somatic Stem Cells

Somatic stem cells (SSCs) have been found in all tissues studied and are also known as tissue-specific stem cells or, more commonly, adult stem cells (ASC). The use of the term adult stem cells can be confusing as this group of stem cells also includes those cells isolated from fetal tissues and the placenta. The role of SSCs is to maintain homeostasis and to repair the tissue or organ. The best characterised SSC population is the haematopoietic stem cell (HSC); these multipotent SCs have been extensively studied since the 1960s and have been used clinically, initially as bone marrow transplants and more recently in HSC transplantation, for more than 50 years. Investigation of the haematopoietic system has shown that there is a developmental hierarchy within tissues with the SC at the apex undergoing rare self-renewing divisions to produce progenitors that proliferate

extensively before differentiating to the mature functional cells. In the case of HSC, these comprise all of the blood cell types including B- and T-lymphocytes, red cells, platelets, monocytes and granulocytic lineages [4]. The human HSC can be identified by the presence of the cluster of differentiation CD34 antigen on the cell surface in the absence of CD38 and lineage-specific markers; however the search for SC populations in other tissues has been hampered by the lack of specific markers. There are some HSC proteins that are also commonly found on different SC populations including stem cell antigen-1 (Sca-1) which has been used to prospectively identify and isolate SCs from diverse tissues including the mammary gland, skeletal muscle, skin and cardiovascular tissues including vascular endothelium and the heart [5]. Similarly, CD133 and ABCG2 were initially identified on HSC but are now commonly used in other tissues including those of the cardiovascular system [6, 7]. Unlike hESCs, HSCs may be used in an autologous (extracted from and returned to the same individual) or allogeneic manner and are unencumbered by the ethical concerns that surround hESC.

4.4 Ex Vivo Culture of Stem Cells

Once isolated SSCs are compromised by their inability to undergo long-term self-renewing division ex vivo, this makes it difficult to maintain these populations in culture and limits the amount of expansion that can be achieved, which constrains the usage for SSCs for both experimental and clinical use. For example, despite decades of research, there are very few, if any, clinically proven methods for the expansion of HSCs ex vivo [8], and transplantation is still hampered by the need for one-to-one donation as a single collection is only sufficient for one transplant and the cells cannot be isolated, expanded and stored for future use by multiple recipients. In situ, SSCs live in a specific and highly regulated local microenvironment or niche, within which their behaviour remains tightly regulated by surrounding cells and various paracrine and autocrine factors along with the local concentration of nutrients including O_2 [9]. Hence it is perhaps then not surprising that once removed from this highly supportive environment, they do not self-renew in isolation in the laboratory. Significant effort is focussed on understanding the contribution of the niche to SSC behaviour and recapitulating these signals to allow the reconstruction of ex vivo niches that might be used to successfully expand SSCs whilst retaining their original capacity for self-renewal and differentiation [10].

In contrast to SSCs, when maintained carefully in the right conditions, hESCs seem to be capable of indefinite self-renewal in culture. As a result hESCs can undergo significant expansion to generate large numbers of cells. This occurs as hESCs fail to undergo replicative senescence that normally limits cells, including SSCs, to a maximum of about 40–50 division cycles. In contrast, hESCs have been maintained for many hundreds of doubling times whilst maintaining normal karyotype. Replicative ageing of a cell is normally measured by erosion of the telomeres at the end of each chromosome [11], but the length of telomeres can be maintained by the enzyme telomerase, which resets the replicative clock. Telomerase is highly expressed in hESCs allowing them to avoid senescence and to continue to proliferate in culture. Whilst self-renewal is recognised as the key to cellular longevity, the mechanisms governing this process remain poorly understood. Critical regulators have been identified including Oct4, SOX2 and Nanog. These transcription factors form a self-regulating core network that is modulated by external factors including bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF-2), Wnt and the transforming growth factor (TGFβ)/activin family of growth factors [12]. Human ESCs are an excellent model in which the process of self-renewal can be fully elucidated. By better understanding the molecular mechanisms involved, we may gain valuable insights which may be applied to SSCs in order to facilitate their maintenance and expansion ex vivo to extend their potential for therapeutic use.

4.5 Induced Pluripotent Stem Cells

As shown in Fig. 4.2, both SSCs and hESCs have certain qualities that limit their clinical utility. For example, expansion of SSCs remains limited. In contrast, hESCs may be expanded significantly but are not accepted by some individuals and groups because of their controversial origin. In 2006, Professor Shinya Yamanaka and his research group generated a new type of SC that has changed

	Somatic Stem Cells	Embryonic Stem Cells	Induced Pluripotent Stem Cells
Origin	Autologous or Allogeneic	Allogeneic*	Autologous or Allogeneic
Expansion	Poor	Potentially unlimited	Potentially unlimited
Differentiation	Limited lineages	All cells of the body	All cells of the body
Ethics	Few issues	Pro-life concerns	Few issues

Fig. 4.2 A comparison of the properties of human SSC, ESC and IPSC. Note: * refers to hESC generated from embryos, not derived by somatic cell nuclear transfer (SCNT)

the face of SC biology and the potential to develop cell-based therapies. Yamanaka built on the work of John Gurdon in the 1960s which showed that any cell had the potential to generate an entire organism by removing the nucleus of a fertilised frog's egg and replacing it with a nucleus from an adult frog's intestine. After the nuclear transfer, the modified egg developed into a normal frog showing that the DNA from the intestine cell still had all of the information to generate the entire new animal [13]. Yamanaka extended this by choosing a list of 24 genes that were known to play important role in self-renewal, proliferation and stem cell identity and using retroviruses to insert them into fibroblasts from the skin of a mouse. They initially found that the full set of 24 genes was able to induce pluripotency, and by using an all-minus-one approach, they were able to define the minimal set of four genes that were necessary and sufficient to reprogramme fibroblasts to pluripotency; these were *OCT3/4 (POU5F1)*, *KLF4*, *SOX2* and *c-MYC*. Colonies of reprogrammed cells that morphologically resembled ESC grew from the cultures of fibroblasts, and unlike the postmitotic starting cells, the induced cells were capable of self-renewal and expansion in hESC conditions. They were also capable of differentiation to all three germ layers of the embryo in vitro or in teratoma assays and when implanted into a mouse blastocyst contributed to all tissues to produce chimeric mice. The cells were dubbed induced pluripotent stem cells or iPSCs [14]. In 2007 the Yamanaka lab repeated this work using human fibroblasts with the same four factors [15], whilst James Thomson's group also reported the reprogramming of human cells using *OCT3/4* and *SOX2* in combination with *NANOG* and *LIN28* [16]. Since 2007 the generation of iPSC has swept through the field of stem cell biology and is now a routine and established technique in laboratories around the world.

The ability to generate pluripotent stem cells from adult tissue has addressed some of the major issues with the use of hESC. Human iPSC lines can now be produced from skin or blood cells that are taken from donors with informed consent rather than from embryos, thus avoiding the difficult ethical debate around the origin of hESCs. Further, as the cells are taken from an individual, the resulting cell line will be genetically identical to that person and could be used as an autologous source to differentiate and transplant cells of any lineage, potentially avoiding the need for tissue matching and antirejection drugs. The use of cells from individual donors also facilitates the generation of iPSC lines with specific characteristics, for example, the presence of a disease-causing genetic mutation or the expression of common human leukocyte antigen (HLA) haplotypes. This ability to choose the specific characteristics of an iPSC line has enabled research into diseases including those of the cardiovascular system [17], correction of defined genetic mutations [18] and also the generation of 'super donor' lines for transplantation [19].

Despite these significant advantages, some of the problems with hESCs are shared by iPSCs. Further, the method to induce iPSCs generates a range of other issues. For example, the reprogramming of somatic cells is achieved using viruses which integrate into the host genome and may cause insertional mutagenesis, disrupting the

expression of essential genes or switching on unwanted genes/oncogenes. c-Myc is itself a well-characterised oncogene. Technological advances and modern techniques now avoid the use of integrating viruses and to enable the oncogene c-Myc to be replaced with alternatives [20, 21]. Further, as both iPSCs and hESCs are pluripotent, they share the capacity for spontaneous multilineage differentiation that must be suppressed by the culture conditions in order to maintain their capacity for self-renewal and proliferation. Once implanted into a host, both cell types have the capacity to form teratomas, tumours that may be benign or malignant and are comprised of mixed differentiated tissue. In fact, the capacity to form teratomas containing cells from all three germ layers is used as a test of pluripotency when a new hESC or iPSC line is made. Loss of this capacity is seen when cells begin to differentiate and lose pluripotency. Therefore, there remains a risk that if any undifferentiated iPSC or hESC persisted in a culture of differentiated cells that are to be used therapeutically, those residual pluripotent cells could form a teratoma in the recipient. Finally, it is now clear that many of the cell types derived from hESCs/iPSCs have a fetal or even embryonic phenotype, rather than that of the mature, adult cell type. For example, hESC-/iPSC-derived cardiomyocytes are small and have a profile which more closely resembles fetal cardiomyocytes in terms of their electrophysiological properties and metabolic profile. Such differences raise the possibility that hESC-/iPSC-derived tissues may not integrate sufficiently if transplanted into adult tissue and therefore fail to restore organ function. As a result, efforts are now being made to further mature the hESC/iSPC cells in vitro, either by maintaining them for longer time periods, growing them in 3D cultures or by stimulating additional maturation with growth factors and small molecules to mimic development [17, 22–26].

For these reasons, the translation of hESC and iPSC into clinical therapies has been slow. The world's first hESC-based trial started in 2009 and used oligodendrocyte precursor differentiated from hESCs as a therapy for spinal cord injury. The trial was initiated by an American company called Geron, treating the first patient in 2010, and was intended as a safety study to test for side-effects including teratoma formation and immune response to the transplanted cells. By 2011, nine patients had been treated with no reported serious adverse effects; however the company chose to re-prioritise their efforts and stopped the trial [27]. There are currently a number of trials that are using hESC-derived retinal pigmented epithelial (RPE) cells to loss of sight due to macular degeneration, and in 2014 the first iPSC trial started in Japan, also using RPE cells [28]. These trials are all currently Phase 1 with safety rather than efficacy as the primary endpoint.

4.6 Stem Cells and the Cardiovascular System

4.6.1 SSC in the Heart

The identity and role of SCs in the CV system are currently the subject of much debate. The heart demonstrates minimal regenerative capacity following injury, resulting in severe morbidity and mortality after ischemic damage due to myocardial infarction. This lack of intrinsic regenerative capacity was thought to reflect the lack of a resident stem cell population. In 2005 the group of Ken Chien reported that they had detected a progenitor population, termed cardioblasts, in the post-natal myocardium of rodents and humans that could be identified by the expression of Islet-1 (Isl-1) [29]. Subsequently other groups reported different populations of cardiac precursors that may be activated after injury [5, 30]. More recent studies however bring the idea that the heart harbours a resident stem cell population into question. Lineage-tracing studies in rodents fail to demonstrate that c-kit-positive cells (which in bone marrow marks progenitor cells) exist and/or function as an endogenous cardiomyocyte-producing stem cell [31]. Importantly, Li et al. demonstrate that whilst these cells are able to differentiate into cardiomyocytes during early heart development, they lack this ability in the adult heart after injury, i.e. myocardial infarction [32]. The issue is by no means without controversy, with others questioning the lineage-tracing techniques [33, 34] and providing alternate data to support the existence of resident, endogenous, cardiac stem cells [33, 35]. However, the inability to robustly demonstrate intrinsic regeneration or to demonstrate engraftment/transdifferentiation makes the existence of an endogenous stem cell less likely. To contribute to the uncertainty, a number of seminal papers have been questioned

and withdrawn from the literature due to concerns regarding the data [36, 37]. The American Heart Association, in 2017, presented a consensus statement to address such issues and identify important areas which required clarification and further research [35]. Despite this, there is no doubt that in murine models and in specific human studies, the administration of bone marrow/progenitor cells contributes to repair and regeneration of the myocardium, but the exact mechanism(s) remains elusive and is discussed in further detail below (see ▶ Sect. 4.7).

4.6.2 Generation of Cardiomyocytes from PSC

Many studies have drawn on the knowledge of embryonic development to help identify and isolate cardiac progenitors; similarly, developmental biology provides the blueprint for those who aim to generate cardiac progenitors and functional cardiomyocytes from hESC or iPSC [23, 38–40]. The use of pluripotent SCs is particularly attractive for heart therapy as large areas of muscle are often affected by ischemic injury and the intrinsic progenitor population to self-renew is so poor. Banks of iPSCs and hESCs could potentially be used to generate large numbers of cardiomyocytes that could be stored, ready for use following acute myocardial infarction, or iPSCs could be used to make autologous treatments in more chronic disease states, such as in heart failure. As the heart is a complex structured tissue comprised of spatially distinct areas with different function, e.g. ventricular, atrial and pacemaker cells supplied by an extensive vascular network, much work is now focussed on engineering multicellular grafts including patches and the re-cellularisation of whole organs, to ensure that the appropriate cells are maintained in the correct temporal and spatial distribution to attain maximal function [41]. In addition to growing hESC-/iPSC-derived cardiomyocytes as potential therapeutics, this source of cells offers a new and exciting way to study disease and to identify novel pharmacological strategies to treat them. As iPSCs are generated from a known individual, cells may be taken from patients with specific genetic conditions. The tissue to be reprogrammed thus carries the relevant mutation, thereby enabling iPSC lines to be derived and differentiated into the affected cell type. For example, there are now iPSC lines that can be matured into CM in vitro, and their responses mimic those seen in the disease. Multiomic studies (proteomic, genomic, metabolomic, etc.) can reveal the genetic, metabolic and functional differences between the affected cells and a normal counterpart in order to identify new drug targets. Further novel drugs can be tested for efficacy and toxicity in this human assay system [42, 43]. Indeed it is hoped that iPSC-derived cells could replace animal use for some toxicology studies and lead to the development of personalised therapy with safer drugs at lower cost [44, 45]. These studies are somewhat confounded by the heterogeneity of subtypes (atrial-, ventricular- and nodal-like) that arise in typical cultures and also by the immature, embryonic/fetal characteristics of PSC-derived cardiomyocytes [46]. However, recent studies using different strategies to mature the cells in vitro have started to demonstrate promising results. For example, the combination of 3D culture and mechanical stimulation, causing contraction of immature PSC-derived cardiomyocytes, has been demonstrated to cause metabolic maturation and generating cells that more closely resemble adult cardiomyocytes with greater potential to model diseases associated with mitochondrial abnormalities or cardiac metabolism [25]. Furthermore, embryonic stem cell-derived cardiomyocytes and progenitors, whilst demonstrating similar efficacy to bone marrow-derived cells, demonstrate greater efficiency and effects upon improving cardiac contractility in rodent myocardial infarction studies, suggesting they may be a more desirable cell type for improving cardiac function post injury [47]. More recently, studies in non-human primates have generated promising results for the use of PSC-derived cardiomyocytes for heart repair [48], and in France Philippe Menasché has initiated a first-in-human trial wherein clinical-grade hESC-derived cardiovascular progenitor cells have been implanted to assess their safety in patients with severe ischemic left ventricular dysfunction [49].

4.6.3 Role of SC in Vascular and Other Tissues

As for cardiac SCs, there is also considerable deliberation about the identity and location of the endothelial stem or progenitor cells. Initially it

was thought that the main reservoir of endothelial progenitors (EPCs) was the bone marrow, with some circulating in the blood, and that EPCs were mobilised in response to hypoxia. However, the currently accepted paradigm is that these circulating EPCs are less important than originally thought and that EPCs residing in the vascular wall, and/or perivascular cells, are the predominant source of vascular maintenance and repair [50–56]. Progenitors have been identified in all three layers of the blood vessel, the intima, media and adventitia, and are capable of contributing to endothelium and smooth muscle. There are also populations that resemble multipotent mesenchymal stromal cells (MSC) that may directly contribute to, or provide support for, revascularisation. Such cell types include perivascular cells (pericytes), which have been extensively reviewed in [57]. EPCs and pericytes from various sources are being tested for their capacity to induce revascularisation in in vivo models and also in a number of early-phase clinical trials. One important issue surrounding the use of such cells is the realisation that the number of circulating EPCs (bone marrow derived) not only correlates strongly with the conventional risk factors for coronary artery disease, including chronic kidney disease and diabetes mellitus, but their diminution also portends additional risk including cardiovascular death, beyond that identified by the Framingham profile [25]. As a result, identification of methods to enhance both cell number and function ex vivo is being pursed at the preclinical stage and in clinical trials in order to ascertain whether this will overcome the effects of cardiac risk factors upon cell number and function. One such method involves the restoration of eNOS (endothelial nitric oxide synthase) activity. Nitric oxide (NO), the product of eNOS activity, is a vasodilating factor with several beneficial actions on the vasculature. NO prevents platelet aggregation, leukocyte extravasation and smooth muscle proliferation whilst contributing to endothelial repair and prolonged endothelial cell survival. Results from experiments with eNOS knockout mice (eNOS−/−) have established that the NO pathway plays a critical role in EPC-mediated endothelial maintenance and neovascularisation. Importantly, restoration of eNOS activity and protein expression achieved by transfection of EPCs restores EPC functionality and improves neovascularisation [58]. The impact of eNOS overexpression is being assessed in clinical trials following myocardial infarction and in patients with primary pulmonary hypertension [59]. Table 4.1 provides a list of clinical trials involving EPCs and MSCs.

Pluripotent stem cells are also capable of differentiation into endothelial cells using methods that are suitable for clinical use [60, 61], as well as into pericytes [62, 63]. This would provide a therapeutic alternative for the treatment of peripheral limb ischemia, acute myocardial infarction and stroke, in order to restore vasculature to infarcted territories. Unfortunately, this approach may be hampered by limited cell engraftment; hence alternate means to improve cell retention such as encapsulation or development of biocompatible matrices are being developed [64, 65].

4.7 Early Translation of Stem Cell Therapies

In the late 1990s, the prevailing hypothesis regarding SSCs was that the ability to differentiate might not be restricted to the tissue that they arose from. Preclinical studies demonstrated that when SSCs from one tissue were transplanted into damaged tissue of another organ, the SSCs would adopt the identity of the new host tissue and contribute to its restoration. This was termed 'stem cell plasticity', and various studies demonstrated transplanted bone marrow (BM); a mixed tissue rich in HSCs, EPCs and other populations including mesenchymal stromal cells (MSCs) was particularly effective contributing to repair of the liver, skeletal muscle, brain and heart, amongst other tissues. More stringent isolation of different SSC populations and other technical advances in the 2000s lead to alternative explanations for the phenomena observed, and the hypothesis of plasticity fell out of favour [66]. However, there was sufficient evidence to support a number of clinical trials using BM in order to repair the myocardium following infarction or in individuals with impaired cardiac function and from a variety of aetiologies who developed heart failure. A number of small trials were undertaken, mostly designed to assess safety; however a number also assessed efficacy primarily by assessing cardiac function

Table 4.1 Summary of ongoing clinical trials employing EPCs/MSCs for ischemic heart disease. Details of all trials can be found using the Clinical Trials ID at ► www.ClinicalTrials.gov

Study	*n*	Cell source	Condition	Design	Delivery	Clinical Trials ID
Acute Myocardial Infarction						
RELIEF	135	Autologous BM	Acute MI	Phase 3	IC	NCT01652209
CIRCULATE	105	Allogeneic	Acute MI	Phase 2/3	IC	NCT03404063
HUC-HEART	79	Autologous/ Allogeneic	Pre-CABG	Phase 1/2	IM	NCT02323477
ENACT-AMI	100	Autologous EPCs and EPCs transfected with human eNOS	Acute MI	Phase 2	IC	NCT00936819
BAMI	350	Autologous BM	Acute MI	Phase 3	IC	NCT01569178
Chronic Ischemic Heart Disease						
Jerome et al.	NYD	Autologous BM	Chronic Ischemic CM (wean from LVAD)	Phase 1	IM	NCT02460770
MESAMI2	90	Autologous BM	Chronic Ischemic CM	Phase 2	IM	NCT02462330
Dai et al.	45	Autologous BM	Chronic Ischemic CM	Phase 1/2	Collagen Scaffold	NCT02635464
CONCERT-HF	144	Autologous	Ischemic CM	Phase 2	IM	NCT02501811
Tresukosol	24	Autologous	Ischemic CM	Phase 2	IC	NCT00384514
CardiAMP	250	Autologous BM	Ischemic CM post MI	Phase 3	IC	NCT02438306
Non-Ischemic Cardiomyopathy						
Hu et al.	30	Umbilical Cord	Idiopathic Dilated CM	Phase 1	IM	NCT01219452
Olson et al.	45	Allogeneic	Anthracycline-Mediated CM	Phase 1	IV	NCT02408432
Fernandez-Avilez	70	Autologous	Idiopathic dilated CM	Phase 1/2	IM	NCT01957826

BM bone marrow, *IC* intracoronary, *IV* intravenous, *IM* intramyocardial, *CABG* coronary artery bypass grafting, *NYD* not yet determined, *CM* cardiomyopathy

as measured by left ventricular ejection fraction [67–69]. As a result, interpretation of the data remains confounded by the small numbers of participant in most trials, variations in protocol including the use of different cell populations from BM, time of delivery, number of cells used and route of administration amongst others. Also, few of the trials have been randomised and/or placebo controlled. It is now accepted that this improvement is not due to the transdifferentiation of BM cells into cardiomyocytes but is likely due to indirect or environmental effects including the production of growth factors that (a) stimulate revascularisation and (b) modulate

the local immune/inflammatory response to injury, leading to improved survival and recovery of the endogenous tissue [70, 71]. These initial studies demonstrate that the approach, although undefined, is feasible and at this early stage has not been associated with significant safety concerns; however larger and longer-term studies are required to definitely address the safety concerns.

Despite this, meta-analyses have been performed that show a small increase in cardiac function in those patients who had received autologous BM cells after myocardial infarction or heart failure [72–74]. Importantly, the functional improvements noted in the REPAIR-AMI trial persisted out to 5 years following BM infusion and were associated with a significant reduction in death, nonfatal MI or revascularisation ($p = 0.03$) when compared to placebo [75]. Meta-analysis of such trials has provided conflicting results with one showing that BMC transplantation reduces the incidence of death, recurrent myocardial infarction and stent thrombosis in patients with ischemic heart disease [74], whilst a subsequent meta-analysis by Gyongyosi et al. found no effect of cell-based therapies upon clinical events or ventricular function [76]. Possible explanations include different patient populations, different cell preparations and protocols utilised (different timing of cell administration as an example). Future, larger multicentre, double-blinded placebo-controlled trials with the statistical power to detect small effects are needed to definitively decide the benefit of these therapies (see ◘ Table 4.1).

4.8 Direct Reprogramming (or Transdifferentiation) Strategies

Basic scientific research into the developmental origins of CV tissues and the differentiation of cardiomyocytes and vascular lineages from diverse sources, combined with the seminal findings of Yamanaka et al., have altered the future for CV therapies [77]. Rather than treating disease with pharmacological therapies, arterial grafting, stents and mechanical assist devices, it is hoped that blood vessels and cardiac muscle may be repaired and replaced with stem cell-derived tissues in order to affect a cure. The methods discussed above offer the realistic hope of cell therapies; perhaps the most exciting prospect though combines some of these approaches. In 1987 it was reported that insertion of the muscle-specific gene MyoD into fibroblasts could cause their transdifferentiation into muscle cells. It has subsequently been shown that the enforced expression of master transcription factors (TFs), or combinations of TFs, can reprogramme many adult cells to a different fate [78–81]. This method of direct reprogramming has been used to convert fibroblasts into cardiomyocytes [80, 82, 83] and more recently into endothelial cells [84]. Induced cardiomyocytes (iCM) have been generated from human cells in vitro, and excitingly, studies on murine cells have generated iCM both in vitro and in vivo, introducing the possibility that fibroblasts residing in the heart could be reprogrammed in situ to iCM by the addition of messenger or microRNA or even by small-molecule drugs that mimic the action of the TFs [85–89].

We are undoubted entering a new era of medicine, where repairing tissues and curing diseases using cell therapies will become a viable alternative to managing symptoms with drugs. There are many strategies being developed using stem cells from various sources and their derivatives; the cardiovascular system has been used as one of the first test beds, and recent developments promise great progress to treat these endemic and life-limiting diseases.

Conclusions and Clinical Perspective

- The field of cellular-based therapies is rapidly moving forward with a myriad of stem cell populations under investigation for their regenerative properties
- Early clinical studies show some potential benefit using bone marrow-derived cells as a regenerative therapy to treat post-myocardial infarction-induced cardiac dysfunction
- Large, multicentre, placebo-controlled trials are lacking to definitely demonstrate efficacy of this approach
- Better characterisation of cell types along with a more detailed understanding of the molecular mechanism(s) for repair will aid in targeting future therapeutic strategies using the optimal cell population, for the particular disease of interest

Gaps in Knowledge

- Current and future placebo-controlled, blinded and large-scale trials should help elucidate the true potential of bone marrow cell therapy in ischemic heart disease and heart failure.
- More work is required to generate specific cardiomyocyte subtypes (atrial-, ventricular- and nodal-like) with fully adult phenotype. These more representative cells may improve the capacity of PSC-derived cells in drug testing and toxicology assays and in disease modelling.
- Additional laboratory studies and preclinical testing are needed to determine whether PSC-derived cardiomyocytes, delivered alone or in combination with vascular elements in tissue-engineered grafts, can be used safely and effectively in clinical trials.
- Additional work is required to characterise endogenous cell-based populations in different organs and identify the mechanism of repair using cell-based strategies, with a focus upon single-cell analysis.

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The Lymphatic System

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_5

Key Concepts

- The lymphatic system drains the ultrafiltrate continuously generated in the microvascular bed, thus maintaining tissue and plasma volume homeostasis.
- It includes lymph nodes and lymphatic vessels. Vessels centripetally acquire a muscular layer, which spontaneously and rhythmically contract to drive lymph forward, in addition to extrinsic propulsion produced by tissue movements.
- The elementary pumping units, separated by valves, are finely regulated. Their impairment results in defective fluid drainage from tissues, i.e. oedema. Classically, any oedema caused by impaired lymph flow is called *lymphedema*.
- Lymphedema is primary, when due to pathologic development of the lymphatic vessels, or secondary, when the lymphatic vessels are damaged by external agents or conditions.
- Beyond classic insults, such as lymphadenectomy for cancer treatment, recent evidence points to an impairment of lymphatic function in inflammatory conditions, as well as in cardiovascular risk factors and diseases.

5.1 Introduction

A key functional need has driven the development of a cardiovascular system: the delivery of metabolic substrates to the cells within large organisms. Overall, this is achieved by the heart, generating the pumping force to maintain the flow of blood through multiple vascular conduits and, ultimately, by the microcirculation (the capillary bed and the microvessels immediately upstream and downstream), where the exchange of this substrates takes place. At this level, the interplay between plasma, the highly organised microvascular wall, the perivascular interstitial space, and the primary force sustaining the circulation, i.e. blood pressure, generates filtration. This phenomenon is governed by the Starling-Landis principle, whereby the rate is proportional to the hydraulic pressure gradient between plasma and interstitium minus the corresponding oncotic gradient (P_p-P_i and $\pi_p - \pi_i$, favouring and opposing filtration, respectively). The traditional, *arteriovenocentric* interpretation of the above principle has assumed a 90% reabsorption of extravasated fluid into the venous end of the microvascular bed, where hydraulic forces drop (Fig. 5.1a); however, modern evidence, based on direct measurements of P_i and π_i, demonstrates that the net sum of forces along the entire length of well-perfused microvessels consistently favours filtration over absorption in most organs and conditions (with few exceptions represented by intestinal mucosa, renal peritubular and lymph node capillaries and the early phases of vasoconstriction, e.g. after haemorrhage (Fig. 5.1b) [1]). As a consequence, fluid balance must be achieved through other mechanisms that provide constant drainage of the ultrafiltrate and filtered plasma protein: this is accomplished by the lymphatic system. By transporting lymph back to the central venous blood, lymphatic vessels represent the frequently neglected support for the arteriovenous circulation: they complete the extravascular circulation of fluid and maintain tissue and, to some extent, plasma volume homeostasis. In fact, a substantial volume of lymph is generated and transported every day (4–8 L), even in the face of a vanishing small filtration fraction in most tissues (i.e. the fraction of plasma water that escapes during one transit through the capillary, ~0.1 to 0.3%). Thus, the lymphatic system has a vital, rather than ancillary role in cardiovascular physiology [1–3].

In addition to preservation of fluid balance, which will represent the primary focus of this chapter, the lymphatic system serves two other major functions: nutrition, being responsible for most fat absorption in the gut, and immune surveillance, by providing an entry site for soluble antigens and tissue-resident immune cells, as well as a low flow system, with checkpoints represented by lymph nodes, suitable for the generation of an immune response. As briefly outlined below, both of these apparently unrelated functions are by no means trivial to cardiovascular physiology and disease.

5.2 Functional Anatomy

In light of these fundamental roles in whole-organism homeostasis, the ubiquitous distribution of the lymphatic system comes as no surprise: lymphatic vessels or lymphatic-like structures are

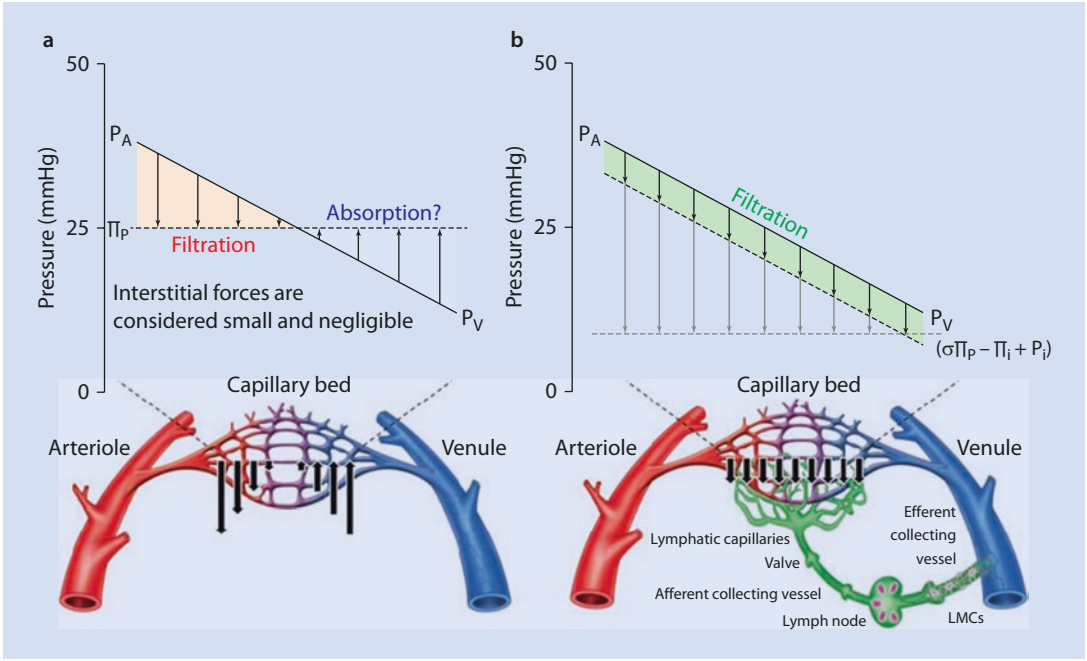

Fig. 5.1 Starling-Landis equilibrium and the role of the lymphatic system in the circulation. (*Panel* **a**) traditional arteriovenocentric model assuming interstitial forces negligible and, therefore, reabsorption of fluids at the venous end of the microvascular bed. (*Panel* **b**) reappraisal of the above by including direct measurement of hydrostatic (P) and oncotic (Π) pressures; calculated (grey arrows) and actual filtration values (black arrows), as vascular glycocalyx modulates Π_i across the capillary bed. Fluid filtered as a constantly positive sum of forces is drained by the lymphatic system to central venous blood via lymphatic capillaries, afferent collecting vessels, lymph nodes, efferent collecting vessels and ducts. P_A, P_V and P_i, arterial, venular and interstitial hydrostatic pressures, respectively; Π_p and Π_i, plasma and interstitial oncotic pressures, respectively; σ = reflection coefficient; LMC, lymphatic muscular cells. (Adapted from: Levick and Michel [1]; Potente [25, 26])

found in almost every vascularised tissue, including the brain and the eye.

Lymphatic vessels are unidirectionally and hierarchically organised into series of blind-ended lymphatic capillaries, pre-collecting vessels and collecting vessels that, via chains of lymph nodes, ultimately drain into final ducts (i.e. the thoracic duct and the right lymphatic duct) where the lymph is returned to the blood circulation via the subclavian veins (Fig. 5.1b).

5.2.1 Initial Lymphatic Vessels

Lymphatic capillaries (or initial lymphatic vessels; diameter 20–70 μm) are composed of a single layer of oak-leaf-shaped lymphatic endothelial cells (LECs) with an incomplete basement membrane and no pericytes. In contrast to blood vascular endothelial cells, LECs are joined together by VE-cadherin in "button-like" patterns. They overlap and are attached to the extracellular matrix by anchoring filaments: this distinct architecture generates a primary system of clefts and valves suitable for the controlled entry of solutes, macromolecules and cells. In particular, changes in intraluminal and external pressures (generated by skeletal muscle contraction, by excess interstitial fluid or by movement of skin or structures around the vessels) pull the endothelial cells apart and open the junctions between them.

5.2.2 Collecting Vessels

The initial lymphatic network drains into larger collecting vessels. These vessels are less permeable owing to a continuous basement membrane and zipper-like interendothelial junctions. As with veins, they also have bi-leaflet valves to direct lymph centrally and prevent backflow and are covered by sparse peri-endothelial lymphatic

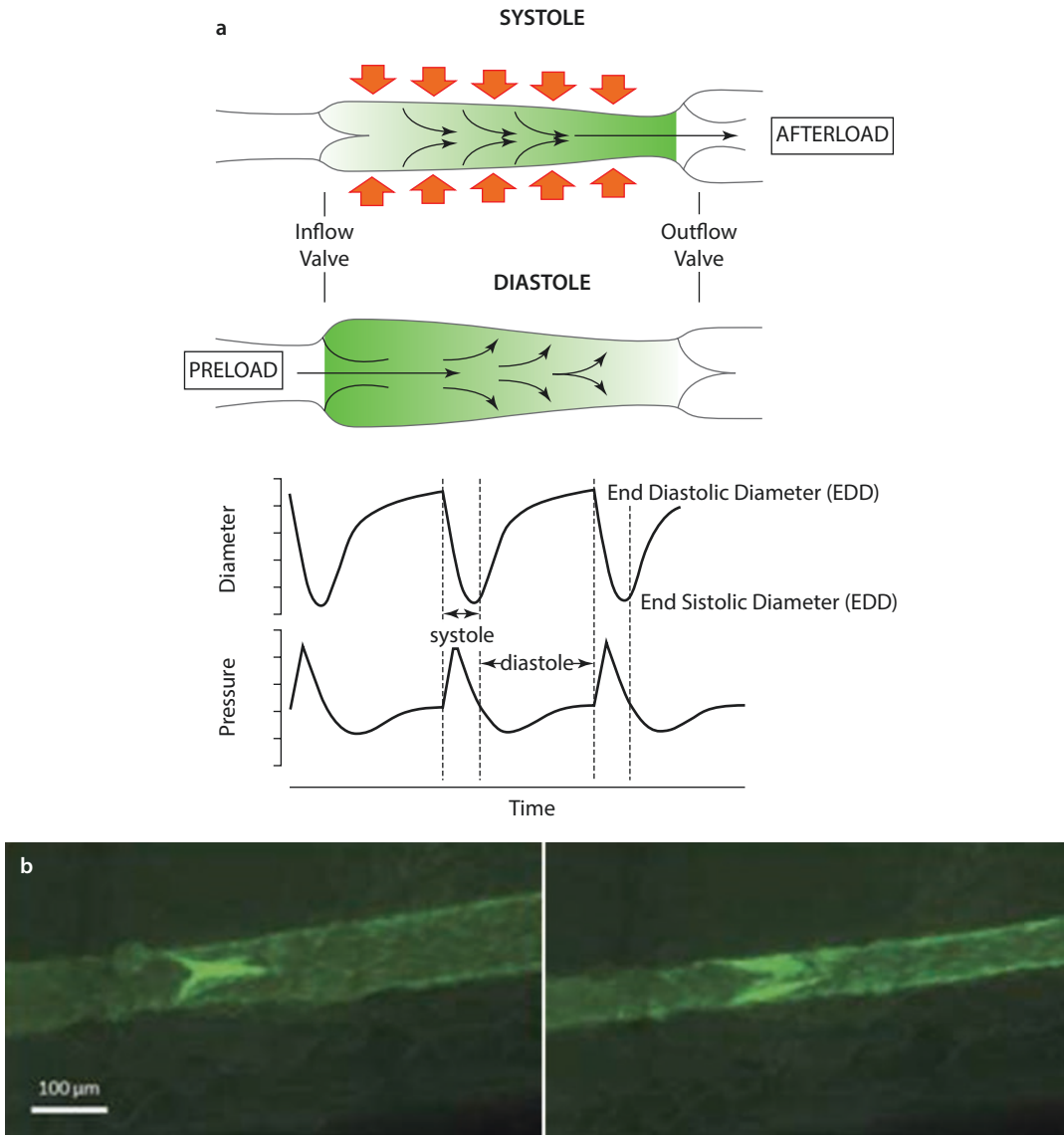

Fig. 5.2 Lymphangion contraction cycle. (*Panel* **a**) schema of the systolic-diastolic phases of lymphangion contraction, with representative pressure and diameter traces. (*Panel* **b**) opening and closing of a lymphatic valve corresponding to the different phases of the contraction cycle (end-diastole for the downstream lymphangion, left; mid-systole of the upstream lymphangion, right). (Adapted with permission from Chong [27])

muscle cells (LMC) (Fig. 5.1). The development of this coverage depends on the vessel hierarchy, regions and species: in human skin, for example, there are nonmuscular pre-collectors (diameter 70–150 µm) that connect the subpapillary initial lymphatics with collecting vessels (150–500 µm) deeper in the dermis; in their centripetal progression, the initial circular layer expands to reach the longitudinally, circularly and obliquely oriented three layers present in larger vessels and ducts. LMCs are nonstriated, but they share biochemical and functional characteristics with both vascular and cardiac muscle: importantly, they spontaneously and rhythmically contract to drive lymph forward (*vide infra*). The segment of a collecting lymphatic vessel between two intraluminal valves, or lymphangion, is the "elementary pumping" [4] and key functional unit of the lymphatic system (Fig. 5.2).

5.2.3 Lymph Nodes

Lymph nodes are the immunosurveillance checkpoints where interstitial fluid and associated antigens, carried by lymphatic vessels, antigen-presenting cells, and lymphocytes, converge. LECs within lymph nodes not only regulate the cell trafficking necessary to mount an immune response within the parenchyma, through expression of various chemokines and adhesion molecules, but they can also act as antigen-presenting cells involved in the induction of peripheral tolerance [5].

Even if a detailed discussion of the immunological events that transpire in a lymph node is beyond the scope of this chapter, the importance of an intact lymphatic network to provide a route into (afferent collecting vessels) and out of (efferent collecting vessels) these checkpoints appears obvious. The high density of the lymphatic vasculature in "barrier" organs, such as skin, respiratory and gastrointestinal tracts, continuously exposed to foreign antigens and microorganisms, supports this concept. Accordingly, conditions where the lymphatic system is locally impaired, i.e. in lymphedema (see later), are prone to recurrent infections due to a deficient primary immune response [3].

5.2.4 Large Conduits

Eventually, collecting vessels emerging from chains of lymph nodes converge into two large lymph vessels: the thoracic duct and the right lymphatic duct. The former carries about three quarters of the total efferent lymph and, importantly, receives the chylomicron-rich lymph formed in the intestinal lacteals and collected into a saccular dilatation at its lower, abdominal end. Both ducts drain into the venous circulation via lymphovenous valves located at the junction of the subclavian and jugular veins. Despite some additional, minor peripheral lymphovenous communications, this anatomy finds antecedents in early embryogenesis, when lymphatics sprout from the cardinal vein and later join with a non-venous-derived superficial plexus [6].

5.3 Modulation of Lymphatic Flow

5.3.1 Extrinsic and Intrinsic Determinants

Progression of lymph through the lymphatic system against an opposing hydrostatic pressure gradient requires extrinsic and intrinsic propulsion forces.

Extrinsic forces result from intermittent compression of the lymphatics by tissue movements, like skeletal muscle contractions, intestinal peristalsis, movement of the skin, or pulsation of adjacent arteries: in the lower limbs, the extrinsic component due to skeletal muscle contraction accounts for approximately one-third of lymph transport. Deep breathing/exercise is also thought to stimulate lymph movement through the thoracic duct, but there is a lack of robust evidence. A forward direction of flow is guaranteed by the one-way valves, as in the venous system.

Intrinsic propulsion is generated by the active and rhythmic pumping of the LMC layer that line the collecting vessel network. Mechanisms and modulators of this distinctive activity have been extensively reviewed elsewhere [4, 7] and are summarised below.

5.3.2 Intrinsic Lymphatic Pumping and Its Regulation

5.3.2.1 Molecular Bases of Contraction

Lymphatic muscle is characterised by a pacemaker-generated action potential (AP), generated by the summation of spontaneous transient depolarisations. These are regulated by several types of ion channels similar to those that control pacemaking in the sino-atrial node [4]. Contraction depends on the opening of voltage-operated calcium channels (predominantly L-type Ca^{2+} channels [8, 9]), leading to Ca^{2+} influx. In a calmodulin-dependent manner, Ca^{2+} regulates the balance of myosin light chain kinase (MLCK)/myosin light chain phosphatase (MLCP) activity controlling myosin light chain phosphorylation, which triggers MLC shortening. In analogy to vascular smooth muscle cells, tone and spontaneous contraction of LMCs are inhibited by NO, produced by LECs via

endothelial/inducible nitric oxide synthase (eNOS and iNOS, respectively) in response to various stimuli, such as shear-stress, or by other cells, such as inflammatory cells [4, 10]. When translocated to LMCs, NO activates soluble guanylyl cyclase and downstream cyclic GMP-dependent protein kinase, which enables dephosphorylation of myosin light chain and relaxation [7].

5.3.2.2 Lymphangion Mechanics

The synchronisation of the above cellular mechanisms within the functional unit of a lymphangion bears striking similarities with the mechanics of the cardiac pump: its activity can be viewed as a sequence of systolic (contractile) and diastolic (filling) phases; valves open and close secondary to the generated pressure gradients. Accordingly, end-systolic and end-diastolic pressures and diameters (ESD and EDD), as well as ejection fraction (EF), contraction frequency (FREQ) and their product, pump output, can be defined (Fig. 5.2). As in the heart, lymph pumping is subject to multiple regulatory mechanisms.

- *Preload*. In a relationship similar to the Frank-Starling law that is observed in the heart, lymphatic segments can increase their output in response to an increase in more distal input, with a peak that approximates 5 cmH_2O of pressure. This is achieved by modulating FREQ, which is highly sensitive to distension, and diameters.
- *Afterload*. Similarly, lymphatics adaptively pump against increased loads (due to gravity, increased central venous pressures and/or partial outflow obstructions), up to a failing threshold that approximates 11 cmH_2O above preload pressure: in response to a rise in outflow pressure, lymphangions respond with an increase in contractility (positive inotropy) (Fig. 5.2).

  Very importantly, the *in series* architecture of lymphangions within the lymphatic vasculature guarantees a much higher propulsion force than individual lymphangions would generate: the above pressure adaptation potential (and limits) must therefore be considered as additive, allowing limb lymphatics to pump, in aggregate, 40–50 mmHg against a resistance. To this end, contraction synchrony within and across lymphangions is a necessary condition. Such electrical coupling presumably involves connexins in both LECs and LMCs, but their expression and function have yet to be fully elucidated.
- *Neurohormonal modulation*. Large lymphatic vessels are innervated by sympathetic adrenergic fibres: through α-adrenergic stimulation, they increase both tones, FREQ and EF. This is particularly useful during conditions like haemorrhage, when an increase in lymphatic pumping function facilitates the transfer of interstitial fluid into the depleted circulation. Also muscarinic stimulation would otherwise elicit a positive chronotropic effect on LMCs, but this is counteracted by the inhibitory effect of NO release by LECs; similarly, an increase in NO endothelial production is induced by σ_1 receptor activation. Serotonin effects on lymphatic contraction appear to be species- and receptor expression- specific. Substance *P*, a chemical mediator of inflammation, promotes tone generation and increased FREQ; at variance, the anti-inflammatory vasoactive intestinal peptide (VIP) inhibits lymphatic pumping and hyperpolarises the LMC membrane potential.

In summary, lymphatic vessels actively generate propulsive forces thanks to a highly specialised structural and functional organisation [4]. Many of the detailed molecular and mechanical properties of LMCs are still elusive, but their future modulation is a promising avenue for therapeutic interventions that might improve lymphatic function locally or systemically.

5.4 Oedema and Lymphedema

As discussed, a primary function in cardiovascular physiology is the preservation of fluid balance. An impairment in such balance at the tissue level results in excess interstitial fluid, i.e. hydrostatic oedema. Oedema can be localised to specific organs or cavities (such as the peritoneal or pleural spaces), but the most common site is within the peripheral subcutaneous space, which offers a relatively high compliance to distension compared to other tissues, like muscle. Nevertheless, even minimal amounts of oedema in an organ can remarkably impact local homeostasis and

function [11], and, in the long term, this process can culminate in proliferation of parenchymal and stromal elements, excessive deposition of extracellular matrix substances and adipose tissue.

Despite being a key sign of disease, clinical appreciation of oedema tends to fall short in two ways: (1) the intrinsic limits of the physical examination to identify clinically important oedema before a considerable volume (litres!) of extracellular fluid has accumulated and (2) the failure, in most cases, to appreciate the central role of lymphatic drainage itself as a determinant or a contributor to oedema.

Strictly speaking, oedema develops when the microvascular (capillary and venular) filtration rate exceeds lymph drainage (◘ Fig. 5.1) [3]. Considering the plasticity of the lymphatic system in response to increased need (discussed above), but also the multiple factors that can potentially impair its function (*v.i.*), the lymphatic component of the equilibrium clearly cannot be ignored.

In other words, all chronic oedema represents an absolute or relative incapacity of lymphatic function to cope with microvascular filtration, either as a consequence of a microvascular filtration rate that is high (due to increased hydraulic pressures, as in heart failure or venous thrombosis; to a plasma colloid osmotic pressure that is reduced, as in nephrotic syndrome, malnutrition or liver disease; or to increased endothelial wall permeability, as in inflammation), or of impaired lymph flow (in conditions traditionally called "lymphedema"), or of the two combined (which is probably more common than generally appreciated, as discussed below). As suggested "it may be better to always consider the presence of chronic edema as synonymous with the presence of lymphedema" [3], intended as relative lymph drainage failure. If so, we might better appreciate the potential, and in some cases the need, for early intervention with strategies other than solely diuretics.

5.4.1 Traditional Classification and Pathological Mechanisms

Lymphedema, even in the traditional use of the term, accounts for an enormous global disease burden. Precise estimates of its prevalence range widely according to age, gender and aetiology: depending on the latter and on clinical presentation, lymphedema is classified as primary or secondary.

5.4.1.1 Primary Lymphedema

Primary lymphedema is generally due to a congenital or inherited condition associated with pathologic development of the structure and/or function of lymphatic vessels. Primary lymphedema often presents in childhood, sometimes as part of a syndrome, but later presentations in adulthood also occur: age of onset is still a key determinant for clinical classification [12].

In the recent years, causal genetic mutations in human lymphedema have been identified; this has been paralleled by a better understanding of the role of specific proteins in lymphatic biology. For example, vascular endothelial growth factor receptor (VEGFR3), mutated in some cases of a congenital-onset form primary lymphedema (Milroy disease) and accounting for about 25% of primary human lymphedemas, was identified as fundamental in lymphatic vasculature expansion. Similarly, mutations in FOXC2, a transcription factor downstream of VEGFR3 signalling, have been associated with pubertal-onset lymphedema and associated with both lymphatic and venous valve failure, emphasising the embryological link between the two vascular systems.

Research in lymphatic biology has now revealed >50 genes involved in the specification, expansion and maturation of lymphatic vessels: prospero homeobox 1 (PROX1) transcription factor, integral membrane glycoprotein podoplanin (PDPN) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) are the most commonly used for molecular imaging of LEC, just to mention a few. A more detailed molecular and clinical discussion is beyond the scope of this chapter, and the reader is referred to other reviews [3, 5]. However, it is important to highlight that gene mutations, when identified, are now part of the current classification of primary lymphedema.

5.4.1.2 Secondary Lymphedema

Secondary lymphedemas arises from damage or physical obstruction of the lymphatic system from a specific external stimulus or agent: they represent the majority of cases.

The most common cause worldwide is filariasis, due to infection by the nematode *Wuchereria bancrofti*, which specifically targets and dwells in lymphatic vessels and LNs for years, resulting in extensive fibrosis and obstruction. An estimated 95 million people in the endemic areas of Africa, South America and Southeast Asia have filarial lymphedema.

In the developed world, the most common, although not exclusive, cause for secondary lymphedema is malignancy or its treatment: its prevalence is estimated in >1% of all cancer survivors [13], but in breast cancer, up to 15–20% are at clinical risk of lymphedema. This concept of risk (*v.i.*) relates to the evidence that development of lymphedema is not dependent only on the obstruction to lymph drainage following removal of axillary lymph nodes: a systemic "predisposition" appears to discriminate affected individuals from the 80% to 85% that will remain free of swelling.

Accordingly, the general perception of lymphatic "damage" in secondary forms as a solely anatomical issue is probably simplistic. In fact, a growing body of literature has now identified potential roles for lymphatics also in other diseases: while a physical obstruction or disruption of the lymphatic vasculature is not as obvious as in oncology, lymphatic function or, in most cases, dysfunction can be an important mechanistic determinant in a broad array of diseases [14]. In rheumatoid arthritis (RA), for example, after an initial expansion phase that parallels the distinctive inflammatory flare, a subsequent chronic phase is characterised by structural and cellular changes in the draining lymph nodes, an overall decline in lymphatic vascular contraction and, ultimately, clearance. This goes hand-in-hand with altered immune cell trafficking. Targeting lymphatics has therefore been suggested as a potential therapeutic intervention in RA [10].

Evidence suggesting a similar link between an underlying lymphatic dysfunction and "traditional" cardiovascular (CV) diseases has just started to accumulate. While it is generally accepted that in some clinical conditions, such as obesity or heart failure, the longstanding haemodynamic lymphatic overload eventually results in a gradual functional deterioration, thereby reducing overall transport capacity, the potential direct impact of CV risk factors and diseases on the lymphatic system is the focus of the next section.

5.5 The Lymphatic System in CV Risk Factors and Disease

5.5.1 Ageing

With increasing life expectancy in developed countries, there is an increasing proportion of older adults within the population: remarkably, this group accounts for nearly three quarters of the overall cardiovascular disease burden (see also ▶ Chap. 28).

Ageing is an ineluctable CV risk factor associated with multiple biological changes, and lymphatic function is no exception to this rule. In isolated rat thoracic duct segments, tone and contraction amplitude were both reduced by ageing. Similarly, age-related negative chronotropy was observed. Consequently, partial or complete failure to provide the adequate transport of lymph through the duct may occur [15]. Additionally, inhibition of LEC NO release with N-Nitro-L-arginine methyl ester hydrochloride (L-NAME) induced a higher increase of total pumping output in aged compared to young animals, as if in ageing the physiological inhibitory action of NO was constant, rather than dependent upon the phasic contractions. Further mechanistic insights into the topic are described in *Gashev, LRB 2013*, but they are mostly limited to evidence derived from animal studies.

Human data are scant. Functionally, ageing was shown to be inversely associated with lymphatic pumping, and this might relate to a general reduction in the innervation observed in collecting vessels and ducts from elderly subjects when compared to younger ones. Anatomically, an old report from Russian investigators described the destruction of the elastic elements and atrophy of muscle cells in the thoracic duct wall in the elderly, resulting in the development of "duct sclerosis". How this might link with the concept of "lymphaticosclerosis", observed in the limb collecting vessels of severe lymphedema, associated with collagen deposition and the morphological shift of LMC layer changes toward a hypocontractile phenotype, is currently unknown but could share similarities with that of "premature vascular ageing" in CV disease.

5.5.2 Fat Metabolism and Obesity

The well-established role for lacteals (intestinal lymphatics) in the absorption of fat is paralleled by the evidence of excess fat deposition (and not just fluid accumulation) as a definite clinical characteristic of lymphedema. This suggests a role for the lymphatic system in peripheral tissue lipid homeostasis beyond the gut itself.

A mouse model with leaky lymphatic vessels ($Prox1^{+/-}$) was shown to develop adult-onset obesity without changes in caloric intake or energy expenditure: the predominant accumulation of fat around the lymphatics suggested that the leakage of lymph (and the associated lipoproteins) is the key mechanism promoting adipocyte hypertrophy. The concept of permeability of collecting lymphatics (barrier function) and its modulation has been extensively reviewed [4]: intriguingly, hypercholesterolemic $ApoE^{-/-}$ mice also revealed lymphatic structural changes associated with gross leakage.

An additional role for lymphatics in relation to fat homeostasis has also been suggested in reverse cholesterol transport (RCT), the primary route for HDL efflux from atherosclerotic plaques [5]: such a phenomenon implicates active transcellular mechanisms of lipid transport into the vessels, in contrast to the traditional mechanism of passive paracellular passage of fluid, macromolecules, and immune cells.

In humans, obesity is a strong risk factor for the development of postsurgical breast cancer-related lymphedema, and lymphatic drainage of macromolecules is reduced in obese compared to lean subjects. According to available evidence, the relationship between obesity and lymphatic vessels dysfunction is likely to be bidirectional, but the exact mechanisms remain to be elucidated. Similarly, it is still unknown whether inducing peri-plaque lymphangiogenesis could enhance RCT and reverse atherosclerosis.

5.5.3 Diabetes

Evidence for defective lymphatic function in diabetes is currently limited to preclinical studies.

Compared to wild type, diabetic (*db/db*) mice revealed an impaired barrier function of collecting vessels. The dramatic loss of lymphatic vascular integrity in diabetes could be rescued by increasing NO substrate availability or by limiting the degradation of its downstream effector cGMP. Notably, NO had an opposite effect on the permeability of healthy, nondiabetic mice [4].

More recently, the direct effects of insulin resistance induced by prolonged hyperglycaemia and hyperinsulinemia in LMCs were assessed: by induction of inflammatory signalling, alterations in cellular bioenergetics and increased phosphorylation of myosin light chain, the contractile function of LMCs was ultimately impaired [16]. In support of this evidence, a high-fructose-fed, nonobese rat model of metabolic syndrome showed a significant reduction in lymphatic pumping, mostly driven by a reduction in contraction frequency [4].

5.5.4 Hypertension

The distinct involvement of the lymphatic system in hypertension was first described in association with new concepts related to tissue sodium homeostasis [17]. Titze and his collaborators demonstrated the existence of hypertonic skin salt accumulation in rodents in response to a high-salt diet. This phenomenon implicates multiple regulators: glycosaminoglicans in the interstitium, as the binding site for the excess sodium; resident immune cells, secreting VEGFc in response to activation of a tonicity-responsive transcription factor (TonEBP); and ultimately the lymphatic network, undergoing dynamic changes in response to VEGF-C secretion via VEGFR3. This interplay offered a buffering mechanism, whereby the high sodium load was counteracted by an expansion of the lymphatics to provide local drainage. Blockade of any of the above determinants resulted in excess skin sodium accumulation and a salt-sensitive hypertensive phenotype. Further investigations by independent investigators showed that a high salt diet also modulates the mechanical activity of murine collecting vessels [18]. Moreover, enhanced cardiac lymphangiogenesis induced by VEGF-C reduced myocardial fibrosis and macrophage infiltration, decreased blood pressure and preserved myocardial function in a salt-sensitive rat model of hypertension; VEGF-C blockade produced opposite effects.

While demonstration of an increased skin sodium content in association with ageing and

uncontrolled or secondary hypertension has also been provided by 23Na-MR imaging in humans, a similar demonstration of parallel changes in the cutaneous lymphatic system is currently lacking.

Recently, a significant increase in renal lymphatic vessel density in both salt-sensitive and salt-independent (L-NAME) rodent models of hypertension was observed; further enhancing organ-specific lymphatic expansion in the kidney reduced renal inflammation and completely prevented the development of hypertension in both models [19].

5.5.5 Myocardial Infarction

Myocardial oedema has been known for more than two decades to be a negative determinant of systolic and diastolic function, and description of heart lymphatics dates back to the late 1920s. However, only in the recent past has lymphatic plasticity in response to injury been appraised; in particular, despite the endogenous cardiac lymphangiogenic response occurring after myocardial infarction, the remodelling and dysfunction of collecting ducts have been shown to contribute to chronic tissue oedema and inflammation, thus aggravating cardiac fibrosis and dysfunction in a rodent model. Therapeutic lymphangiogenesis showed promising potential by improving all the above [20].

5.6 Lymphatics and Inflammation

The role of lymphatics in inflammation deserves a dedicated discussion. In its broadest sense, inflammation is a complex cascade of biological phenomena in response to a wide range of stimuli, ranging from pathogens to autoantigens to damaged endogenous cells. As mentioned, an important function of the lymphatic system is host defence, and this is accomplished not only by serving as a delivery system for soluble antigens and cells to lymph nodes but also by an active interplay with most of the inflammatory response effectors. In this regard, we already alluded to the direct involvement of LECS in adaptive immunity, by antigen-presentation or by expression of ligands to guide other immune cells [6]; additional supporting evidence is offered by the profound predisposition to skin bacterial infections observed in lymphedema [3]. Studies of transcriptional profiling of both murine models and human lymphedema confirmed the key involvement of pathways involved in inflammation [21–23].

In general, lymphangiogenic factors are produced by macrophages and granulocytes in inflamed tissue. Teleologically, inflammation-associated lymphangiogenesis (IAL) facilitates the resolution of tissue oedema and promotes immune cell mobilisation; similarly, modulation of function of the already- existing vessels is crucial, but often neglected, as recently reviewed [24]. Correspondingly, disturbances in antigen and immune cell trafficking compromise tissue immunosurveillance, predisposing to uncontrolled infections, such as cellulitis/erysipelas in lymphedema, or chronic inflammation, as for the aforementioned case of rheumatoid arthritis.

Low-level inflammation is now a well-established contributor to obesity, diabetes, metabolic syndrome, hypertension and cardiovascular disease in general (please see also ▶ Chaps. 23 and 24): this further reinforces the relevance of any lymphatic system impairments observed in these conditions and the need for additional research.

5.7 Treatment

Regardless of aetiology and the pathophysiologic aspects previously discussed, current therapeutic approaches for "lymphatic diseases" treat the consequences of lymphatic dysfunction, i.e. lymphedema, which generally requires lifelong care and attention.

Management of lymphedema depends on clinical severity, according to the International Society of Lymphology (ISL) Classification [11]. The table below summarises the main stages of lymphedema; remarkably, they include a preclinical stage 0, which may be referred to as an "at risk" state in those individuals known to have an impaired lymphatic system, e.g. following lymph node removal or radiotherapy to regional lymph nodes. However, it may also be anticipated or assumed in people with any of the conditions mentioned above (or their combination), known to overload/impair the lymphatic system, e.g. repeated episodes of cellulitis, obesity, hypertension, poor mobility, venous insufficiency and chronic heart, renal or liver failure (◘ Table 5.1).

Table 5.1 Clinical stages of lymphedema

Stage	Characteristics
0 (latent or preclinical)	Absence of swelling; potential subtle subjective symptoms
1	Visible, soft and pitting oedema that may subside with elevation. Patients may report swelling, tightness, feeling of fullness, bursting and discomfort and be aware of jewellery and clothes feeling tighter
2	Increased swelling that does not resolve on elevation, as the build-up of fat cells and the matrix of extravascular spaces over time replace the fluid element of the swelling. Tissues become increasingly firm with pitting only possible with strong, sustained pressure. Skin becomes progressively dry, scaly, cracked, thickened with hyperkeratosis, then possibly patches of lymph blisters, papillomatosis and lymphorrhea
3	Swelling is severe at this stage, with extensive skin changes as above. There is distortion of limb shape due to uneven tissue fibrosis, with bulges and deepened skin folds

5.7.1 Management of Stage 0 and 1 Lymphedema

Managing lymphedema is fairly straightforward and effective in the early stages with a high potential for patients to quickly become self-managing under professional monitoring. Key elements of treatment are the:

1. Initiation of a skin care programme to maintain the skin as an effective barrier to infection.
2. Initiation of regular activity/exercise: this positively impacts lymphatic function and helps to resolve obesity-associated perilymphatic inflammation, beyond the extrinsic contribution to propulsion.
3. Early application of well-fitting compression garments.
4. Potential benefit from a simplified form of manual lymphatic drainage.

5.7.2 Management of Stage 2 and 3 Lymphedema

In later stages a more intensive approach is needed, but the aim is always to achieve and maintain a stable state following maximum improvement with a high level of self-management. The likelihood of a good result decreases as the duration of untreated lymphedema and its severity increase. However, treatment is still essential to prevent complications and disease exacerbation and to maximise the patient's quality of life. Intensive treatment is referred to as Decongestive Lymphatic Therapy (DLT) or Complex Decongestive Therapy (CDT). DLT involves:

1. Skin care programme to heal any wounds, stop leakage, eradicate infection and improve skin integrity.
2. Exercise programme to enhance the effectiveness of compression therapy and encourage lymph movement.
3. Lymphedema Compression Bandaging (LCB). There are key differences in compression bandaging for lymphedema: it involves the use of short stretch bandages to provide a low resting pressure and high exercise pressure; bandaging includes the digits and continues beyond the extent of the swelling; bandaging is reapplied two to five times weekly, depending on the treatment philosophy, to maintain the level of compression.
4. Manual lymphatic drainage (MLD), complemented by simple lymphatic drainage self-administered by the patient or a caregiver, has traditionally been a component of DLT and there is some evidence for the effectiveness of this approach as a four-component treatment. However, evidence in relation to MLD treatment alone is lacking.

Some modern reconstructive microsurgical procedures (performed at the time of lymph node excision during oncological surgery, or shortly

thereafter) are showing promise in enhancing residual lymphatic function, in particular lymph node transfer and lymphaticovenous anastomosis. Liposuction may also be helpful in removing excess adipose tissue in selected patients with stage 2–3 lymphedema which has achieved maximum benefit from conservative measures [11]. However it still requires lifelong use of strong compression 24 h a day.

Importantly, diuretics are of limited use in the treatment of lymphedema. The initial fluid loss induced by diuretics comes from the intravascular space, with a consequent re-equilibration due to interstitial fluid mobilisation to maintain plasma volume. With lymphatic obstruction/dysfunction, this mobilisation is limited, and diuretics can therefore promote volume depletion and/or electrolyte imbalance; therefore, their use is discouraged [11]. Whether newer diuretic-like agents such as SGLT 2 inhibitors, with additional pleiotropic effects on muscular cells and well-documented beneficial impact on other cardiovascular conditions, could simultaneously improve lymphatic function and ameliorate lymphedema is an intriguing hypothesis worth additional investigation.

5.7.3 Future Therapeutics

It appears evident from the above that most of current therapies for lymphedema are palliative in nature, in that they replace an impaired lymphatic system for the drainage of lymph, without cure. Even more advanced physical and/or surgical approaches, reviewed elsewhere [11], make no exception to this limitation.

Only recently, a better understanding of lymphatic biology has begun to provide hope that a specific pharmacology is possible. Advances in the investigation of molecular pathogenesis of both primary and secondary forms of lymphedema have been paralleled by considerable progress in the techniques for anatomical and functional imaging of the lymphatic network, although many limitations still exist (recently reviewed in *Sevick-Muraca* et al, *JCI 2014*). Despite the obvious need for further progresses, they offer tools for quantification of lymphatic function and, therefore, for the design of robust clinical studies.

After promising results of therapeutic lymphangiogenesis in a variety of animal models, a phase 1 study of VEGF-C gene therapy for lymphedema following mastectomy is currently ongoing. Whether other lymphatic-modulating drugs, potentially targeting the mechanical properties of LMCs, could provide any benefit in conditions where functional, rather than anatomical, recovery is needed is a question requiring further research. If so, however, the close relationship between the lymphatic system and cardiovascular disease would suggest tremendous opportunities for their application.

Conclusion and Clinical Perspectives

- The lymphatic system plays a crucial, rather than ancillary, role in cardiovascular homeostasis. Highly sophisticated anatomy and physiology are central to this function.
- By frequently ignoring the lymphatic active and continuous contribution to the drainage of excess fluid from tissues, our appreciation and full understanding of oedema in pathophysiology might be inadequate. Fluid removal and other functions served by the lymphatic system, such as lipid trafficking and immune/inflammatory modulation, are key determinants in the development of cardiovascular disease.
- Similarly, cardiovascular disease and risk factors can predispose to overt lymphedema when additional insults damage the lymphatic system.
- Current treatments for lymphatic disease are almost entirely limited to mechanical palliation.
- Further improvements in the understanding of lymphatic biology will help unravel the specific associations with cardiovascular conditions and give hope for the development of a tailored pharmacology.

Gaps in Knowledge

- More research is required to understand the mechanisms regulating lymphatic function and the interplay between lymphatics and common cardiovascular disease.
- Specific interventions mechanistically targeting lymphatic dysfunction are currently lacking.

- Further advances in anatomical and, more importantly, functional imaging should stimulate the design of clinical studies with appropriate comparison across research groups.
- Most importantly, the almost neglected importance of the lymphatic system should be reappraised by the whole medical and scientific community.

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Imaging the Vascular Wall: From Microscope to Virtual Reality

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_6

Key Concepts

- Fluorescent probes cover the entire visible light spectrum but must be chosen very carefully if several are to be combined in a single sample.
- The vascular wall of small blood vessels can be efficiently visualised in 3D using confocal or multiphoton microscopy.
- Fluorescence-based microscopy can be used with either live or fixed blood vessels.
- Modern games technology (software and virtual reality headsets) can now be used to examine complex vascular structures.

6.1 Introduction

Contraction and relaxation of the vascular wall represents the coordinated activity of at least four cell types (endothelial, smooth muscle, adventitial fibroblasts, adipocytes). The balance of activity of each cell, and the influence of autonomic nerve fibres, determines the degree of vascular tone. Therefore, measuring an agonist-induced contraction (in vitro) tells us nothing about the activity of individual cells or their relative agonist sensitivity. Work by Graham and Keatinge in 1972 [1] demonstrated that the inner layer of smooth muscle in sheep carotid artery was more sensitive to a range of agonists than was the outer layer of smooth muscle. The agonists included noradrenaline, 5HT, histamine and angiotensin II. The conclusion from the work was that inner layers of smooth muscle needed to be more sensitive to noradrenaline as they are further away from the source of the neurotransmitter since the autonomic nerve fibres are largely confined to the adventitia/medial border. This observation raises interesting questions about the distribution of hormone receptors within the vascular wall and also about possible heterogeneity of cells even within the one type. Smooth muscle cell heterogeneity has been suggested since the early 1970s based on the contractile, proliferative (non-contractile) and secretory (collagen production) role of these cells in the vascular wall. It has been further suggested that contractile and non-contractile phenotypes are two extremes of a broad spectrum of possible types of smooth muscles [2].

Answers to the questions raised above can perhaps be addressed using imaging techniques that visualise the blood vessel wall during contraction or examine the distribution of various receptors within and among the cells of the vascular wall. The development of the first generation of isometric wire myograph enabled the mounting of semi-transparent segments of rat mesenteric artery. Using a phase contrast objective (i.e. no staining), it was possible to visualise, in live vessels, wall thickness, smooth muscle cell outlines and, in some cells, nuclei and mitochondria [3]. Unfortunately, phase contrast and interference contrast methods for imaging the vascular wall do not provide the stark contrast, between objects, that is required for automated or semiautomated image analysis software packages. Fluorescent dyes and ligands do provide such a dramatic contrast and desired signal to noise ratio.

This chapter will provide an overview of the use of fluorescent probes for studying both the structure and function of the vascular wall. Aspects of image analysis, image correction and 3D image processing will also be touched on. A brief mention is made of nonfluorescent 'classical' histological stains.

6.2 Nuclear Stains

Fluorescent nuclear stains are a starting point for visualising the vascular wall. Stains are available across the entire visible spectrum from ultraviolet (UV) to far red and every colour of the spectrum in between. Personal experience has shown that it is difficult to predict how efficient a particular stain will be for a given blood vessel. As an example, SYTO 62 (a red fluorescent nuclear stain) works well in some vessels but can be faint and unstable in others. In contrast, bisbenzimide (Hoechst 33342) is a UV nuclear stain which appears to work well, with excellent stability, in most blood vessels. The message here is that it requires a little trial and error to find the right nuclear stain for any particular laser wavelength and blood vessel type.

The cells of the vascular wall (excluding the perivascular fat) can be considered as belonging to three distinct groups or tunics. These are endothelium, smooth muscle and adventitia. Fortunately, each cell group has a distinctively shaped nucleus and orientation relative to the

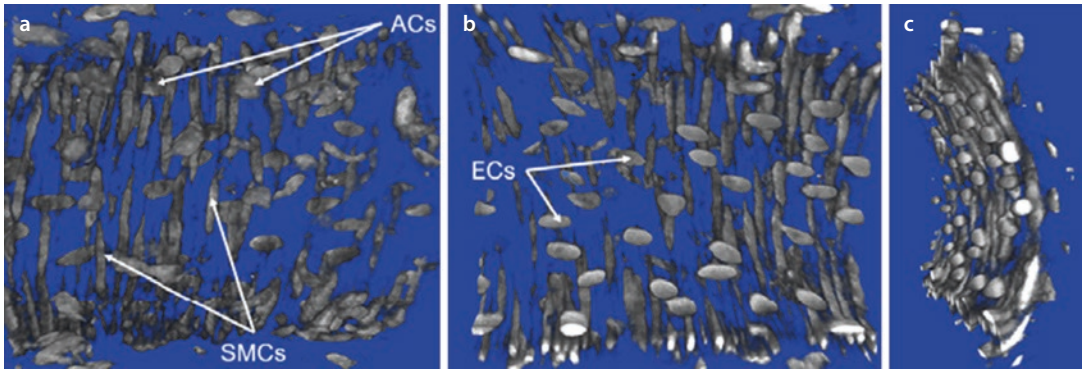

Fig. 6.1 Nuclear staining (SYTO 62, 1–10 μg/ml) of a mouse mesenteric artery. **a** Viewed from the outer surface, adventitia cells (ACs) have irregular circular nuclei, whilst the smooth muscle cell (SMC) nuclei are aligned perpendicular to flow and can have several layers. **b** Endothelial cell nuclei (ECs) have an oval-like shape and align with the flow. **c** The data set is shown from the side looking along the axis of flow

axis of blood flow. Therefore, nuclear stains are perfect for examining cell number, arrangement, orientation and (depending on choice of stain) viability within the vascular wall [4]. The adventitial cells have a roughly circular but often irregularly shaped nucleus. The cells will be a mix of fibroblasts, macrophages and vascular stem cells. Some cell nuclei appear to be aligned with the position of the autonomic nerves and may be associated with non-myelinating Schwann cells. All adventitia cell nuclei are located on the outer region of the external elastic lamina. The tunica media contains the smooth muscle cells and is sandwiched between the internal and external elastic lamina. The smooth muscle cells have a very distinctive elongated shape. In most blood vessels, the smooth muscle cells are aligned perpendicular (i.e. 90°) to the axis of blood flow. However, it is not uncommon for individual cells to lie at a slight angle (±10°). A characteristic diagonal pattern of organisation can often be observed (Fig. 6.1, [5]). The orientation of the smooth muscle is commonly referred to as circular. However, coronary, pulmonary and hepatic vessels from rodents can show marked deviation from purely 'circular'. Endothelial cells are aligned with the axis of flow and are positioned on the inner surface of the internal elastic lamina. The nuclei are oblong, thin and easily identifiable by their shape, arrangement and orientation.

Nuclear staining can be used to identify several normal and pathological features of vascular structure. Often, the functional response of a small artery may indicate a dysfunctional endothelium. Nuclear staining, and viewing, of an unfixed arterial segment can be achieved within an hour in the lab. A reduction in coverage or removal of endothelial cells markedly affects vascular function and can easily be assessed using wide-field fluorescence or confocal microscopy.

Small arteries taken from hypertensive patients or animal models can exhibit a thickened and/or rearranged media resulting from tissue remodelling [6]. Conversely, vessels taken downstream from a critical limb ischaemic block exhibit reduced medial cell number [7]. A striking characteristic of mesenteric arteries taken from hypertensive rats is the proliferation of adventitial cells compared with normotensive controls [8].

Following identification of suitable dyes, nuclear stains provide a quick and easy means of quantifying and examining certain aspects of vascular structure.

6.3 Intracellular and Extracellular Staining

In some cases, it may be necessary to examine the size and shape of individual smooth muscle cells within the wall of a live artery, either slide-mounted or myograph-mounted. There are two basic approaches: (a) stain the individual cells and (b) fill the extracellular space with a non-cell-permeant fluorescent dye (Fig. 6.2). Both approaches can be combined with nuclear staining. The disadvantage of both methods is the large amount of fluorescence that needs to be visualised.

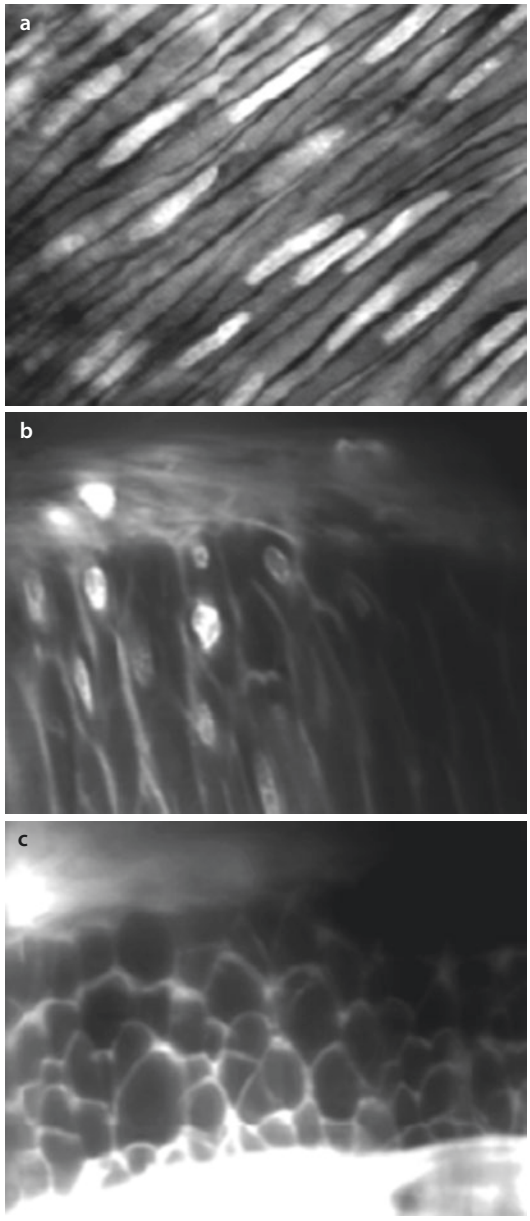

Fig. 6.2 Intracellular and extracellular staining of unfixed rat mesenteric tunica media. **a** BCECF AM (5 μM) combined with dihydroethidium nuclear stain (DHE, 1 μM). BCECF is a green fluorescent pH indicator but can be used as an effective cell-permeant intracellular stain. **b** Extracellular space is filled with non-cell-permeant 5,6-carboxyfluoresceine (5,6 CF, 1 mg/ml) combined with DHE. The cells can be seen curving round the edge of the vessel. **c** 5,6 CF staining viewed at the edge of the vessel and thus showing the cell profiles in cross section. The wall thickness can be easily measured as can the 3–4 layers of smooth muscle cells. (Images taken from C. Daly PhD Thesis, University of Glasgow 2000)

Therefore stained vessels can only be effectively viewed by using confocal (or multiphoton) laser scanning microscopy to reduce the out-of-focus glare that blurs the focal plane of interest. If stains are carefully chosen and applied, the medial smooth muscle cells can be viewed through the tunica adventitia (Fig. 6.2a, b) or in profile at the mid-point of the lumen (Fig. 6.2c) [9].

6.4 Fluorescent Ligands

Antibodies for studying vascular structure can be of tremendous value. However, they tend to require access to intracellular targets (even when targeting GPCRs), and therefore vessels need to be fixed. Fluorescent drugs offer the possibility of examining receptor distribution, and receptor recycling, in live cells and tissues [10, 11]. One consideration is the influence that a fluorescent side chain may have on the affinity of the host ligand. Another is the relatively low fluorescent yield that can be expected when fluorescent drugs are used at, or around, their Kd values (i.e. low concentrations) to maintain selectivity. This will generally necessitate increasing the gain on any image detector (photomultiplier or camera) which will increase photon noise in the image. A variety of image analysis techniques can be used to average or smooth the image. This can usually be done within the microscope capture software or post-capture using ImageJ or similar analysis software. Combining fluorescent drugs can be extremely informative and has indicated that smooth muscle cells are heterogeneous in their expression of specific receptor types [12]. A particularly interesting observation is the high concentration of beta-adrenoceptors in smooth muscle cells at the vascular branch points in mouse mesenteric artery [12]. The possibility of individual smooth muscle cells having a preference for the expression of one particular adrenergic receptor (i.e. α- or β-) requires further study. It should be noted however that fluorescent ligands indicate binding sites and not necessarily functionally coupled receptors. Therefore, pharmacological studies using nonfluorescent, highly selective, ligands should be used in combination with their fluorescent counterparts (Fig. 6.3).

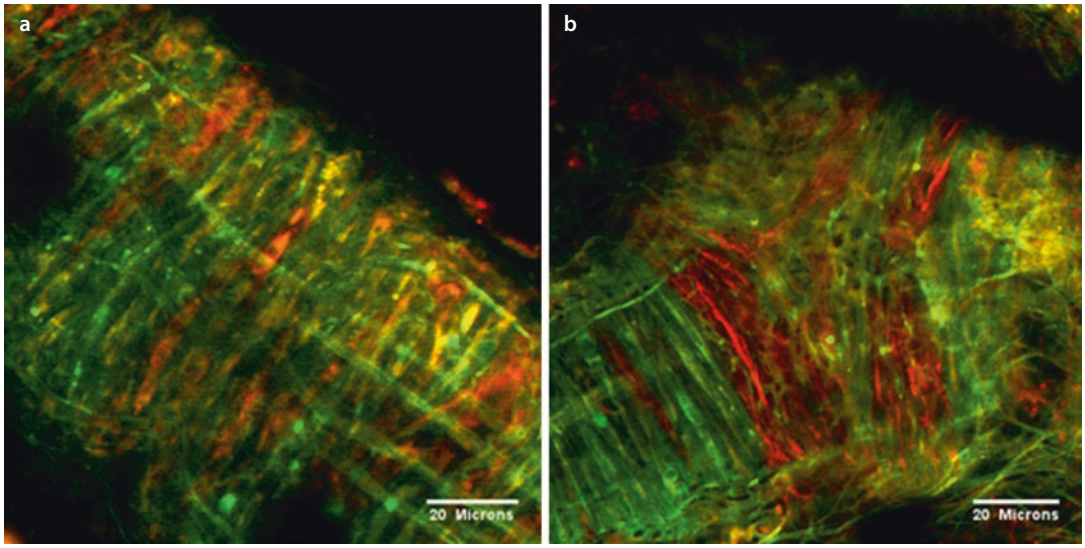

Fig. 6.3 Mouse mesenteric artery incubated with fluorescent prazosin (QAPB, 0.1 μM) and fluorescent CGP12177 (Fluo CGP, 0.1 μM). QAPB is a green fluorescent ligand for α_1-adrenoceptors. Fluo CGP is a red fluorescent ligand for β_1- and β_2-adrenoceptors. **a** Combined fluo-ligand staining in the tunica media shows some cells that are exclusively α_1- (green), some which express high amounts of β-adrenoceptors (red) and some which express both α_1- and β-adrenoceptors (yellow). **b** High expression of β-adrenoceptors can be seen at the arterial branch point

6.5 3D Reconstruction and Visualisation

Confocal microscopes offer the opportunity to collect perfectly aligned serial optical sections along the z-axis (depth) of a tissue. The method used to collect the data has a marked effect on the quality of any 3D reconstruction. Important factors to consider are axial step size (usually 0.1–1 μm), frame averaging (affects the time taken to complete the scan), depth attenuation (see below), immersion medium (air, oil or water) and numerical aperture (NA) of the objective (1–1.4). These are just a few of the factors that can influence the accuracy of 3D reconstruction. Depth attenuation is a significant problem when imaging thick-walled vessels such as the aorta or carotid arteries. In addition to signal fading deep within the specimen, the deepest bound fluorophores can also bleach as the beam scans down through the preparation. Therefore, it is often advisable to start the scan from the deepest point and focus out. The specimen will always be brighter on the outside and so can withstand a degree of photobleaching. Post-processing can be used to alleviate some of the problems.

A variety of 3D methods exist. The simplest is the z-projection (maximum, minimum or average). This method can be thought of as an overlay (combination) of all images in a stack. The maximum intensity projection tends to be the most popular. Ray tracing, alpha blending and volumetric rendering are phrases used to describe the method shown in Fig. 6.4. Voxels (3D pixels) are created from the 2D stack of images. Each voxel is assigned a colour and an opacity value. This can be particularly CPU intensive and will probably require a PC fitted with a high-end graphics card, if the model is highly complex and a fast rotation is required.

The data in Fig. 6.4 was rendered using AMIRA. Another popular package is IMARIS. However, both software packages offer very limited animation output. Movie builder modules are confined to tilt, turn, fly-through, limited lighting and a single camera. To transfer the 3D model to further software for animation purposes, the data needs to go through a process of thresholding, segmentation and iso-surface generation [13].

Fluorescence and confocal microscopy has a resolution of around 0.3 μm in the x-y plane. However, along the z-axis, the resolution is much poorer and can be of the order of 0.5–1 μm. This creates a spherical aberration in which structures are artificially elongated along the z-axis. This can be corrected using digital deconvolution. The

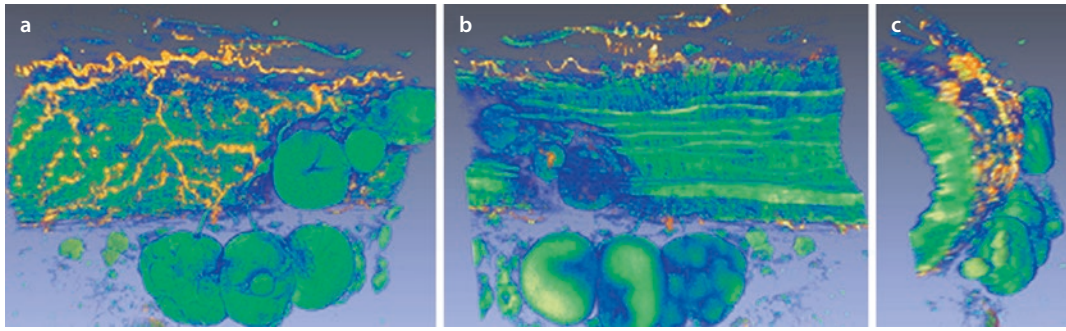

Fig. 6.4 A mouse mesenteric artery has been stained with QAPB (fluorescent prazosin – green channel) and PGP9.5 (antibody to nerve tissue – yellow channel). **a** The vessel is viewed from the top surface showing the sympathetic nerves in the adventitia. Several large round fat cells have been left intact following removal of the majority of the perivascular adipocytes. **b** The vessel is viewed from the inner surface. The internal elastic lamina shows green due to autofluorescence and should not be confused with QAPB binding. **c** The vessel is shown from the side

details of this process are beyond the scope of this chapter, but there exists a huge literature on the subject for those that are interested.

6.6 Animations and Virtual Reality

The popularity of modern games and the availability of sophisticated animation software (Maya, 3Ds Max, Cinema 4D) and game design software (UNITY, Unreal, Lumberyard) have opened new possibilities for visualising microscope-based data. Once a confocal data set has been converted to a wireframe surface (mesh), it can be read by many different CGI-based packages. Some work is required to reduce the mesh geometry whilst still maintaining anatomical accuracy. 'Instant Meshes' is a particularly useful application for mesh reduction. Once the data is imported into an animation package, it can be lit, textured, manipulated and sculpted. Additional characters can be added to the scene (i.e. neurotransmitters, proteins, hormones etc.). Figure 6.5a shows a confocal data set depicting the sympathetic innervation of perivascular adipose tissue (PVAT). This is one still image from a 2-minute animation describing autonomic control of PVAT. A selection of animations can be viewed on the authors' YouTube channel accessed via ▶ www.cardiovascular.org.

Another advantage of having confocal data sets in the form of an iso-surface mesh is that this data type can be read by games engines such as UNITY. This means that interactive applications/games can be created using microscope-based data (Fig. 6.5b). Furthermore, the games engine UNITY can export directly to a virtual reality head-mounted display (VR HMD) such as the HTC VIVE (Fig. 6.5c).

6.7 Nonfluorescent Stains

There are a huge variety of stains that could be used for studying vascular structure. This chapter has focussed exclusively on fluorescent probes that would be suitable for confocal, multiphoton and super-resolution microscopy. Classical histological stains can also provide excellent resolution, and the reader is encouraged to experiment with haematoxylin (DNA/RNA) and eosin (cytoplasm). This pair of stains is commonly referred to as H&E. Another commonly used histological stain is Verhoeff–Van Gieson stain for elastin. This stain is particularly effective for visualising the internal elastic lamina.

6.8 Record Keeping

A final word on something that may seem obvious but cannot be overstated – image manipulation is relatively easy using Photoshop and similar applications. As an imaging specialist, you are likely to collect a huge quantity of image data. Often those images will be shared with colleagues and collaborators. It is not unusual for the image to change hands several times within a group of collaborators. Therefore, you can quickly lose ownership of an image that could be adjusted, cropped, rotated and worse. It is therefore essential that you

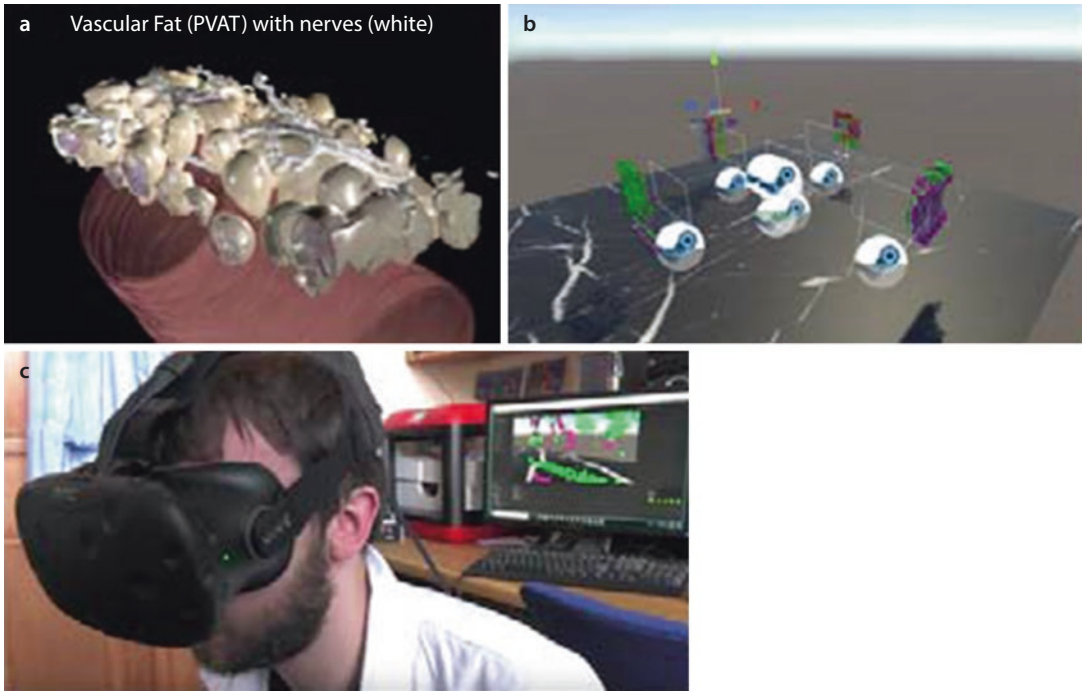

Fig. 6.5 Visualising vascular structure using 3D animation software, games engines and VR head-mounted displays (HMD). **a** A confocal data set of perivascular adipose tissue (gold coloured cells) with sympathetic innervation (white strands) is imported into Autodesk Maya and used as the main set for a 3D movie describing PVAT function. **b** Three similar data sets and a protein structure are arranged within a 3D scene using the games engine UNITY. **c** The UNITY scene can be visualised using a VR HMD

keep accurate records and copies of all original images so that these can be quickly located should the need ever arise. Journals are now carefully inspecting all submitted images for suspected forgery, and it is crucial that you can source the original if requested by the editorial office.

Conclusion

Thin-walled vessels such as the mesenteric arteries and cerebral blood vessels lend themselves very well for optical examination. A standard confocal microscope should be able to penetrate through the entire depth of the wall in a small mesenteric artery. Larger vessels such as the aorta and carotid artery present problems of depth penetration, and often it may be necessary to slit open the vessel and view from the inner surface (if a wet mount, unfixed, preparation is being examined). For fluorescence-based microscopy techniques, there is an enormous range of probes for just about every cellular component. The key to success is in knowing which laser lines a microscope has available, as this determines exactly which probes can be visualised.

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Clinical Cardiovascular Imaging

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_7

Key Concepts

- Imaging plays a key role in cardiovascular diagnosis and intervention.
- There is no single modality that comprehensively evaluates all aspects of vascular disease in all body regions.
- Medical imaging technology and methods are still evolving at a rapid rate, and optimal choice of imaging modality requires understanding of relative benefits of available methods.
- Quantitative imaging biomarkers are being developed in clinical research studies and are increasingly replacing conventional visual (qualitative) assessment of medical image data.

7.1 Introduction

After Röntgen's discovery of X-rays, a series of important discoveries in the domain of physics and chemistry followed over the ensuing decades to extend the reach and impact of medical imaging way beyond the initial plain X-ray radiographs. Many of the seminal scientific contributions that have been central to the development of medical imaging were recognised by Nobel Prizes in physics, chemistry and medicine, including Röntgen (X-rays, 1895), Purcell and Bloch (nuclear magnetic resonance, 1952), Cormack and Hounsfield (CT scanner, 1979) and Lauterbur and Mansfield (MRI, 2003), to mention a few.

The history of cardiovascular imaging can be traced back to the first radiographs of the chest performed by Dr. John McIntyre at Glasgow Royal Infirmary in 1896 when he modified Röntgen's technique to allow lower kilovoltage imaging of soft tissues. The chest X-ray is the most commonly performed imaging investigation worldwide and remains a cornerstone of cardiovascular diagnosis giving basic information about heart size and the manifestations of heart failure such as pulmonary oedema and pleural effusions. Dr. John McIntyre also pioneered dynamic imaging by obtaining images of the movements of a frog's legs in 1897 at a time before the moving images of cinema had become widely known to the public. This paved the way for the cine-radiography that forms the basis of all invasive catheter angiography today.

For many years, invasive catheter angiography with its attendant risks was the only method to obtain more detailed information on the vasculature than plain radiographs could provide, even though only the outline of the lumen is visualised directly. However, non-invasive imaging of the cardiovascular system using ultrasound, CT and MRI is now the primary diagnostic means of evaluation with invasive angiography largely reserved as a platform for intervention. Non-invasive imaging assesses not only the lumen but also the vessel wall and vascular function. Even the coronary arteries with their rapid, complex motion can now be non-invasively assessed for both structure and function.

Medical imaging science and technology are still undergoing rapid development, propelled by the recent improvements in information technology and semiconductor electronics. In parallel to the improvements in speed, sensitivity and robustness of the hardware used for image data collection, recent improvements in image reconstruction and analysis algorithms benefitted from availability and affordability of high-performance computers.

As modern medical imaging produces digital image data, a natural extension of traditional qualitative (visual) assessment is quantitative imaging: a set of methods for acquisition and analysis of medical images, which enable extraction of robust, reproducible and objective imaging biomarkers. The translation between basic and clinical science is greatly enhanced and accelerated by the use of quantitative medical imaging, which acts as a catalyst in this process by reducing the required sample size or by detecting tissue and organ changes that fall below visual assessment thresholds. For example, quantitative indices of perivascular inflammation derived from coronary computed tomography angiography (CCTA) have recently been shown to correlate with cardiac mortality and could therefore be used for stratification of preventative interventions in the near future [2].

Before considering the details of individual imaging techniques and clinical applications, it is useful to look at the reasons behind the sustained growth of the use of imaging in modern medicine and huge ongoing investment in optimising existing technologies and the development of new methods. Consider a generalised pathogenesis cascade presented in Fig. 7.1.

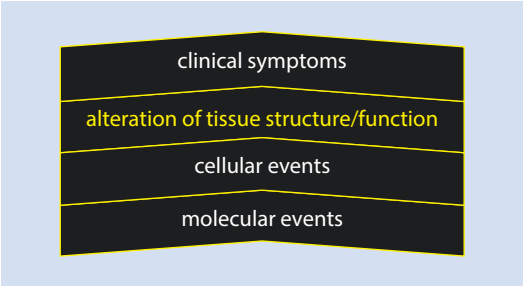

Fig. 7.1 A generalised model of pathogenesis cascade, starting from initiating molecular and cellular events through alteration of tissue composition and function and culminating in the onset of structural tissue and organ changes and appearance of clinical symptoms. Imaging detects early, potentially reversible signs of disease progression at tissue level

The alterations in tissue structure and function occur as a result of a series of molecular and cellular events but ahead of the onset of clinical symptoms. Medical imaging, and quantitative imaging in particular, allows objective and non-invasive measurement of tissue characteristics at the stage within the pathogenesis cascade where the potential for successful treatment is far greater than in the established disease, where the degree of irreversible damage is often significant. Furthermore, biomarkers which reflect this tissue stage of pathogenesis are particularly attractive because it can be argued that intervention becomes necessary only when cellular and molecular self-repair homeostasis feedback mechanisms start to break down. It is at this crucial stage of the pathogenesis cascade that imaging provides unique insight into the nature and extent of the disease process and has the potential to detect the earliest signs of transition between health and disease that warrant intervention. Another important application of imaging is to act as a gatekeeper against unnecessary invasive interventions as it can detect that the hypothesised pathogenesis pathway doesn't correspond with imaging findings.

Even the simplest medical imaging procedures generate vast amounts of data. Conventional image analysis methods arguably utilise only a fraction of information contained within the acquired data. With recent developments in artificial intelligence and machine learning algorithms, the amount of information extracted from individual images, and in particular from large-scale longitudinal and cross-sectional imaging studies, is set to expand significantly. This will provide a step change in deepening our understanding of the aetiology of many highly prevalent diseases and help open up new avenues for risk assessment and design of optimal prevention and treatment strategies [3, 4].

7.2 General Classification of Medical Imaging Methods

Imaging is defined as a process of creating a physical likeness or representation of objects. In the context of medical imaging, this narrow definition can be directly applied to *morphological imaging* which provides information about geometric properties of organs and tissues and allows the measurement of dimensions and distances between different structures. However, medical imaging also encompasses methods that allow visual representation of various physiological processes (*functional imaging*) and assessment of tissue properties (*tissue characterisation*).

Across these three imaging subcategories, there is a further classification based on the way image data is collected and represented. Two-dimensional (2D) imaging refers to in-plane representation of an individual cross section through the object of interest (as in different tomographic imaging modalities) or a projection image (as in conventional X-ray radiographs and X-ray angiography). Three-dimensional (3D) imaging allows imaging data to be reconstructed in an arbitrary plane as a series of 2D slices or allows the construction of 3D models using volume rendering methods. Further dimensions can be added by introducing a time-resolved component to either 2D or 3D basic sequences (dynamic imaging). It is possible to add extra dimensions to provide multiparametric (multidimensional) image datasets acquired at the same location but with different types of contrast, sensitised to provide complementary layers of information about tissue morphology, function and composition.

Images can be derived using intrinsic tissue properties, or, when the contrast presented by native tissue properties is insufficient to provide desired diagnostic information, exogenous contrast agents can be introduced to provide so-called contrast-enhanced images. Furthermore, contrast-enhanced images can be static or dynamic (time-resolved).

Another important subcategorisation relates to the physiological state during an imaging procedure: the majority of diagnostic and research images are acquired at rest, i.e. under normal physiological conditions. However, it is possible to acquire images under stress, either induced by exercise or a pharmaceutical agent (e.g. the vasodilator adenosine). Stress/rest imaging is usually performed in pairs, and diagnostic information is derived from the difference in imaging features between the two states (e.g. blood perfusion reserve, derived as a ratio of estimates of perfusion at stress and rest).

Further classification of imaging techniques is based on their invasiveness. Most commonly, the term *invasive* refers to procedures that involve insertion of catheters or probes inside the body. The use of a peripheral intravenous cannula for contrast administration is generally not classed as 'invasive' and is common for the non-invasive methods. These non-invasive imaging procedures are subclassified as those that employ ionising X-rays or gamma rays (and require a justification process under law) and those that involve application of non-ionising radiation (e.g. ultrasound or MRI).

7.3 Overview of Cardiovascular Imaging Modalities and Applications

Apart from these general classifications based on dimensionality, physiological state, etc., the principal distinction between various imaging methods is related to the nature of the physical process used to create a visual representation of living tissues. The system under observation (organ/tissue) is probed or excited by a physical signal that is modified by the object. The image is created (reconstructed) from the observed changes in the 'messenger' physical signal. MRI, CT and PET use specific segments of the electromagnetic spectrum as their messenger signal, unlike ultrasound, where the physical probe consists of sound waves.

This principle applies to most imaging methods (often referred to as imaging modalities), from the most rudimentary ones (such as Röntgen's plain hand X-ray radiograph acquired in 1895) to the most advanced and modern MRI and CT systems. Nuclear medicine techniques rely on direct administration of molecules containing physical messengers (radiotracers), and the extent of their uptake in different tissues is detected to produce tomographic depiction of their spatial distribution.

Two principal groups of cardiovascular imaging applications are cardiac (including heart chambers and valves) and blood vessel imaging (including angiography, blood flow assessment, vessel wall and plaque imaging). Cardiac imaging is used in the evaluation of non-atherosclerotic diseases (e.g. cardiomyopathy, valvular and congenital heart disease). In atherosclerotic diseases (e.g. coronary artery disease), imaging protocols can employ both blood vessel imaging (primary pathology) and cardiac imaging, to investigate functional impact of atherosclerotic lesions on cardiac structure and function.

7.4 Invasive Catheter Angiography

This procedure involves the use of X-rays in a time-resolved dynamic (cine or fluoroscopic) mode and the administration of radio-opaque contrast medium that creates dynamic image contrast. This is classed as an invasive imaging procedure, because it requires insertion of a combination of a thin wire and catheter into a blood vessel with its attendant potential for complications.

Direct puncture of the blood vessel of interest with injection of a contrast medium through the needle to outline the vascular lumen was the basis of all initial angiography but carried risks, especially puncturing the carotid arteries for imaging the cerebral circulation or direct aortic punctures. This has now been largely supplanted by variations on the Seldinger technique. In this procedure the puncture of a 'safe' vessel (such as the femoral or radial artery) remote from the vascular bed of interest is followed by the insertion of a guidewire over which shaped catheters can be exchanged and directed allowing the vessels of interest to be accessed with much less risk.

For coronary arteriography an arterial catheter will be directed to the coronary vessels under fluoroscopic X-ray control and contrast medium injected to visualise the coronary arterial lumen and delineate any stenotic disease. The injection of contrast with cine (dynamic) imaging will reveal the consequences of steno-occlusive disease by enabling observation of slowing of the contrast column, obstruction to flow and any

collateral pathways. For example, in coronary arteriography the concept of TIMI (thrombolysis in myocardial infarction) flow grading is widely used as a method for evaluation of flow in acute myocardial infarction and its response to thrombolysis.

Once a catheter is in place within a vessel of interest, it can be used for further, more sophisticated flow evaluation using pressure wire measurements, e.g. the measurement of fractional flow reserve (FFR), where the pressure drop across a lesion is measured under maximal vasodilation and FFR is computed as a ratio between distal and proximal pressure readings [5]. A clear advantage of invasive angiography is that with a catheter in place, this can be used as a platform for intervention using angioplasty balloons and the placement of stents to treat flow-limiting stenoses.

7.5 Ultrasound

In ultrasound imaging the physical messenger signal consists of sound waves that are modified by the object to create a series of echoes. Echoes therefore contain the information about the geometry and composition of structures encountered by the messenger waves. Ultrasound can non-invasively assess both the heart and accessible vessels in real-time at high spatial resolution although a suitable acoustic window is required, and deep vasculature may be obscured by intervening bone or air. Ultrasound has advantages in that it employs no ionising radiation, has very high spatial resolution and can be performed at the bedside.

7.5.1 Doppler Ultrasound

The Doppler effect is used in ultrasound to ascribe velocity to moving blood and is fundamental to both echocardiography for cardiac imaging and more general vascular ultrasound studies. The strength of Doppler ultrasound is its very high temporal resolution which allows determination of what can be very transient peak velocities. However, ultrasound precision depends upon the angle of insonation with increasing errors as the beam becomes less parallel to the flow under interrogation, and as with all ultrasound techniques, a good acoustic window is required limiting access to deep vessels.

For accurate quantification of flow, ultrasound is also limited in that the Doppler angle needed for maximum accuracy precludes evaluation of the cross-sectional area of the vessel in question. Measurement of stenosis with Doppler ultrasound relies upon assumptions based upon of the changes in peak velocity across the region of concern and application of the continuity and Hagen–Poiseuille equations.

7.5.2 Intravascular Ultrasound (IVUS)

Invasive arteriography with injection of contrast only outlines the vessel lumen; however, by employing a catheter that includes a miniaturised ultrasound probe, the vessel wall can be evaluated 'from inside'. The images obtained are of high resolution and can delineate the layers of the vascular wall and plaque components outside of the lumen with good concordance to autopsy and pathology. The problem of additional expense and examination times compared to arteriography alone means that IVUS is largely used in research trials in coronary revascularisation or for evaluation of the most complex lesions.

7.5.3 Ultrasound Echocardiography

When used to study the heart, ultrasound is referred to as echocardiography. Echocardiography (often abbreviated as Echo) is a versatile and widely available technique capable of producing detailed images depicting the morphology of the heart chambers and valves. Echo is used to assess cardiac function (including ejection fraction) and in the assessment of myocardial infarction, heart failure and congenital and valvular heart disease. Due to its non-invasiveness, echo is particularly well suited to applications in fetal, neonatal and paediatric cardiology. Apart from traditional transthoracic echo, there is also transoesophageal echocardiography which can provide more detailed assessment of the heart, although this is a relatively invasive procedure. Doppler techniques are employed in echocardiography to assess the blood flow through the heart chambers and valves.

Echocardiography can also be performed under stress, to detect abnormalities in heart wall motion. The application of ultrasound contrast agents (microbubbles) generates additional contrast that can be used to improve the delineation of boundaries between blood lumen and surrounding tissues or to assess microvascular perfusion. Strain imaging provides a normalised index of cardiac muscle deformation and significantly increases sensitivity to earliest manifestations of both ischaemic and non-ischaemic cardiac pathologies [6].

7.5.4 Computed Tomography

In computed tomography, the messenger physical signal consists of X-rays, but unlike traditional X-ray radiography, where X-rays produce a projection 2D image, CT generates a set of transverse cross-sectional images (2D slices). This is achieved by mounting pairs of X-ray sources and detectors on a rotating gantry, with the patient positioned at the centre of rotation. The contrast is generated by the modulation of the messenger signal by the tissues with varying degrees of opacity to X-rays. Additional exogenous contrast media can be used to highlight the architecture of blood vessels, for example. CT is an inherently quantitative imaging technique as it produces images that are calibrated to reflect individual image element (voxel) radiodensity (Hounsfield number). In this respect, CT differs markedly from MRI, where conventional images are displayed using an arbitrary grey-level scale.

7.5.5 CT Angiography

The advent of helical CT scanners, with fast enough rotation times to allow capture of vascular anatomy during the first pass of contrast medium, enabled dedicated CT assessment of the vasculature in most body areas. However, synchronisation to heart motion with electrocardiographic gating was required before the heart and specifically the coronary arteries could be imaged successfully with CT. An illustration of coronary artery CT scan demonstrating a normal epicardial coronary artery tree is presented in Fig. 7.2 (left panel).

Advances in detector design and reconstruction techniques have dramatically reduced radiation doses in recent years allowing increased applicability of CT angiography [7]. Meanwhile the results of large prospective trials are propelling CT coronary angiography in particular into evidence-based guideline recommendations supporting widespread clinical practice [8, 9].

7.5.6 CT Perfusion

The assessment of bulk flow using CT is possible indirectly but not employed clinically. For example, analysis of repetitive time series during con-

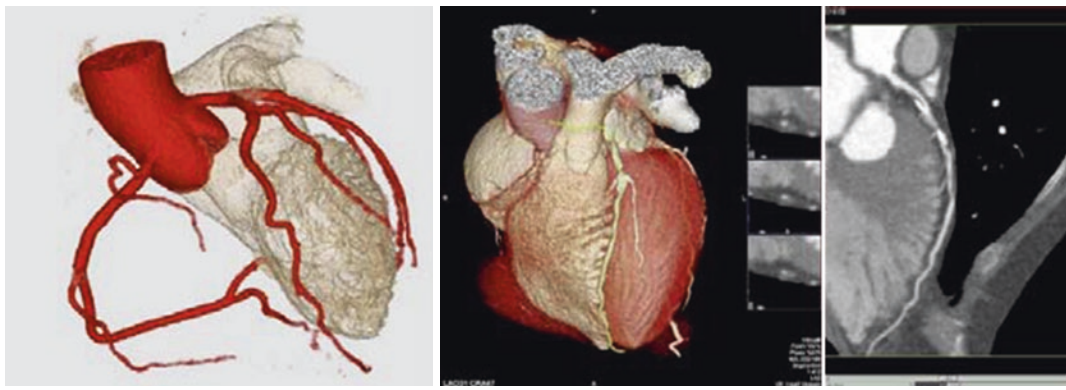

Fig. 7.2 Coronary artery CT – volume-rendered overview of the coronary arteries and heart with transparent cardiac chambers isolating normal coronary arteries (left panel); volume-rendered overview of a CT scan of the coronary arteries and heart with accompanying curved planar reformat of diseased left anterior descending (LAD) coronary artery. LAD has mixed calcified and non-calcified plaque causing significantly stenotic disease (right panel)

trast passage through the thoracic vasculature has been shown to correlate with cardiac output. On the other hand, CT assessment of solid organ perfusion has been validated and entered clinical practice in limited circumstances such as brain perfusion for assessment of acute stroke. ECG-gated techniques for myocardial perfusion have also been developed but remain in the research domain.

7.5.7 CT-Derived Fractional Flow Reserve

Non-invasive fractional flow reserve calculation based upon CTA anatomical datasets has recently been introduced into clinical practice following clinical validation studies for coronary artery evaluation. This technology applies computational fluid dynamics to the three-dimensional coronary CTA dataset with limited assumptions made about boundary and inlet conditions and the volume of the myocardium subtended for the vessel of interest. Larger studies will be needed to determine the place of this in day-to-day practice, but the technique holds great promise.

7.5.8 CT Vessel Wall and Plaque Imaging

CT has no difficulty visualising vessels in any part of the body. However, the spatial and contrast resolution is relatively poor such that the normal vessel wall component layers cannot be evaluated. Plaque manifests as lipid attenuation, intermediate attenuation or calcification. Unfortunately for intermediate attenuation material, fibrous plaque is not differentiated from haemorrhagic/thrombotic components, and beam hardening artefacts exaggerate calcified parts. An example of a coronary CT scan demonstrating mixed morphology left anterior descending coronary artery plaque is presented in Fig. 7.2 (right panel).

7.6 Nuclear Medicine

In nuclear medicine imaging procedures, the images are created from gamma rays emitted by exogenous contrast agent (intravenously or orally administered radiopharmaceuticals). Radiopharmaceuticals contain a radioisotope linked to a tracer, selected to reflect a specific biological target. Location and intensity of gamma radiation (physical messenger) are detected by the gamma cameras. In clinical practice, the most commonly performed cardiac nuclear medicine procedure is single-photon emission computed tomography (SPECT), whereas positron emission tomography (PET) is still primarily used as a research tool. A relatively recent new technological development is the introduction of hybrid systems (PET-CT and PET-MRI), which combine the advantages of high-resolution tomographic data obtained by MRI and CT with functional, metabolic assessment provided by PET.

7.6.1 Single-Photon Emission Computed Tomography

Assessment of myocardial perfusion using SPECT requires injection of radiopharmaceuticals containing radiotracers such as technetium-99m and thallium-201. SPECT is used clinically to evaluate myocardial perfusion, viability and function (when combined with ECG-gating). Its main application is evaluation of functional status of the heart muscle in coronary artery disease to assess the need for invasive intervention.

7.6.2 Positron Emission Tomography

Positron emission tomography alone does not localise or evaluate structure but measures its metabolic activity, and hence it needs to be combined with either CT or MRI to anatomically localise any abnormality. Despite its numerous advantages (including the ability to derive absolute measure of metabolic activity), the use of PET is still hampered by the practical complexity of the imaging procedure. Positron-emitting radioisotopes have short half-lives, which makes their availability problematic although this also makes them more versatile and capable of tracking a wide range of biological targets. One example includes assessment of glucose metabolism in the myocardium using 18F-2-fluoro-2-deoxyglucose (FDG)-PET.

7.7 Cardiovascular Magnetic Resonance Imaging

7.7.1 The Basics of MRI

The main features of MRI, which make it an extremely powerful tool for the evaluation of the cardiovascular system, are its non-invasiveness, superior soft tissue contrast, high spatial and temporal resolution, as well as the availability of safe and efficient contrast agents. A comprehensive treatment of physical basis and technical aspects of MRI can be found in standard textbooks such as [10] or review articles [11, 12].

MRI is based on spatially encoded nuclear magnetic resonance (NMR) signal. NMR involves selective absorption and re-emission of the electromagnetic energy in the radiofrequency (RF) range by a sample of nuclei with non-zero magnetic moments subjected to a strong static magnetic field. The fact that NMR involves energy transition in the RF (non-ionising) range accounts for its non-invasive nature and contributes to suitability for biomedical applications.

An NMR signal can be derived from the nuclei of isotopes such as ^{1}H, ^{13}C, ^{19}F and ^{31}P (all of which possess a non-zero nuclear magnetic moment). Magnetic resonance spectroscopy (MRS) is concerned with the study of the NMR signal derived from these nuclei. Due to its superior biological abundance in living tissues and high strength of its nuclear magnetic moment, ^{1}H (hydrogen proton) is used in a vast majority of MRI applications. The following very brief description will therefore be restricted to proton (^{1}H) NMR, and its aim is solely to illustrate the source of often-quoted "superior MRI soft tissue contrast".

When placed inside a strong static magnetic field, a collection of ^{1}H protons form a net magnetic moment. Following an excitation by an RF field with a characteristic resonant (Larmor) frequency, a measurable, time-varying NMR signal is induced in a receiver RF coil. The resonant nature of this phenomenon is a consequence of the quantum nature of energy transitions. The dynamic behaviour of the system of protons in response to the controlled perturbation and energy transition determines the properties of the time-varying NMR signal. NMR signal is characterised by three intrinsic NMR parameters: proton density (ρ), spin-lattice or longitudinal relaxation time (T1) and spin-spin or transverse relaxation time (T2).

In living tissues, NMR signal is derived from mobile hydrogen-containing molecules. The primary tissue properties not only reflect the number of ^{1}H protons inside mobile hydrogen-containing molecules (through proton density ρ) but also characterise the mobility of these molecules (through relaxation times T1 and T2). Therefore, NMR tissue properties reflect tissue characteristics at the molecular level and provide a powerful tool for assessing not only the anatomical features of tissues but also their biochemical and pathological status. The range of values that these parameters can assume in both normal and pathological tissues is greater than that of the parameters used in other imaging modalities. Furthermore, the dependence of NMR signal intensity on T1 and T2 is exponential, which further increases the dynamic range of NMR and accounts for superior sensitivity of spatially encoded NMR (MRI) for imaging living tissues.

Numerous scientific and technological advances in the field of hardware and software design have transformed MRI from an exotic, expensive imaging modality, with crude spatial resolution and prohibitively long scanning time, to a widely used clinical diagnostic and investigational tool. Major technological developments include the design of highly homogenous wide-bore superconducting magnets for clinical applications with typical magnetic field strength of 1, 1.5, 3 or 7 Tesla whole-body clinical systems, the development of powerful gradient systems, with ultrashort rise times, as well as improvement in the performance of the RF coils, and application of parallel imaging. All these developments have contributed to significant improvements in signal-to-noise ratio (SNR) and increased spatial and temporal resolution in modem MRI systems.

As a result of these technological and methodological advancements and driven by the need to eliminate image degradation by motion artefacts (both physiological and gross body motion), several rapid imaging techniques were developed in the early 1990s. They include fast gradient echo, fast spin echo and echo-planar imaging. Rapid acquisition techniques have dramatically reduced MRI acquisition times. An important result of the availability of rapid imaging techniques is the possibility of dynamic scanning with temporal resolution of the order of 1 s or even below 0.1 s on

high-performance systems. However, it is important to note that MRI is still an inherently slow technique compared to X-ray and ultrasound and thus highly vulnerable to motion artefacts.

Another landmark in the development of MRI was the design of effective and safe contrast agents which improved the capacity of MRI to depict lesion morphology in situations where the inherent tissue contrast is not sufficient to enable accurate delineation of pathological tissue. The first MRI contrast agent approved for clinical applications was gadopentetate dimeglumine (Gd-DTPA). Gadolinium-based contrast agents (GBCAs) selectively alter MRI signal intensity throughout their distribution volume (blood plasma and extravascular extracellular fluid). The effect that GBCAs exert on MRI signal intensity results from the paramagnetic properties of the Gd^{3+} ion and its spatial relationship to the chelate, whereas the pharmacokinetics is determined by the in vivo behaviour of the chelate. Physiological variables that determine tissue microcirculation and water compartmentalisation have a direct influence on the resulting local bulk tissue concentration of a GBCA following intravenous administration.

In conventional (static) MRI applications, GBCAs are used to delineate tissue morphology since they selectively enhance highly perfused and permeable tissues with a high extracellular volume. The introduction of the fast imaging sequences opened the possibility of monitoring contrast kinetics dynamically, with high temporal and spatial resolution, thus providing not only morphological but also functional information.

Signal intensity in MRI, expressed in arbitrary units, is dependent not only on the fundamental tissue properties but also on the imaging system field strength, the design of acquisition method, the geometry and performance of the RF coils, the receiver gain setting and the method used for mapping the detected signal onto a grey-level (display) scale. A direct comparison of MRI signal intensity obtained using different acquisition sequences is therefore difficult. In clinical practice (as well as in clinical research), MRI exam consists of a set of procedures (sequences) that together form an MRI protocol. Each sequence is designed and optimised to display a chosen set of spatial, temporal and contrast characteristics, depending on the type of information being sought. A single hour-long MRI protocol can therefore consist of images that have different dimensions and that take anything from a couple of seconds to 10 min or more to acquire.

Individual MRI sequences are designed to preferentially influence signal intensity by one of the characteristic tissue parameters (this is known as image weighting). This gives rise to T1- or T2-weighted images, diffusion-weighted images or images sensitive to flow, for example. Furthermore, in contrast-enhanced MRI, image weighting is designed to maximise the effects of the introduction of exogenous contrast agents. This process of image optimisation is complicated by the fact that underlying contrast parameters often act in opposition to one another (e.g. T1 and T2). Another important consideration for the design of clinical and clinical research protocols is that of the interplay between spatial and temporal resolution and signal-to-noise ratio (SNR). Prolonged acquisitions can bring about improved SNR that can be traded for improved spatial resolution but with concomitant increase in overall acquisition time and increased vulnerability to motion artefacts.

7.7.2 MR Angiography

Initially the variable signals observed with MRI on the early scanners in vessels with flowing blood posed a confounding problem. However, it was soon grasped that time-of-flight effects and phase differences could be exploited to image both the vascular lumen and quantify flow – this became known and magnetic resonance angiography (MRA). Non-contrast time-of-flight MRA (TOF MRA) while a significant advance at the time was hampered by long acquisition times (especially for large vascular territories such as the legs), in-plane flow saturation effects and limited signal return from vessels deep in the body. Nevertheless, with more advanced 3D implementations allowing small voxel sizes, TOF MRA has found a productive niche in the evaluation of the intracranial vasculature.

The use of gadolinium-based contrast for vascular imaging with MRI was first reported in 1991. An example of a multi-station GBCA-enhanced aortoiliac and lower limb arterial vasculature is presented in Fig. 7.3. The subsequent development of this as a 3D technique resulted in its widespread adoption, and contrast-enhanced MRA (CE-MRA) has been the predominant form of MRA since [13, 14].

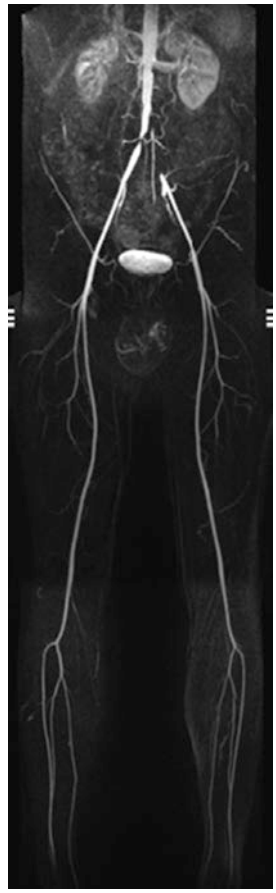

Fig. 7.3 Multiple station contrast-enhanced MRA of aortoiliac and lower limb arterial vasculature. Patient with aortic bifurcation disease including tight stenosis right common iliac origin and left common iliac artery occlusion. Normal infra-inguinal arterial run-off

7.7.3 MRI Assessment of Blood Flow

Phase-contrast MRA (PC-MRA) techniques have become invaluable in the investigation of bulk flow and essential in much cardiac MRI where congenital heart disease with shunts is being investigated. However, accurate placement of the 2D imaging planes can be time consuming and challenging for correct evaluation. Recently the development of four-dimensional PC-MRA, allowing collection of a temporally resolved 3D flow fields with very simple planning, promises to revolutionise the use of PC-MRA [15]. An example of a 4D flow evaluation of thoracic aortic dissection is presented in Fig. 7.4.

With PC-MRI technique, the velocity of flowing blood is mapped proportional to its phase shift following application of alternating gradients of equal and opposite direction. Static tissue will yield null signal, while the phase shift of moving blood will be proportional to the encoding gradient. Measurement of these phase shifts across the whole cross section of the vessel of interest will yield instantaneous flow. Subsequent integration at multiple time points across the cardiac cycle will then result in a flow curve from which parameters such as total and net forward flow, backward flow and regurgitant fraction can be derived. The major advantage of PC-MRI is the ability to map flow at any site with accurate evaluation of cross-sectional area; the main limitation is its reduced temporal resolution compared to ultrasound such that transient peak systolic velocities are less accurately characterised.

7.7.4 MR Plaque Imaging

The advantage of MRI for plaque evaluation is the ability to characterise all different components using a multiparametric approach such that haemorrhagic, fibrous and lipid parts can be discriminated. The difficulties are gaining appropriate spatial resolution in clinically acceptable scan times, and the imaging of deep mobile vessels such as the coronary arteries remains very challenging. MRI also has a role in the evaluation of the vasculitides being able to image the thickened and enhancing vessel wall in these conditions.

7.7.5 Cardiac MRI

Cardiac MRI (CMR) comprises a set of imaging sequences designed to provide information about various aspects of myocardial morphology, function and myocardial tissue properties. Physiological (respiratory and ECG) motion presents a major challenge for CMR and requires special consideration. Some procedures are performed during a series of breath holds to remove effects of blurring caused by respiratory motion. Others are performed using respiratory gating – e.g. images are acquired only within a specific respiratory window. To eliminate the effects of ECG motion, image acquisition is synchronised with the ECG trace to trigger signal collection at a specific point in the cardiac cycle [11].

The principal components of clinical CMR protocols are designed to assess ventricular

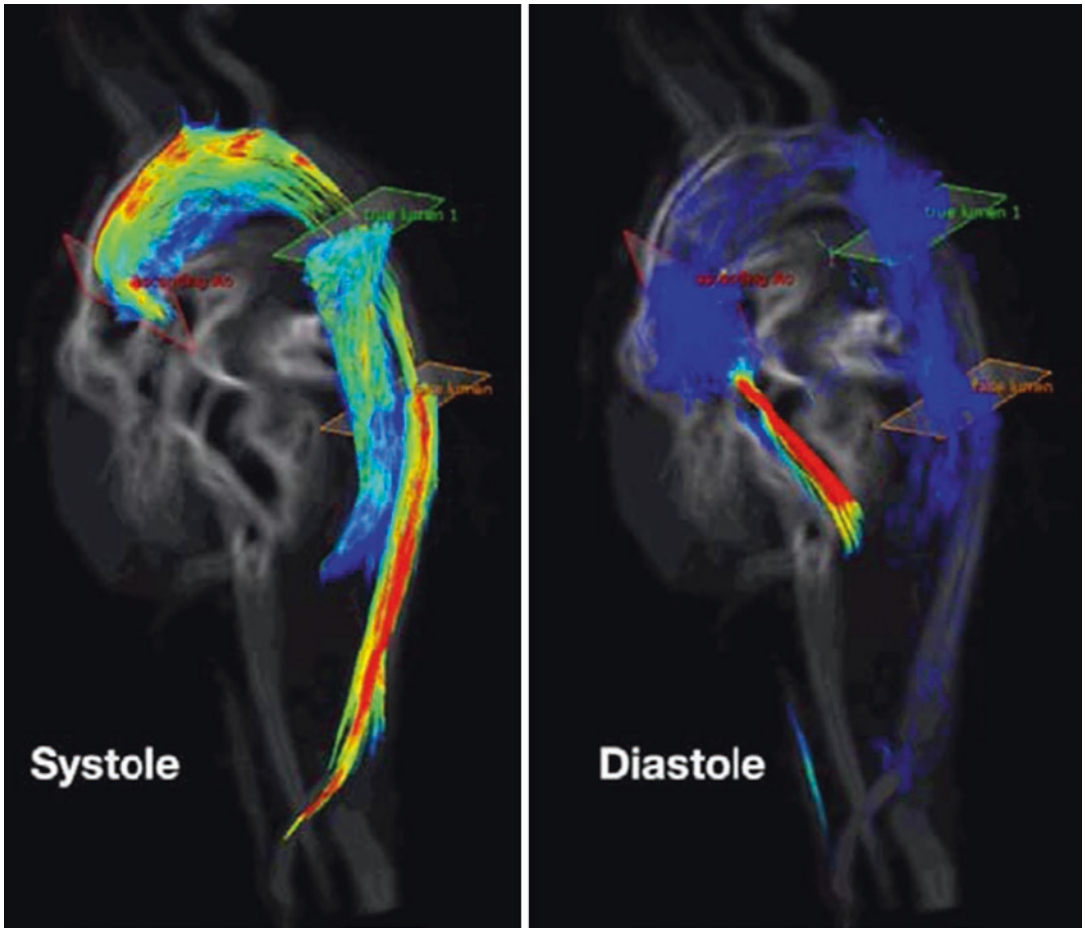

Fig. 7.4 Systolic and diastolic frames from a 4D phase-contrast flow acquisition in a patient with type B thoracic aortic dissection plus aneurysmal ascending aorta with secondary aortic valve incompetence. Images rendered with streamlines, colour coding denotes velocities of flow streamlines generated from emitter planes in ascending aorta and 2 points beyond arch in descending aorta. Blue for low velocity through rainbow to red for highest velocities. Higher flow rates in true lumen of descending aorta dissection channel in systole, jet of high-velocity aortic valve regurgitation in diastole

volumes and function (cine MRI), to provide assessment of regional scar or fibrosis (late gadolinium enhancement – LGE) and microvascular perfusion (first-pass dynamic contrast-enhanced MRI).

Advanced CMR components include relaxation times mapping (T1, T2 and T2*) to reveal fibrosis, oedema or iron depositions, respectively [16]. T1 maps acquired before and after contrast can be used to calculate maps of extracellular volume. Other advanced CMR methods include myocardial tagging, strain imaging [17], flow measurement [15] and coronary MR angiography [18]. Less extensively explored are techniques such as diffusion-weighted MRI and arterial spin labelling (ASL) for non-contrast assessment of myocardial perfusion.

CMR techniques vary widely in terms of their robustness, level of validation and availability of reliable normal ranges [19]. Cine MRI evaluation of LV and RV volume, mass and function, for example, is considered the reference standard [20], whereas quantitative myocardial perfusion [21] and T1 and T2 maps still require the use of local reference values.

CMR has benefitted more than any other area of clinical MRI applications from recent advancements in MRI signal acquisition, reconstruction and the development of quantitative image analysis methods. As the speed and quality of individual components increases, the number of CMR indications is widened to encompass patients who would be unable to comply with hour-long

examination or patients with arrhythmias. The time savings can also be used to increase clinical throughput. However, the acceleration of standard components of the CMR exam also offers a possibility to include additional functional evaluation of the heart function or composition to rudimentary CMR protocols [18] or even to include MRI evaluation of other tissues and organs, when the investigation of cardiovascular co-morbidities is of interest.

Conclusion and Clinical Perspectives

- There are a number of established medical imaging techniques that are used in everyday clinical practice of cardiovascular medicine to provide invaluable information that informs patient management.
- Imaging techniques vary not only in terms of the type of information they provide but also in terms of their complexity, availability, cost, invasiveness and safety profile.
- Optimal use of medical imaging requires a rational choice of imaging modalities (based on understanding of their advantages as well as limitations) to maximise patient benefit and avoid unnecessary invasive interventions.
- Imaging can provide evidence of earliest tissue manifestations of cardiovascular disease that can be used to assess risk and guide optimal therapy.

7

Gaps in Knowledge

- Emerging imaging methods hold immense promise but require robust validation before they are introduced into clinical practice.
- Quantitative imaging biomarkers are still often vendor dependent, and this limits their use in large-scale multicentre clinical trials that are crucial for providing novel insights into aetiology, risk factors, and prognosis and for evidence-based introduction of novel therapies.
- Future development of quantitative imaging biomarkers based on artificial intelligence and machine learning algorithms will require formation of large-scale shareable image databases to maximise their reproducibility, sensitivity and specificity.

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Pharmacology and Signalling

Contents

Vascular Signaling

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_8

Key Concepts

- GPCRs and RTKs are the two major classes of cell surface transmembrane proteins regulating intracellular signaling in vascular smooth muscle cells (VSMCs).
- GPCRs are integral membrane proteins responsible for the conversion of extracellular signals into cellular responses through complex intracellular signaling pathways. Vasoactive peptides, hormones, and neurotransmitters bind to GPCRs.
- RTKs promote signal transduction of several growth factors that induce cellular proliferation, migration, differentiation, metabolism, and survival. RTKs are important drug targets. Inhibitors of RTKs (RTKi) are increasingly being used as anti-angiogenic drugs in the treatment of cancer.
- Crosstalk between GPCRs and RTKs leads to diverse and complex signal transduction, which influences vascular function.

8.1 Introduction

The vascular wall is composed of endothelial cells (ECs), vascular smooth muscle cells (VSMCs), fibroblasts, and extracellular matrix components which dynamically change or reorganize under physiological and pathological stimuli. Smooth muscle cells (SMC) and matrix, under physiological conditions, provide structural support to the vasculature and are responsible for functional characteristics of the vessel wall, including contraction, relaxation, growth, remodeling, and repair. VSMCs also play an important role in vascular dysfunction associated with pathological conditions, including atherosclerosis, restenosis, aortic aneurysm, and hypertension. VSMCs are controlled mainly by two types of cell surface receptors: single membrane-spanning receptor tyrosine kinases (RTK) for growth factors and G protein-coupled receptors that comprise a seven-transmembrane-helix (7TM) domain coupled to heterotrimeric G proteins. Numerous local and systemic factors, including vasoactive peptides, such as angiotensin II (Ang II), endothelin-1 (ET-1), bradykinin, serotonin, and thrombin, among others, regulate VSMC function by interacting with ligand-specific GPCR receptors. Growth factors such as VEGF, FGF, IGF-1, and PDGF bind to RTKs and regulate signaling pathways that typically influence cell growth, important in processes that contribute to vascular hypertrophy and remodeling in cardiovascular disease. This chapter focuses on GPCRs and RTKs and their signaling pathways and how they regulate VSMC function.

8.1.1 G Protein-Coupled Receptors (GPCRs)

GPCRs are integral membrane proteins that bind specific ligands and which convert extracellular signals into cellular responses through activation of intracellular signaling pathways. Vasoactive peptides, hormones, and neurotransmitters bind to GPCRs. The 7TM region, which forms the structural core, binds to specific ligands and transduces this information to the intracellular region through conformational changes. The intracellular region interfaces with cytosolic signaling proteins and induces activation of signaling pathways. GPCRs interact with G proteins, which are composed of three protein subunits, Gα, Gβ, and Gγ. Both the Gα and Gγ subunits anchor the G protein to the plasma membrane. In the inactive state, Gα is bound to Gβγ dimer and guanosine diphosphate (GDP). G protein-mediated signaling starts by the binding of an agonist molecule to the extracellular region of the GPCR, leading to changes in its conformation and consequently its activation. It is also described that some GPCRs may also work as a guanine nucleotide exchange factor (GEF) that promotes the exchange of GDP to guanosine triphosphate (GTP), which is associated with the Gα subunit. The activated GPCR catalyzes exchange of GTP for GDP on the Gα subunit. During these conformational changes, the Gβγ dimer is dissociated from Gα, and now free Gα and Gβγ have their own second messengers to initiate intracellular signaling responses. Thereafter the activated Gα-GTPase hydrolyzes the bound GTP (Gα-GTP) to GDP and inactivates the G protein complex by reassociating the Gα with Gβγ. GDP is again bound to Gα (Gα-GDP) in the G protein complex, and the activation and inactivation cycle is completed [1–3].

Gα subunits are grouped into four subfamilies: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$. All Gα subunits have four GTP binding sites and hydrolyze GTP. G proteins containing the $G\alpha_s$ subunit drive cyclic adenosine monophosphate (cAMP) production by adenylate cyclase (AC). The $G\alpha_i$ group includes eight members closely related by structure. Three $G\alpha_i$ ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$) members inhibit adenylate cyclase. $G\alpha_{i3}$, which also goes by the name $G\alpha_k$, stimulates receptor-regulated K^+ channels. The $G\alpha_q$ subfamily activates phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 induces the release of calcium (Ca^{2+}) from intracellular stores, propagating Ca^{2+}-dependent signaling. Also, Ca^{2+} acts with DAG to turn on certain isoforms of protein kinase C (PKC). This family, also known as $G\alpha_{q/11}$, includes $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_q$, and $G\alpha_{11}$. $G\alpha_{15}$ is specifically expressed in hematopoietic cells. The $G\alpha_{12}$ subfamily has two members, 12 and 13, and both promote the Ras homolog RhoA, a small GTPase that regulates actin reorganization. Much less is known about the Gβ and Gγ subunits [1, 3, 4].

8.1.1.1 GPCR-Mediated Signaling Pathways Regulating Vascular Smooth Muscle Contraction

The classic vasoconstrictors, such as Ang II, ET-1, and vasopressin, act through GPCRs coupled to G proteins of the Gq/G_{11} and G_{12}/G_{13} and to a lesser extent to the G_i family. Upon ligand-receptor binding, Gq/G_{11} activates β-isoforms of PLC, causing formation of IP_3 and release of intracellular stores of Ca^{2+}. This is a central process in the regulation of VSMC contraction initiated by increased intracellular Ca^{2+} mobilization and phosphorylation of myosin light chain (MLC). MLC is phosphorylated by Ca^{2+}-calmodulin-activated MLC kinase (MLCK), culminating in increased interaction and crossbridge formation of myosin and actin, with consequent contraction and increased vascular tone (Fig. 8.1). MLC is dephosphorylated by MLC phosphatase (MLCP). Of importance, Ca^{2+} transmembrane influx is also stimulated through calcium channels, such as voltage-dependent L-type Ca^{2+} channels and transient receptor potential (TRP) cation channels [2–4, 6].

Whereas MLCK regulation is Ca^{2+}-dependent, MLCP is regulated by Ca^{2+}-independent signaling pathways, involving the small GTP binding protein RhoA. RhoA stimulates Rho-kinase which, in turn, phosphorylates and inhibits MLCP. G proteins G_{12}/G_{13} link receptors to the RhoA/Rho-kinase pathway in VSMCs. Once activated by the binding of vasoconstrictors to receptors, G_{12}/G_{13} activates Rho guanine nucleotide exchange factors (RhoGEFs), which promote active RhoA formation and consequently vasoconstriction. Gq/G_{11} can activate RhoA indirectly through Ca^{2+}- and Jak2-dependent activation of the RhoGEF. For example, Ang II through AT1 receptors induces RhoA-dependent VSMC contraction. In addition, some GPCRs couple to G proteins of the G_i family, which is associated with pro-contractile activity of their ligands through release of the βγ-subunits, resulting in the activation of PLC β-isoforms or by inhibiting adenylyl cyclase, which leads to reduced intracellular cAMP levels [2, 4, 7].

G_q/G_{11}- and G_{12}/G_{13}-mediated signaling has been studied in smooth muscle-specific $G_{\alpha q}/G_{\alpha 11}$- and $G_{\alpha 12}/G_{\alpha 13}$-deficient mice. Isolated vessels from these animals showed that several pro-contractile receptors use both G_q/G_{11}- and G_{12}/G_{13}-mediated signaling transduction pathways to increase vascular smooth muscle tone. In contrast, α_1-adrenergic receptors were identified as signaling exclusively through G_q/G_{11} to promote contraction. Findings using the same approach revealed that basal vascular tone and basal blood pressure require G_q/G_{11}-mediated signaling in VSMCs, whereas during hypertension an increase of vascular tone requires activation of both G_q/G_{11} and G_{12}/G_{13}. Regulation of vascular smooth muscle relaxation is mediated primarily through pathways that involve nitric oxide (NO), cAMP, and cyclic guanosine phosphate (cGMP) [4, 6, 8, 9].

8.1.1.2 G_s- and G_i-Mediated Signaling

G_s-coupled receptors increase cAMP levels, which interfere with Ca^{2+}-dependent and Ca^{2+}-independent Rho/Rho-kinase-mediated signaling pathway, leading to a relaxation of vascular smooth muscle. The major mediators of cAMP-induced relaxation in VSMCs are protein kinase A (PKA) and protein kinase G (PKG), which are activated by cAMP (Fig. 8.1). Molecular mechanisms whereby PKA and PKG inhibit pro-contractile signaling include inhibition of G_q/G_{11}-mediated signaling by phosphorylation and activation of the regulator of G protein signaling 2 (RGS2), phosphorylation and inhibition of MLCP,

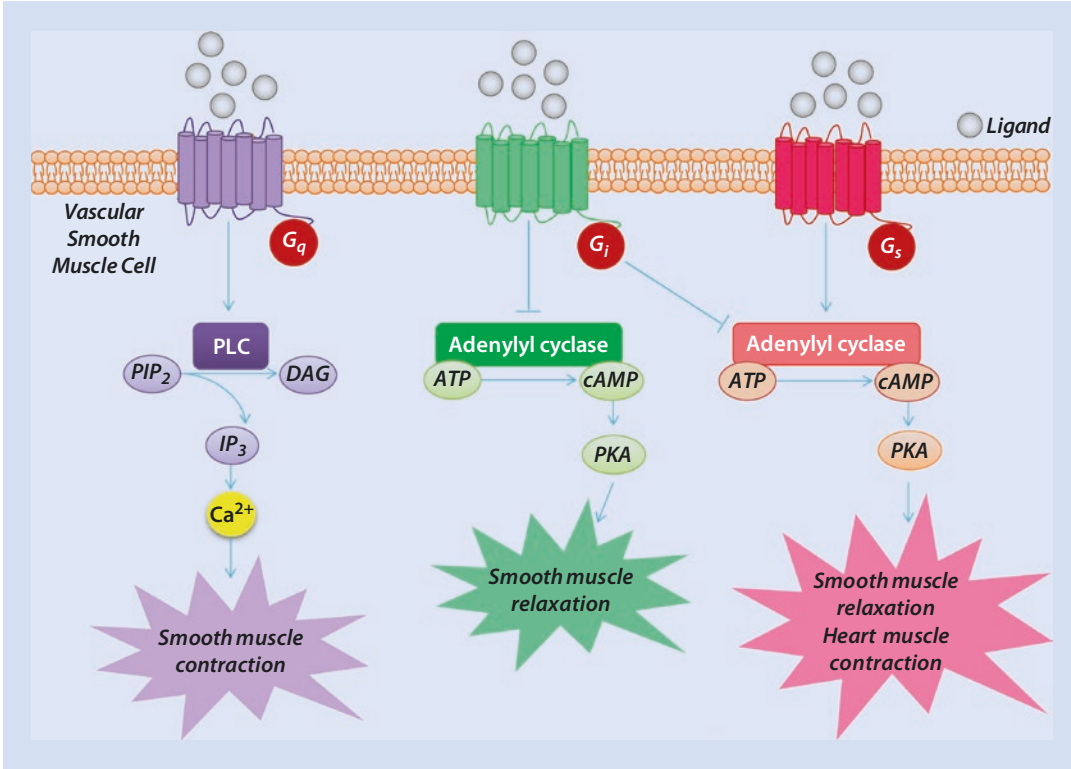

Fig. 8.1 Schematic of GPCR-mediated signaling pathways in vascular smooth muscle cells (VSMCs). Classically, G proteins mediate signal transduction via GPCRs. Signal transduction is initiated when ligands bind to GPCRs. G_s activates adenylyl cyclase leading to the production of cAMP and PKA activation, which promotes VSMC relaxation. G_q controls the activity of PLC, which hydrolyzes PIP_2 to generate IP_3 and DAG. IP_3 and DAG in turn lead to an increase in the intracellular concentrations of free Ca^{2+} and the activation of a number of protein kinases and pathways to induce VSMC contraction. G_i inhibits adenylyl cyclase activation and consequently decreases cAMP levels and PKA activation in VSMCs, which results in VSMC relaxation. G_i can also modulate G_s-activated signaling by decreasing adenylyl cyclase activity

inhibition of IP3-induced release of intracellular Ca^{2+}, and inhibition of RhoA [4, 5, 7].

G_s-mediated increase in intracellular cAMP levels affects both G_q/G_{11}- and G_{12}/G_{13}-mediated signaling. G_s-induced increase in cAMP levels and activation of its effector PKA are associated with disruption of PDGF-induced activation of ERK1/2 in VSMCs, supporting its role in VSMC dedifferentiation. In a rat model of balloon injury of carotid arteries, infusion of cAMP-elevating agents inhibited VSMC growth and proliferation as well as neointima formation. These preclinical results supported clinical studies using phosphodiesterase (PDE) inhibitors as vasoprotective agents. In line with these data, activation of different G_s-coupled receptors in vivo prevented VSMC proliferation and vascular remodeling. Together, these findings indicate that G proteins mediating receptor-dependent stimulation (Gs) or inhibition (Gi) of adenylyl cyclases are involved in the regulation of VSMC plasticity and vascular remodeling (Fig. 8.2) [4, 5, 10, 11].

G_i-coupled receptors have been implicated in VSMC hyperproliferation, migration, and pathological vascular remodeling. Most GPCRs preferentially couple to members of the G_i family, which appears to be the most abundantly expressed heterotrimeric G protein in many cell types. G_i activation is typically associated with inhibition of G_s-stimulated adenylyl cyclase activity and thus reduced cAMP, which has been associated with enhanced transactivation of growth factor receptors and induction of mitogen-activated protein kinase (MAPK) signaling via extracellular-regulated kinase 1/2 (ERK1/2). Because the expression of G_i is relatively high, its receptor-dependent activation results in a relatively higher release of βγ-complex. Activation of G_i is there-

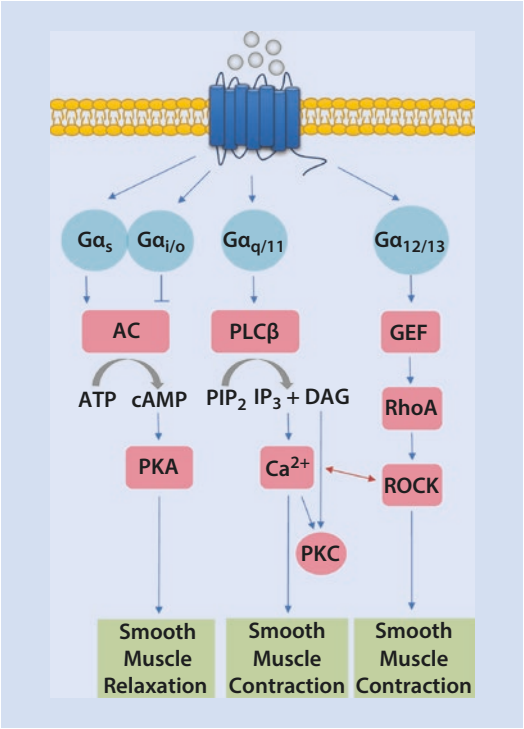

Fig. 8.2 Different subtypes of GPCR and its activated intracellular signaling cascades in vascular smooth muscle cells (VSMCs). G_s leads to the production of cAMP and PKA activation, promoting VSMC relaxation, while G_i inhibits adenylyl cyclase activation and consequently decreases cAMP levels, which also result in VSMC relaxation. G_q hydrolyzes PIP_2 to generate IP_3 and DAG, which modulate intracellular concentrations of free Ca^{2+} and induce VSMC contraction. G proteins G_{12}/G_{13} link receptors to the RhoA/ROCK pathway in VSMC. Once activated by the binding of vasoconstrictors to receptors, G_{12}/G_{13} activates Rho guanine nucleotide exchange factors (RhoGEFs), which promote active RhoA formation and consequently vasoconstriction. Gq/G_{11} can activate RhoA indirectly through Ca^{2+}- and Jak2-dependent activation of the RhoGEF

fore thought to be the major coupling mechanism related to the activation of βγ-mediated signaling processes. For example, agonist-induced MAPK activation and proliferation of VSMCs in vitro was shown to be primarily mediated by G protein βγ-subunits [3, 5–9].

8.1.2 Receptor Tyrosine Kinases (RTKs)

Receptor tyrosine kinases are single-pass membrane receptors with intrinsic tyrosine kinase activity, a process that utilizes transfer of a phosphate group from adenosine triphosphate (ATP) to tyrosine residues in protein substrates. These receptors promote signal transduction of several growth factors mediating effects such as cellular proliferation, migration, differentiation, metabolism, survival, and control of cell cycle. The RTK family is composed of at least 58 receptor types in the human genome including the vascular endothelial growth factor receptors (VEGFR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptors (PDGFR), insulin-like growth factor (IGFR), and fibroblast growth factor receptors (FGFR). They are grouped in distinct classes based on the nature of their cognate ligands and modular composition of their extracellular domain. Although the RTK family is composed of many different members, receptors from this family share the same basic structure. They comprise an extracellular domain, a single transmembrane helix, and a cytosolic domain composed of a juxtamembrane domain (adjacent to the membrane on one side of it) followed by a tyrosine kinase sequence and a C-terminal tail. The extracellular domain contains the site for ligand binding and includes immunoglobulin (Ig)-like domains, cysteine-rich domains, and fibronectin type III-like domains, depending on the type of RTK [12–14].

In general, ligand binding to the extracellular domain leads to conformational changes that induce and stabilize receptor dimerization resulting in autophosphorylation of their cytoplasmatic domain and consequent activation of RTKs. With the exception of the insulin receptor (IR) family, all known RTKs (e.g., VEGFR, EGFR) are monomers in the cell membrane. Other tyrosine residues located on the juxtamembrane and C-terminal domains are phosphorylated serving as docking sites for binding of adaptor proteins. This role is played by proteins bearing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, including proteins with enzyme activity such as Src and PLC and adaptor proteins such as Grb2, which in turn will further activate signaling cascades. Activated PLC results in generation of DAG and activation of PKC with consequent activation of MAPK signaling pathways. In addition, PLC generates IP_3 mediating the increase in Ca^{2+} release from intracellular stores. Ca^{2+} then binds to calmodulin, which in turn activates a

family of Ca^{2+}/calmodulin-dependent protein kinases. In addition, both DAG and Ca^{2+} activate members of agonist the PKC family of protein kinases [12–14].

Recruitment of Grb2 leads to Ras/Raf activation, which induces phosphorylation of ERK1/2 and activation of transcription factors involved in cell growth and proliferation. Additionally, RTKs can activate phosphoinositide 3-kinase (PI3K), promoting protein kinase B (PKB, Akt) activation and induction of cell survival pathways. PI3K-dependent activation of Akt leads to phosphorylation and inactivation of BAD. Phosphorylation of BAD in turn prevents apoptotic cell death by blocking its complex formation with the apoptotic proteins Bcl-2 and Bcl-xl. Thus, RTKs can form signaling platforms that activate several transcription factors regulating diverse cellular processes (Fig. 8.3) [12, 13].

RTKs are regulated by internalization, degradation, or dephosphorylation through protein tyrosine phosphatases (PTPs). In addition to activation of signaling pathways, RTKs influence transcriptional repressors and phosphatases that can decrease signal amplitude. After activation, those receptors are internalized and undergo endocytosis via the clathrin-dynamin pathway; subsequently they are ubiquitinated and targeted to lysosomal degradation. Several RTKs are fundamental for vascular development and maintenance of vascular function such as EGFR, PDGFR, FGFR, IGFR, and VEGFR. In addition to the important role of RTKs in the regulation of vascular development and function, these receptors have been implicated in tumorogenesis. Dysregulation of RTKs is associated with uncontrolled angiogenesis and cancer [14, 16, 17].

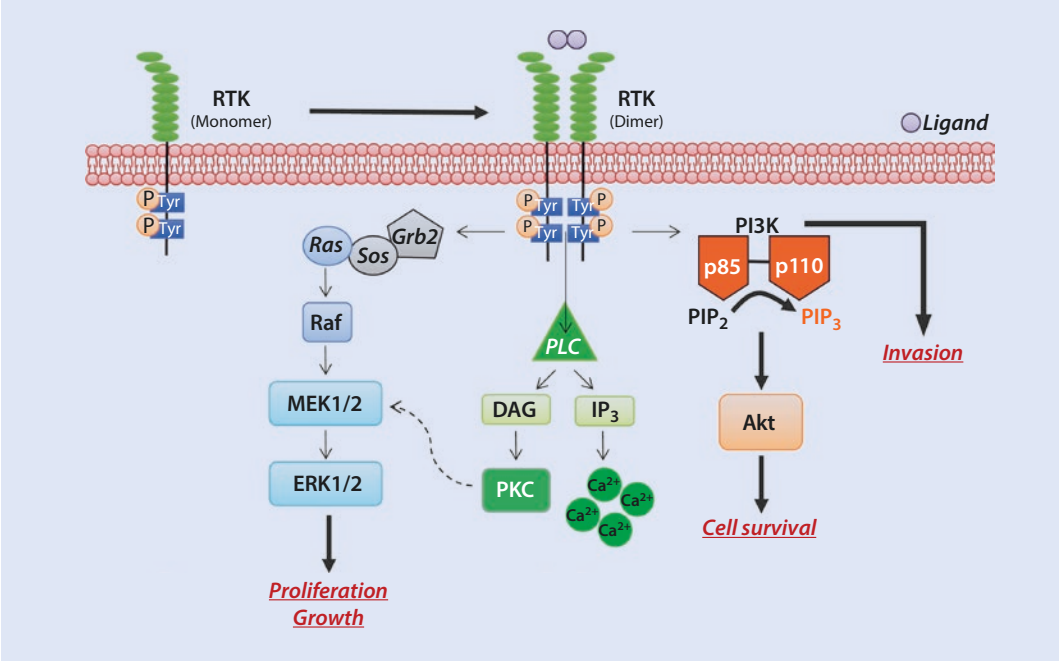

Fig. 8.3 Ligand-activated signal transduction by receptor tyrosine kinase (RTK) complexes. Growth factor binding to its RTK induces RTK dimerization leading to phosphorylation of its tyrosine residues in the intracellular domains. TKR activation induces phosphorylation and activation of multiple downstream signaling pathways including Ras/Raf/MEK/ERK, PI3K/Akt, PLC/PKC, and IP_3/Ca^{2+}. Tyrosine phosphorylated receptors bind to adaptors proteins such as Shc and Grb2 leading to SOS recruitment and Ras/MAPK pathway activation. Additionally, the regulatory subunit p85 binds to the intracellular phosphorylated tyrosine residues of RTKs via its Src homology 2 (SH2) domains and recruits the catalytic subunit p110 to form the fully active PI3K enzyme, which activates Akt, important in cell survival. RTKs also activate PLC, which leads to formation of DAG, which in turn activates PKC and IP_3 and mobilization of intracellular Ca^{2+}. Through these signaling events, RTKs modulate proliferation and growth, cell survival, and invasion

8.1.2.1 RTKs as Important Drug Targets

Several RTK inhibitors have been approved to treat cancers and are currently used in many solid tumors, including renal carcinoma, colon cancer, and brain cancer. These drugs comprise two categories: small-molecule inhibitors and monoclonal antibodies. Because they target the ATP-binding site of protein kinases, many small-molecule RTK inhibitors affect multiple tyrosine kinases in addition to their initially intended target. For example, imatinib was initially identified as a PDGFR inhibitor, but it also potentially inhibits KIT and the non-receptor tyrosine kinase c-Abl. Sunitinib blocks the tyrosine kinase activity of several RTKs and non-receptor tyrosine kinases, including KIT, VEGFR2, PDGFR, and Flt3. On the other hand, EGFR inhibitors such as gefitinib show much greater specificity and are capable of selectively inhibiting EGFR. Monoclonal antibodies that bind to the extracellular domain of EGFR have been used to treat mammary carcinoma, colorectal cancer, and head and neck cancers. Inhibitors of VEGFR, including sunitinib, sorafenib, pazopanib, sunitinib, and vatalanib, are effective antiangiogenic drugs and have significantly prolonged survival in cancer patients. However, these drugs have unwanted secondary effects, including cardiovascular disease. VEGF/VEGFR inhibitors cause an increase in blood pressure in 80–90% of treated patients, and 40–60% of patients develop hypertension. Mechanisms for this remain unclear but may relate to endothelial dysfunction, increased ET-1 production, rarefaction (reduction in vascular density), and oxidative stress [14–18].

8.1.2.2 Transactivation of RTKs and GPCRs

RTKs and GPCRs are key components of vascular signaling, and their crosstalk influences important mechanisms connecting and diversifying signal transduction pathways. They can act in a synergistic way in some cases, whereas in others they have antagonistic interactions to regulate physiological processes. The activation of GPCRs can stimulate the signaling activity of RTKs connecting the broad diversity of GPCRs with the potent signaling capacities of RTKs. This molecular mechanism is termed transactivation and was first described in fibroblasts stimulated with GPCR agonists, which induced a rapid tyrosine phosphorylation of the EGFR. GPCR-induced EGFR tyrosine phosphorylation is comparable with receptor activation by low amounts of EGF, being rapid and transient [3, 4, 12, 16, 18].

Transactivation of RTKs by GPCRs occurs through different molecular mechanisms: activation of membrane-bound matrix metalloproteases (MMPs), such as the A disintegrin and metalloprotease (ADAM) family members, in a ligand-independent manner where GPCR stimulation triggers the activation of several second messengers such as Ca^{2+} ions, PKC, the non-receptor protein tyrosine kinases Src, β-arrestin, and reactive oxygen species (ROS) which, in turn, induce tyrosine phosphorylation and subsequent activation of RTKs [4, 6, 12, 14, 18].

Several MMPs are involved in GPCR-mediated transactivation of different RTKs such as EGFR, VEGFR, and PDGFR, which link to MMP-mediated shedding of ligands causing activation of growth factor receptors. For example, MMP-2 and MMP-9 are involved in the ectodomain shedding of EGFR ligands and, in turn, in EGFR transactivation. Agonist-bound GPCRs also activate the ADAM family, which transactivate different RTKs in several cell lines. ADAMs are a family of metalloproteases that generate diverse bioactive cytokines and growth factors by ectodomain shedding and regulate many important cellular processes, including growth, adhesion, and motility of cells. ADAM10, ADAM12, and ADAM17 are the sheddases of the EGFR ligands in response to various shedding stimulants, such as GPCR agonists, growth factors, phorbol esters, and cytokines. In cardiomyocytes Ang II binds to Ang II type 1 (AT1) receptor, a GPCR, and induces EGFR transactivation through a mechanism mediated by ADAM17 [6, 13, 16, 18].

ROS are also implicated as signaling intermediates in GPCR-RTK transactivation. ROS can activate many protein tyrosine kinases through altering protein-protein interactions; through inactivating by oxidation of the cysteine residue in the catalytic site of protein tyrosine phosphatases, which in turn results in tyrosine kinase activation; and by stimulating proteolysis of regulatory proteins inhibiting tyrosine kinase activity. Increase in ROS production is commonly observed in cells stimulated with GPCR agonists, which is mainly catalyzed by the family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes. In VSMCs, Ang II

induces ROS production via NADPH oxidase activation and EGFR transactivation. In these cells c-Src acts as an upstream regulator of EGFR transactivation and is also required for ROS generation. Moreover, in lung cancer cells, activation of the GPCR formyl peptide receptor induces EGFR transactivation, which depends on c-Src activity. Additionally, several GPCR agonists activate the G_q protein family, which results in production of IP_3 and DAG. The main intracellular targets of DAG are the isoforms of PKC, and IP_3 triggers Ca^{2+} mobilization from endoplasmic reticulum stores leading to an increase of intracellular levels of Ca^{2+}. In VSMCs ROS-induced EGFR transactivation may involve an increase of intracellular Ca^{2+} concentration, which is required for Ang II-dependent EGFR transactivation. In mouse embryonic cells, H_2O_2 increases intracellular Ca^{2+} concentration and the phosphorylation of PKC, ERK, p38MAPK, and c-Jun *N*-terminal kinase (JNK), as well as EGFR transactivation, which in turn induce cell proliferation. Similar to EGFR, PDGFR can be activated not only by its ligands but also by other stimuli in a ligand-independent manner. In VSMCs, for example, H_2O_2 induces ligand-independent tyrosine phosphorylation of PDGFR as well as the association of PKCδ with PDGFRβ and c-Src. Importantly, protein oxidation also tightly controls the activity of RTKs. Many GPCR agonists lead to activation of PKC isoforms, and PKCα especially mediates feedback inhibition of RTK transactivation during GPCR stimulation [4, 6, 8, 13, 15].

The absence of detectable amounts of EGF-like ligands after GPCR stimulation suggests that GPCR-induced EGFR transactivation can be also triggered by intracellular signaling pathways, which require the presence of Ca^{2+}, PKC, and non-receptor tyrosine kinase activation. Several molecular mechanisms promote GPCR-induced activation of Src family kinases, an integral component of the signal transduction machinery used by RTKs, playing a key role in cellular growth and malignant transformation. Conversely Src activity plays a central role in controlling GPCR responses. In many cases Src is associated with GPCRs through direct interaction with cytoplasmic receptor domains or by binding to GPCR-associated proteins, such as heterotrimeric G protein subunits or β-arrestins [4, 6, 9, 15, 18].

Molecular models of GPCR transactivation by RTK ligands depend on the nature of the GPCR-RTK partners and are similar to those employed by GPCR agonists to transactivate RTKs. In some cases, GPCR transactivation occurs in a ligand-dependent manner through synthesis and secretion of a ligand of the transactivated GPCR. This ligand activates the GPCR in an autocrine and/or paracrine manner. In other cases, transactivation of GPCRs by RTK agonists occurs in a ligand-independent manner and requires formation of GPCR-RTK complexes. Several proteins contribute to this event, such as c-Src, which promotes the phosphorylation of cytoplasmic tyrosine residues of GPCRs. This event is crucial for β-arrestin recruitment, which, in turn, promotes internalization of the RTK/GPCR complex and intracellular signaling cascades [4, 14, 15, 18].

Conclusions and Clinical Perspectives

Signal transduction is essential for cellular function. Extracellular signals, which may be stimulatory or inhibitory, mediate cellular responses through ligand-binding to tightly regulated cell membrane-associated receptors, particularly GPCRs and RTKs. Activation of GPCRs typically induces an increase in $[Ca^{2+}]_i$ and activation of MAPKs, important in VSMC contraction-relaxation, migration, and stress responses, while growth factor binding to RTKs plays a key regulatory role in VSMC growth, apoptosis, and differentiation. In addition to ligand-induced receptor activation, ligand-independent factors, such as mechanical forces and pressure, can stimulate receptors. Crosstalk between GPCRs and RTKs leads to complex networking between signaling pathways that ultimately define the cellular phenotype and functional response. Many currently used cardiovascular drugs target GPCRs, for example, Ang II receptor blockers, beta blockers, and ET-1 receptor antagonists. RTKs are also targets for anticancer drugs.

Gaps in Knowledge

GPCRs are the largest transmembrane receptor family found in humans, and it has been estimated that more than half of all modern drugs are targeted at these receptors. Nevertheless, these drugs only target a very small number of GPCRs, leaving an enormous potential for drug developments

within this field. It is also important to better understand the mechanisms that regulate G protein-mediated signaling pathways governing some cell functions (e.g., VSMC). In particular, the spectrum of GPCRs and their G-protein coupling properties as well as their expression levels under specific physiological and pathophysiological conditions are important to be explored. Additionally, the resolution of structural and functional diversity of GPCRs is necessary to develop better disease-targeted drugs. This is challenging because determination of the 3D structure of many GPCRs is still unclear.

Extracellular domain mutations have been described in several RTK families, which result in constitutive receptor dimerization. At the level of the receptor molecules themselves, the importance of direct receptor crosstalk or hetero-/homodimerization for signaling specificity remains unclear, as does the exact role played by internalization and intracellular trafficking. While it is clear that signaling pathways activated by GPCRs and RTKs in VSMCs are interconnected through protein networks, there are still gaps in our knowledge regarding the regulatory processes that connect signaling molecules and how activation of similar pathways can result in different functional cellular responses in health and disease.

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Vasoactive Peptides: Renin-Angiotensin-Aldosterone System

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_9

Key Concepts

- The renin-angiotensin-aldosterone system, through its effector hormone angiotensin II, is an important mediator of cardiovascular hemostasis.
- Overactivity of the renin-angiotensin-aldosterone system is associated with the development and progression of cardiovascular disease.
- The counter-regulatory axis of the renin-angiotensin-aldosterone system adds complexity to the classical renin-angiotensin-aldosterone system and provides endogenous regulation of angiotensin II actions.
- Antagonizing the generation/actions of angiotensin II via drugs is a mainstay of treatment of a range of cardiovascular diseases in patients.
- Next-generation pharmacological interventions are being developed to antagonize the renin-angiotensin-aldosterone system more specifically to avoid side effects in patients.

9.1 The Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is an endocrine system involved in acute regulation of blood pressure (BP) and fluid and electrolyte balance. The classical RAAS is centered on formation of the effector peptide angiotensin II (Ang II) from its substrate angiotensinogen released by the liver (Fig. 9.1). Angiotensinogen is an inactive 14-mer peptide which is constitutively released by the liver and is cleaved by the circulating enzyme renin. Renin is synthesized as an inactive precursor prorenin which is then cleaved and secreted as an active enzyme from afferent arterioles of the renal juxtaglomerular apparatus in response to decreased renal perfusion pressure, decreased sodium levels in the ultrafiltrate of the nephron, or decreased blood pressure leading to sympathetic stimulation of the juxtaglomerular apparatus. The cleavage of angiotensinogen by renin forms angiotensin I (Ang I), a 10-mer, which is subsequently metabolized to the active octapeptide Ang II by angiotensin-converting enzyme (ACE). Alternatively, Ang II can be produced by ACE-independent pathways via cleavage of Ang I by chymases identified in human heart tissue. Aldosterone secretion is stimulated by Ang II, and aldosterone can stimulate mineralocorticoid receptors to mediate sodium resorption, as well as other detrimental cardiovascular effects including oxidative stress, proliferation, and fibrosis. More recently, a counter-regulatory RAAS axis has been discovered which antagonizes the effects of Ang II. This axis centers on the cleavage of Ang II to angiotensin-(1-7) [Ang-(1-7)] by the ACE homologue ACE2 and Ang-(1-7) then acting on its receptor Mas, inhibiting detrimental effects of Ang II, and mediating independent therapeutic effects. Furthermore, other angiotensin peptide metabolites have recently been reported to contribute to RAAS actions. Additionally, the discovery of RAAS components in various tissues led to the characterization of the "local" tissue-specific RAAS which acts independently of the systemic pathway.

9.1.1 Angiotensin-Converting Enzyme

ACE is a zinc dipeptidyl carboxypeptidase and is the major enzyme involved in the formation of Ang II. ACE is non-specific and cleaves not only Ang I but also various other peptides such as bradykinin, substance P, and cholecystokinin. ACE mainly exists as a plasma membrane-bound enzyme on various cell types and is especially abundant in pulmonary endothelial cells and the brush border membrane of the kidney and intestine. ACE shed from the plasma membrane can be detected in various fluids, including blood, urine, and cerebrospinal fluid, and changes in its secretion can be associated with various diseases. The enzyme consists of two homologous catalytic domains, the N- and C- domains (Fig. 9.2). Despite sequence similarity, the domains exhibit important differential functional characteristics and substrate specificities. Both domains cleave bradykinin with equal efficiency, but the C-domain has a higher affinity for Ang I [1]. Furthermore, Ang II is a natural competitive inhibitor at the C-domain and thereby regulates its own production. Several other substrates, such as the peptide acetyl-Ser-Asp-Lys-Pro (ac-SDKP),

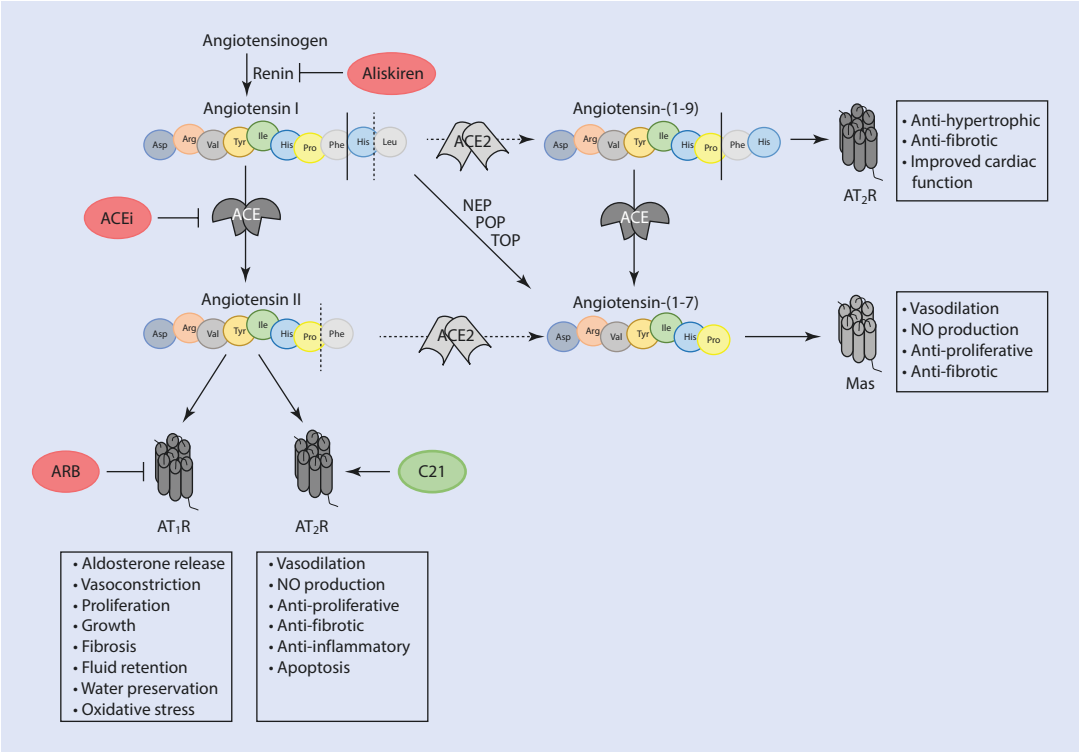

Fig. 9.1 The renin-angiotensin-aldosterone system. Liver angiotensinogen is cleaved by active renin released from the kidney in response to decreased perfusion pressure to form angiotensin I. Cleavage at the Phe-His bond by ACE results in formation of angiotensin II which acts on the AT_1R and AT_2R. Angiotensin I is also cleaved at the His-Leu bond by ACE2 to form angiotensin-(1-9). Angiotensin-(1-7) is formed through cleavage of angiotensin II by ACE2, angiotensin-(1-9) by ACE, or directly from angiotensin I by oligopeptidases (NEP, POP, TOP). Angiotensin-(1-9) interacts with the AT_2R and angiotensin-(1-7) binds Mas. Angiotensin II binds to the AT_1R or AT_2R to mediate pathological or beneficial cardiovascular effects, respectively. Various drugs are effective medicines for treating hypertension and cardiovascular disease including the renin inhibitor aliskiren, ACEi, and ARBs. More recently the first oral agonist of the AT_2R, C21 has been developed and has entered clinical trials to treat pulmonary fibrosis. Abbreviations: ACE, angiotensin-converting enzyme; ACEi, angiotensin-converting enzyme inhibitor; ACE2, angiotensin-converting enzyme 2; ARB, angiotensin type 1 receptor blocker; AT_1R, angiotensin type 1 receptor; AT_2R, angiotensin type 2 receptor; NEP, neprilysin; TOP, thimet oligopeptidase

which has been implicated in hematopoiesis and tissue fibrosis, are predominantly cleaved by the N-domain, and this has important implications for clinical use of ACE inhibitors (ACEi) and potential side effects.

9.1.2 Angiotensin II

Ang II is produced from its precursor Ang I by the cleavage of two residues at the C-terminus to form the active octapeptide. Circulating plasma Ang II levels of healthy normotensive individuals range from 5 to 15 pg/mL with an average half-life of 30 sec in circulation. However, circulating Ang II accumulates in the heart, kidney, and adrenal glands, and this can prolong its half-life to approximately 15 min. In the vasculature, Ang II causes vasoconstriction of small arteries and the renal efferent arterioles. This is concomitant with increased renal sodium reabsorption and the release of antidiuretic hormone from the pituitary gland. In the heart, Ang II increases cardiac contractility, and chronic exposure mediates cardiomyocyte hypertrophy. There are two well-characterized but highly distinct Ang II receptors, the angiotensin type 1 and type 2 receptors (AT_1R and AT_2R, respectively) (Fig. 9.1). Both are seven-transmembrane G protein-coupled receptors (GPCRs) which couple to different signaling pathways mediating contrasting responses.

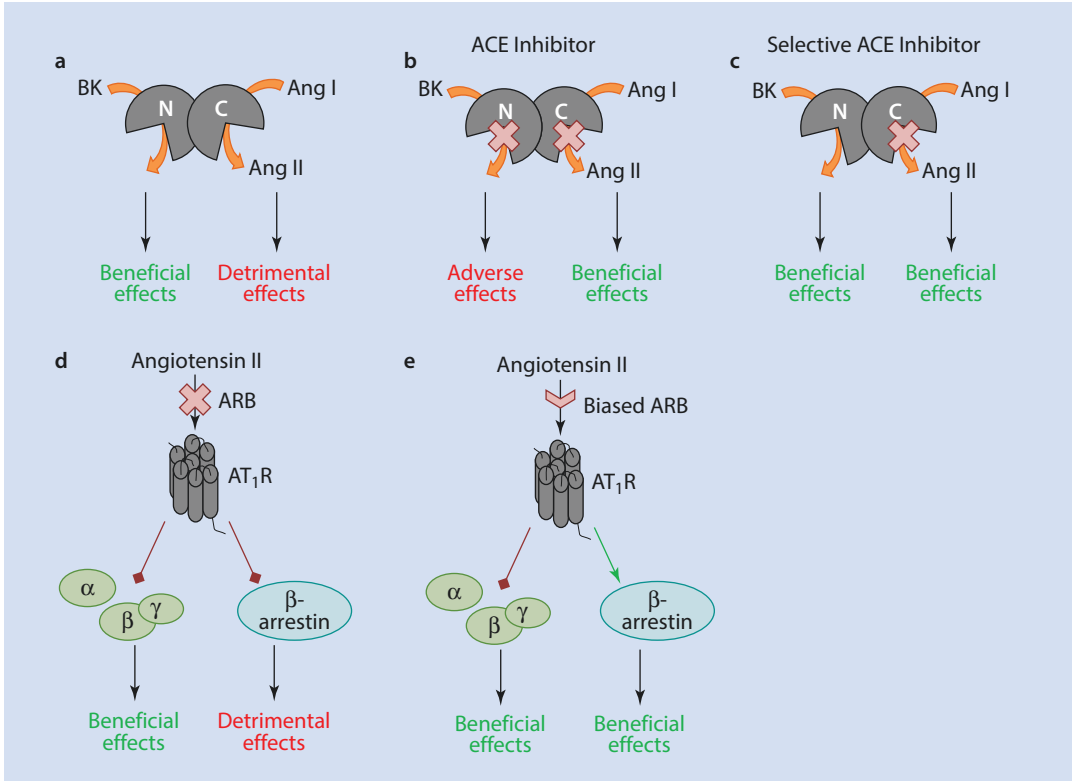

Fig. 9.2 Function of classical and selective ACE Inhibitors. **a** ACE consists of two domains, the N- and C-domain. The C-domain preferentially cleaves angiotensin I (Ang I) to angiotensin II (Ang II) and the N-domain degrades the vasodilator bradykinin. **b** ACEi block both domains inhibiting Ang I conversion to Ang II and thereby mediating beneficial effects. Blockade of BK breakdown leads to side effects. **c** Selective ACEi are designed to target only one domain of ACE. Selective inhibition of the C-domain blocks formation of Ang II but allows breakdown of BK by the N-domain, thereby reducing side effects of ACEi. **d** ARBs block binding of Ang II to the AT_1R and thereby block detrimental G protein-dependent signaling but also inhibit beneficial β-arrestin-dependent pathways. **e** Biased ARBs block Ang II-induced detrimental G protein signaling but allow β-arrestin-mediated signaling, mediating beneficial effects. Abbreviations: ACE angiotensin-converting enzyme inhibitor, Ang I angiotensin I, Ang II angiotensin II, ARB angiotensin type 1 receptor blocker, AT_1R angiotensin type 1 receptor, BK bradykinin

9.1.2.1 The Angiotensin Type 1 Receptor

The AT_1R is a single isoform in humans located on chromosome 3. However, rodents have two isoforms, AT_{1a} and AT_{1b}, with AT_{1a} resembling the human variant. The AT_1R mediates classical Ang II actions and is abundantly expressed in the kidney, vasculature, heart, brain, and adrenal glands, acting to regulate BP and plasma volume [2]. In response to fluid depletion, AT_1R activation rapidly leads to a concerted response to maintain plasma volume by mediating vasoconstriction, aldosterone secretion from the adrenal glands, and renal sodium reabsorption, as well as increased sympathetic tone, cardiac contractility, and stimulation of thirst. In CVD, chronic activation of the RAAS, commonly due to disease-dependent hemodynamic alterations such as decreased cardiac output in heart failure, can lead to fluid retention and thereby increased BP despite absence of volume contraction. Following activation, the AT_1R receptor is desensitized by β-arrestin-mediated endocytosis. However, the long-term stimulation of the AT_1R triggers cardiac and vascular hypertrophy and stimulates deposition of collagens in the heart and vasculature, leading to stiffening and impaired contraction. The AT_1R is selectively bound by AT_1R blockers (ARBs) such as losartan or candesartan, and their use as an antihypertensive medication blocks structural changes in the heart and vasculature independent of BP effects. AT_1Rs

couple to various G proteins, including $G_{q/11}$, G_i, and $G_{12/13}$, leading to signal transduction through phospholipases A_2, C, and D, the release of intracellular Ca^{2+}, and mitogen-activated protein kinase (MAPK) activation. AT_1R signaling can be affected by various regulatory proteins associated with the receptor as well as receptor dimerization. AT_1R dimer formation has been shown to enhance receptor signaling, whereas dimers of AT_1R and AT_2R inhibit Ang II signaling at the AT_1R. The angiotensin receptor-associated protein (ATRAP) plays an important role in the termination and internalization of AT_1Rs through β-arrestin-mediated endocytosis of ligand-bound receptors leading to receptor downregulation and desensitization. In contrast, AT_1R-associated protein 1 (ARAP1) is thought to stabilize the AT_1R at the plasma membrane and increase receptor recycling and has been associated with a role in cardiovascular disease.

9.1.2.2 The Angiotensin Type 2 Receptor

Similar to the AT_1R, the AT_2R is a GPCR encoded by the *Agtr2* gene, located on the X chromosome in most mammalian species and sharing only 34% sequence identity with the AT_1R. The AT_2R is a nonclassical GPCR mediating G protein-independent effects [2]. Activation of the AT_2R antagonizes actions mediated by the AT_1R. This includes vasodilation through the release of bradykinin and the generation of nitric oxide, as well as inducing natriuresis, and it thereby participates in BP regulation. Moreover, AT_2R signaling reduces cell proliferation and induces apoptosis, opposing the pro-proliferative actions of Ang II at the AT_1R. The AT_2R is the predominant isoform during fetal development but rapidly declines after birth. In the adult, it is mainly found in the brain, heart, adrenal medulla, kidney, and reproductive organs. However, AT_2R expression significantly increases in CVD, suggesting a compensatory role for the AT_2R in disease. Unlike the AT_1R, the AT_2R is not internalized on Ang II binding, and there is no evidence of receptor desensitization following chronic Ang II stimulation. The AT_2R has very low affinity for ARBs but has a high affinity for the peptides PD123319 and PD123177 which function as antagonists, as well as CGP42112 which is a partial agonist at the receptor. More recently an oral agonist of the AT_2R, Compound (C)21, has been reported and is a novel promising intervention in CVD treatment (Fig. 9.1). C21 was the first non-peptide oral agonist synthesized for the AT_2R and has been extensively employed to assess the physiological functions of the AT_2R and is now in clinical trials for pulmonary fibrosis [3]. C21 has also been demonstrated to preserve cardiac function post-myocardial infarction (MI) by reducing infarct size and preventing adverse cardiac remodeling and induce vasodilation in experimental models. Three main signaling cascades have been proposed for the AT_2R: stimulation of nitric oxide release and the formation of cyclic guanosine monophosphate; activation of phospholipase A_2; and activation of protein phosphatases such as Src homology region 2 domain-containing phosphatase-1, mitogen-activated protein kinase-1, and protein phosphatase 2A. Recent evidence in rodents suggest that females have higher AT_2R expression and therefore higher AT_2R-mediated effects than males, and this will be interesting to explore further.

9.1.3 Local Tissue-Specific RAAS

The discovery of RAAS components in various tissues, including the heart, kidney, and brain, has led to the characterization of a local tissue-specific RAS acting independently of the endocrine systemic RAAS pathway [4]. In this setting, independent Ang II-forming systems involving cathepsin G, kallikrein, and chymase have been described in several tissues including the heart, kidney, blood vessels, and immune cells. For example, in human blood vessels, it has been estimated that more than half of the generated Ang II is derived via non-ACE pathways, and in the human heart, the serine protease chymase accounts for the majority of locally produced Ang II.

9.2 Drugs Targeting the Classical Axis of the RAAS

Drugs targeting the RAAS are the main first-line treatments in patients with hypertension. The first and rate-limiting step of Ang II generation can be targeted using the renin inhibitor aliskiren (Fig. 9.1). Aliskiren was introduced in 2007 and binds to renin preventing conversion of angiotensinogen to Ang I. Aliskiren is, however,

contraindicated in patients with diabetes or kidney impairment, especially when also taking an ACEi or ARB.

9.2.1 ACE Inhibitors

The first natural ACEi was isolated from Brazilian arrowhead viper venom, leading to the synthesis of synthetic peptide inhibitors targeting ACE. Since the release of captopril in 1981, several ACEi have been developed for clinical use (e.g., enalapril, lisinopril). All ACEi bind and inhibit the ACE catalytic site, preventing cleavage of Ang I to Ang II, decreasing circulating Ang II levels, and reducing aldosterone, which leads to sympathetic activation and vasopressin secretion and elevated levels of the vasodilator bradykinin. As a result of the inhibition of Ang II formation, circulating renin levels are increased due to the lack of negative feedback by Ang II to the kidneys. ACEi are a heterogeneous drug class, differing in structure and pharmacokinetics which determines absorption, half-life, and inhibition kinetics, allowing for optimal treatment of patients with a variety of comorbidities (Table 9.1) [5]. For example, certain ACEi, such as enalapril and lisinopril, are delivered as a prodrug and are only activated when esterified in the liver. The delivery as a prodrug enhances their oral bioavailability compared with active ACEi. It has been suggested that the effect on BP and cardiovascular pathology by ACEi correlates better with the inhibition of tissue rather than circulating ACE. The effect of ACEi is maintained during chronic drug treatment; however, it has been observed that despite chronic ACE inhibition and maintained antihypertensive efficacy, Ang II levels may return to normal or supranormal levels. This "Ang II escape" is thought to be at least in part due to non-ACE pathways of Ang II generation and an increase in ACE transcription and catalytic activity. This would suggest that ARBs may be more effective in treatment of hypertension and preventing Ang II escape; however, studies comparing ACEi and ARBs revealed no difference in efficacy and recommended ACEi as first-line treatment in primary hypertension [6].

Current ACEi have similar affinities for the ACE C- and N-domains leading to side effects such as cough and angioedema, attributed to bradykinin accumulation. The ACE C-domain solely mediates the hypertensive effect of an Ang I bolus infusion, whereas the N-domain is the primary route for ac-SDKP clearance [7]. Therefore, the selective inhibition of one ACE domain could be clinically useful to reduce adverse effects (Fig. 9.2a–c). Selective inhibition of the N-domain would allow accumulation of the anti-fibrotic and anti-inflammatory peptide ac-SDKP and could be employed in fibrotic and inflammatory diseases without affecting systemic BP regulation. In contrast, selective inhibition of the C-domain would prevent Ang I conversion and regulation of BP but allow cleavage of bradykinin and thereby reduce side effects. It has been demonstrated that a C-selective analogue of lisinopril, Lis-W, is equally effective to lisinopril in reducing circulating Ang II levels and lowering BP but does not lead to bradykinin accumulation [8]. These therapeutic options are currently being explored in experimental models and may offer optimal and efficacious therapeutics for hypertension and CVD treatment.

9.2.2 Angiotensin Type 1 Receptor Blockers

With release of losartan in 1995, ARBs are one of the newer classes of drugs for hypertension and act by blocking Ang II binding to the AT_1R, antagonizing pathological effects, reducing BP, and increasing cardioprotection. In contrast to ACEi, ARBs cause increased Ang II levels due to loss of negative feedback through the AT_1R. Theoretically, this may increase signaling through the AT_2R mediating cardioprotective effects. To date, several ARBs have been licensed for clinical use, one of the most recent being azilsartan medoxomil [11]. ARBs vary in structure and have differing pharmacokinetics (Table 9.1). Losartan, valsartan, and eprosartan are surmountable antagonists and can be displaced by high concentrations of Ang II. In contrast, irbesartan, candesartan, and olmesartan are insurmountable antagonists and only dissociate very slowly from the AT_1R and cannot be displaced by Ang II. New developments for hypertension treatment have moved away from single-target ARBs toward dual inhibitors and ARBs with biased agonism. Advances have been made with the development of a drug with dual AT_1R and neprilysin antagonism, LCZ696 [11]. Neprilysin is a neutral endopeptidase which cleaves several vasodilators and vasoconstrictors

Table 9.1 Pharmacological properties of ACE inhibitors and ARBs

ACE inhibitors

Name	Prodrug	Elimination	Dosage (mg)	Bioavailability (%)	Serum half-life (h)	Duration (h)	Peak effect (h)
Benazepril	Yes	Kidney	5–80	<37	10–11	24	2–4
Captopril	No	Kidney	6.25–300	75–91	<2	6–12	1–1.5
Enalapril	Yes	Kidney	2.5–40	60	11	24	4–6
Fosinopril	Yes	Kidney, Liver	10–80	36	11	24	2–6
Lisinopril	No	Kidney	5–40	6–60	13	24	6
Moexipril	Yes	Kidney	7.5–30	13	2–9	24	4–6
Quinapril	Yes	Kidney	5–80	>60	2	24	2
Ramipril	Yes	Kidney	1.25–20	50–60	13–17	24	3–6
Trandolapril	Yes	Kidney, Liver	1–8	70	16–24	24	6–80

ARBs

Name	Prodrug	Elimination	Dosage (mg)	Bioavailability (%)	Serum half-life (h)	AT_1R antagonism
Candesartan (CV11974)	Yes	Hepatic > Renal	4–16	– (42)	3.5–4 (3–11)	Insurmountable
Eprosartan	No	Hepatic > Renal	400–800	15	5–7	Competitive
Irbesartan	No	Hepatic > Renal	150–300	70	11–15	Insurmountable
Losartan (EXP-3174)	Yes	Hepatic > Renal	50–100	33	2 (6–9)	Competitive
Olmesartan	Yes	Hepatic > Renal	20–40	26	13	Insurmountable
Telmisartan	No	Hepatic	40–80	43	24	Insurmountable
Valsartan	No	Hepatic > Renal	80–230	25	9	Competitive

Data taken from Zaman et al. [5]; Brown and Vaughan [9]; Burnier [10]
Abbreviations: *ACE* angiotensin-converting enzyme, *ARB* angiotensin type 1 receptor blocker, *AT_1R* angiotensin type 1 receptor

and can thereby affect BP regulation. It is hypothesized that vasoconstrictor blockade at the AT_1R and dual inhibition of neprilysin lead to vasodilator accumulation and therefore net vasodilation. LCZ696 achieved greater reduction in sitting systolic and diastolic BP than comparable valsartan doses after 8 weeks and is a promising development in hypertension [12].

The concept of biased agonism developed with the discovery that recruitment of β-arrestin to ligand-bound receptors leads to both internalization and induction of β-arrestin-dependent signaling pathways, distinct from classical GPCR responses [13]. This has been characterized for the AT_1R (Fig. 9.2d, e) and includes β-arrestin-mediated cytoplasmic sequestration of signaling

and inhibition of gene transcription, cell survival, and positive inotropy [13]. This has opened a new avenue for drug development of biased AT_1R antagonists that allow beneficial signaling through the AT_1R via β-arrestin while inhibiting the pathological actions mediated through classical GPCR signaling pathways. The β-arrestin-biased AT_1R ligand TRV120027 antagonizes Ang II signaling at the AT_1R but induces β-arrestin recruitment to the AT_1R, reducing BP, increasing cardiac function, and maintaining cardiac and renal performance in disease models [14]. These results are promising for the development of future novel treatments for hypertension and heart failure.

9.3 The Counter-Regulatory Axis of the RAAS

9.3.1 Angiotensin-Converting Enzyme 2

ACE2 is an ACE homologue and is a transmembrane zinc metalloprotease with 40% homology to ACE but not inhibited by common ACEi (Fig. 9.1) [15]. ACE2 has high specificity for Ang II to generate Ang-(1-7) and can also cleave Ang I to produce angiotensin-(1-9) [Ang-(1-9)]. This renders ACE2 an important regulator of the RAAS by degrading Ang II and opposing the actions of ACE. In contrast to ACE, ACE2 is mainly found in the heart, kidney, and testis localized to endothelial and epithelial cells. In the heart and vasculature, ACE2 is an essential regulator of cardiac and vascular function by counteracting Ang II signaling. Polymorphisms in the ACE2 gene have been correlated with increased risk of CVD.

9.3.2 Angiotensin-(1-7) and Angiotensin-(1-9)

Ang-(1-7) is a heptapeptide that is generated by the cleavage of the terminal phenylalanine residue from Ang II by ACE2 and is an endogenous ligand for the G protein-coupled receptor Mas which mediates its beneficial effects (Fig. 9.1). Ang-(1-7) has broad therapeutic effects in the cardiovascular system, and Ang-(1-7) and Mas are being investigated as novel therapeutic targets in CVDs including cardiac and vascular diseases and diabetes (reviewed in [16]).

Ang-(1-9) is a decapeptide that is generated by the cleavage of the terminal leucine residue from Ang I by ACE2 and can be further metabolized to Ang-(1-7) by ACE (reviewed in [16]) (Fig. 9.1). Less well investigated than Ang-(1-7), nonetheless Ang-(1-9) also mediates therapeutic effects and has been reported to be a natural ACEi due to its significantly slower hydrolysis compared with Ang I. Moreover, Ang-(1-9) has been reported to mediate beneficial effects on cardiac remodeling and function in experimental models through a functional effect at the AT_2R.

9.3.3 Other RAAS Peptide Metabolites

In recent years there has been a substantial expansion in our knowledge of the RAAS and with the identification of alternative pathways for Ang II generation; further angiotensin peptide fragments have been identified some of which have been demonstrated to be biologically active. While there is not the space in this chapter to cover these other peptide metabolites in detail, peptides of note include Ang-(1-12), Ang III, Ang IV, and alamandine [17]. Ang-(1-12) is an alternative angiotensinogen-derived substrate for Ang II generation first isolated in the small intestine; Ang-(1-12) is also abundant in heart tissue and is converted to Ang II via chymase, suggesting the existence of renin-independent pathways of Ang II production that may have physiological and pathophysiological relevance. Ang III is generated by aminopeptidase A-mediated cleavage of Ang II leading to lower Ang II levels. Ang III has been reported to be a ligand for the AT_2R and stimulate aldosterone secretion. Ang IV is produced via aminopeptidase N-mediated cleavage of Ang III and is reported to act at the novel receptor, AT_4R, otherwise termed insulin-regulated aminopeptidase. Alamandine is a decarboxylated derivative of Ang-(1-7) reported to be a ligand for the Mas-related GPCR, member D (MRGD), which reduces blood pressure and tissue fibrosis. Overall, these examples highlight the complexity of the RAAS.

Conclusions and Clinical Perspective

Since its discovery, the RAAS has been extensively studied, and with advanced understanding of its role in CVD, drugs targeting Ang II generation and

actions are the mainstay in the treatment of hypertension and other CVD. Development of new next-generation ACEi and ARBs potentially offers more selective and efficacious treatments, with reduced adverse effects. Additionally, development of the first oral AT_2R agonist, C21, to enhance beneficial signaling through the AT_2R has proven successful for the treatment of CVD in preclinical models and has entered phase I clinical trials [3]. Discovery of the counter-regulatory RAAS axis and the beneficial actions of ACE2 and Ang-(1-7) in a variety of diseases has revealed new opportunities for drug development.

Gaps in Knowledge

- The discovery of the counter-regulatory axis of the RAAS has challenged our view of the classical RAAS and added increased complexity to the system with many physiological and pathological roles of this complex system yet to be characterized.
- Next to angiotensin-(1-7), other Ang I and Ang II peptides, such as angiotensin-(1-9), Ang-(1-12), Ang III, Ang IV, and alamandine, have been demonstrated to be biologically active with their physiological and pathological roles yet to be determined.
- The identification of an Ang I-independent pathway of Ang II formation via Ang-(1-12) and chymase, and further angiotensin peptides and receptors, has raised many questions over our current understanding of the RAAS.

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Aldosterone

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_10

Key Concepts

- Aldosterone biosynthesis is a tightly regulated process that is required in order to maintain normal physiological control of cardiovascular function.
- Inappropriately high secretion of aldosterone, as observed in primary aldosteronism (PA), results in hypertension and cardiovascular damage.
- PA is the most common curable form of hypertension, but significant advances are still required to improve its diagnosis and target treatment of its various subtypes more effectively.

10.1 Introduction

In the mid-1950s, James and Sylvia Tait identified a new steroid hormone that acted as a mineralocorticoid, causing reabsorption of sodium and excretion of potassium in the kidney. This hormone, which was named aldosterone, was soon confirmed as having a key role in blood pressure homeostasis through the work of Jerome Conn, who first described a case of primary aldosteronism (PA) in a patient whose hypertension and hypokalaemia were the result of an aldosterone-producing adenoma in the adrenal gland. In the subsequent years, relative aldosterone excess, commonly defined as a raised aldosterone-to-renin ratio (ARR), has been recognised as a frequent causative factor in hypertensive patients, and its correction is proven to have significant therapeutic value. In addition, clinical studies using pharmacological blockade of aldosterone action have demonstrated that this hormone is not limited to blood pressure effects but has a physiological and pathophysiological role in a number of other tissues, including the heart and vasculature. As such, understanding aldosterone synthesis, regulation and action is crucial if we are to reduce cardiovascular risk and better target therapeutic intervention in hypertensive patients [1]. In this article we summarise what has been learned about aldosterone biosynthesis, regulation and action over the last 60 years and highlight those areas that will be key to future scientific breakthroughs and medical treatments.

10.2 Aldosterone Biosynthesis

The adrenal glands are encased in a fibrous capsule and embedded in the fat above each kidney. The gland is composed of a catecholamine-producing medulla surrounded by the steroid hormone-producing cortex. The cortex is made up of three concentric layers, which each consist of distinct cell types and produce different hormones due to the range of steroidogenic enzymes expressed by each [2]. It is the outermost cells of the zona glomerulosa (ZG) clustered just under the fibrous capsule that are responsible for aldosterone biosynthesis. In the rat, the ZG forms a thin unbroken layer, but in the human adrenal cortex, these cells tend to cluster into small clumps called 'baskets' around the edge of the cortex; some may also coat the centripetal vessels that drain towards the medulla. Beneath the ZG lies the much larger and thicker zona fasciculata (ZF), primarily responsible for cortisol biosynthesis in humans (corticosterone in rodents); between the ZF and the medulla is the zona reticularis (ZR), which produces the adrenal androgens dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEA-S).

Aldosterone is synthesised from cholesterol through a series of enzymatic conversions that occur entirely within the cells of the ZG (Fig. 10.1). Aldosterone synthase is a steroidogenic enzyme encoded by the *CYP11B2* gene; it performs the final stages of aldosterone biosynthesis and is therefore essential to its production. As *CYP11B2* expression is confined to the ZG, so too is aldosterone biosynthesis.

All steroidogenesis begins with the conversion of cholesterol to pregnenolone by the P450scc enzyme, which is located on the inner mitochondrial membrane. Delivery of cholesterol from the outer to the inner membrane is performed by the steroidogenic acute regulatory protein (StAR), which is present in all steroidogenic tissues; its function is a rate-limiting step in aldosterone biosynthesis (increasing the conversion of cholesterol to pregnenolone approximately sevenfold) although its mechanism of action is not completely understood. The importance of StAR to steroidogenesis is apparent from mutation of the *StAR* gene, which can result in lipoid congenital adrenal hyperplasia (CAH) characterised by the severe impairment of steroid hormone biosynthesis, elevated levels of ACTH and enlargement of

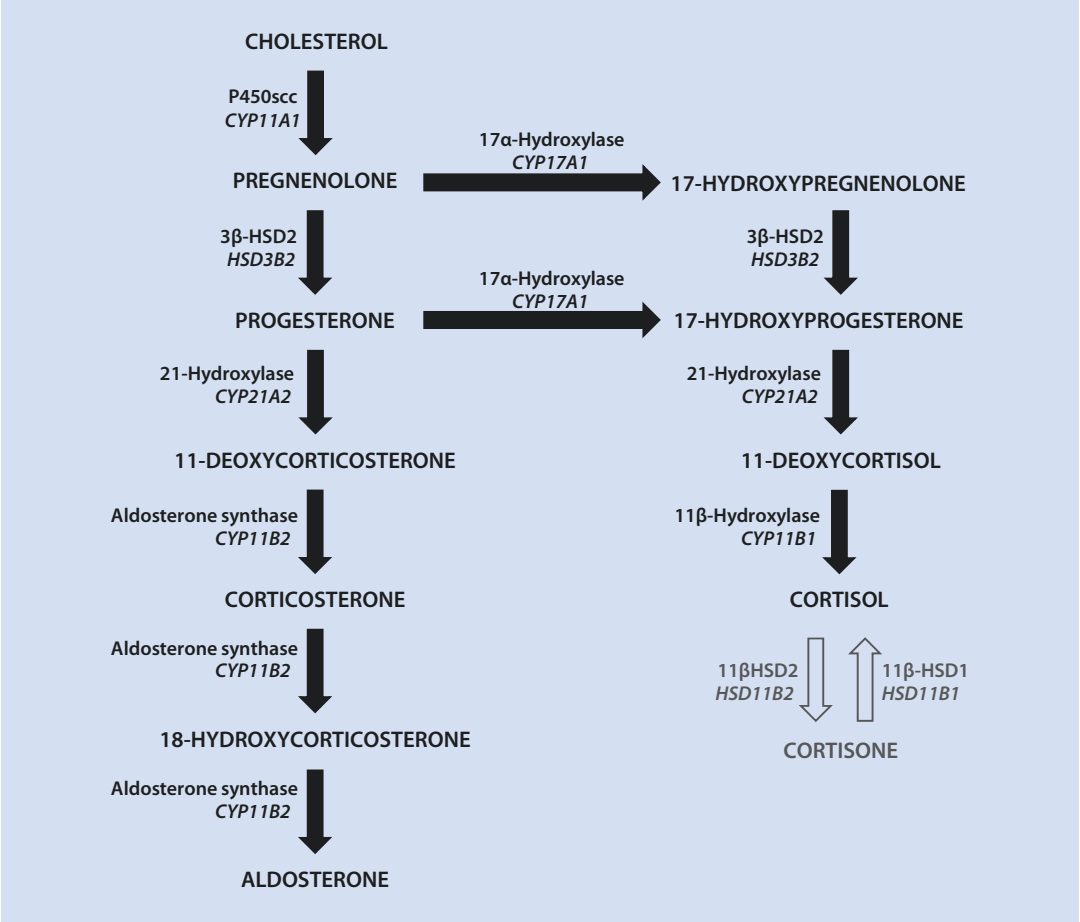

Fig. 10.1 Simplified summary of the human steroidogenic pathways within the adrenal cortex that lead to the production of aldosterone and cortisol. The region-specific production of different hormones is the result of differential enzyme expression throughout the cortex, e.g. the restriction of aldosterone synthase to the zona glomerulosa. In peripheral tissues (indicated by white arrows), cortisol and its inactive form cortisone can be interconverted by the action of the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSD2 is responsible for converting cortisol to cortisone, thereby conferring aldosterone selectivity on tissues where it is expressed; 11β-HSD1 is capable of bidirectional conversion but, as indicated, acts predominantly to form cortisol in the periphery

the adrenal glands, which accumulate high levels of cholesterol and cholesterol esters [2].

Following delivery of cholesterol, P450scc can commence aldosterone biosynthesis. This enzyme, like 21-hydroxylase and aldosterone synthase which function later in the pathway, is a member of the cytochrome P450 enzyme superfamily which uses haem as a cofactor. To form pregnenolone P450scc catalyses three reactions all at the same active site, including cleavage of the C-20 to C-22 bond (the numbers refer to the positions of the relevant carbons on the cholesterol backbone). P450scc is essential to the steroidogenic cell, and loss of its activity – as occurs in rare mutations to the *CYP11A1* gene on human chromosome 15 – prevents steroidogenesis and results in lipoid CAH very similar to that caused by mutation of *StAR*.

The newly formed pregnenolone is released into the cytosol to be converted to progesterone by the type 2 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase enzyme (3β-HSD2) located on the smooth endoplasmic reticulum. This type 2 isoform is expressed in the adrenal gland and gonads and is encoded by the *HSD3B2* gene on human chromosome 1, mutation of which can result in fatal steroid deficiency. Conversion of progesterone to 11-deoxycorticosterone (DOC) is

then performed by 21-hydroxylase, the product of the *CYP21A2* gene, which lies on human chromosome 6p21.1 in tandem with a pseudogene *CYP21A1P*. This genetic arrangement is responsible for a relatively high frequency of mutation at this locus; 21-hydroxylase deficiency is the most common form of CAH which, in severe form, can cause death in untreated newborns owing to aldosterone deficiency.

The resulting DOC is the preferred substrate for aldosterone synthase, and its conversion to aldosterone is the result of three consecutive reactions each catalysed by this enzyme: 11β-hydroxylation of DOC to form corticosterone, 18-hydroxylation to make 18-hydroxycorticosterone (18-OH-B) and, finally, 18-methyloxidation to produce aldosterone. Although the substrate remains bound to the enzyme throughout this process, corticosterone and 18OH-B can be released as by-products. The *CYP11B2* gene encoding aldosterone synthase lies on human chromosome 8 in tandem with the *CYP11B1* gene that encodes the 11β-hydroxylase enzyme and is responsible for the final stage of cortisol production (Fig. 10.1). The two genes are highly similar across their genetic sequences, and this is reflected in their shared 11β-hydroxylation function. However, zonal regulation of these genes' expression is starkly different: as the final steroidogenic enzyme to act in the generation of cortisol, 11β-hydroxylase is strongly expressed in the ZF but not at all in the ZG, while, conversely, *CYP11B2* expression is confined to the ZG. Regulatory differences between these genes also account to a large degree for the contrast between the regulation of cortisol secretion, mainly controlled by the hypothalamic-pituitary-adrenal (HPA) axis and that of aldosterone.

10.3 Regulation of Aldosterone Biosynthesis

The inability of the adrenal gland to store steroid hormones means that they must be produced 'on demand' for secretion immediately after production. For this reason, aldosterone biosynthesis and its regulation are closely related, largely through control of cholesterol supply to the ZG mitochondria and the up- or downregulation of genes encoding the relevant steroidogenic enzymes, particularly *CYP11B2*. The principal regulatory systems determining the degree of aldosterone secretion by the adrenal cortex under normal physiological conditions are the renin-angiotensin system (RAS), extracellular potassium concentration ($[K^+]_e$) and, to a lesser extent, the HPA, although a number of other factors – e.g. leptin, atrial natriuretic peptide (ANP), dopamine and serotonin – have been shown to modify its production [3, 4]. In this section we will focus on the major regulatory systems.

10.3.1 The Renin-Angiotensin System

The RAS has significant influence on fluid balance and blood pressure, mediated in large part through its regulation of aldosterone secretion. The system is activated in response to reduced intravascular pressure or volume, as measured by baroreceptors in the carotid sinus, or to lowered sodium concentrations, which is determined by renal macula densa cells. This stimulates the cleavage of prorenin to form renin and its release in to the circulation from the juxtaglomerular cells located in the afferent arteriole of the kidney. Circulating renin then catalyses the hydrolysis of angiotensinogen secreted by the liver to form the biologically inert decapeptide angiotensin I. The action of angiotensin-converting enzyme (ACE) cleaves a further two residues to yield the active component of this system, the octapeptide angiotensin II (AngII). AngII has several hypertensive actions: it raises blood pressure by direct vasoconstriction – increasing both sympathetic nerve activity and myocardial contractility – and also by enhancing renal salt and water retention through the stimulation of adrenal aldosterone production. In the adrenal gland, AngII acts on the type I AngII receptors (AT1R). Binding of these G protein-coupled receptors, located on the membrane of ZG cells, induces signal transduction that results in the activation within the cell of phospholipase C (PLC). This then stimulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).

Calcium is an important second messenger modulating aldosterone biosynthesis within ZG cells, and AngII induces a rise in intracellular calcium in various ways. For example, IP_3 binds

receptors on the endoplasmic reticulum, resulting in the release of Ca^{2+} from intracellular stores. This initial surge is later supplemented by extracellular calcium, which enters through voltage-dependent calcium channels that are opened upon depolarisation of the ZG cell. At rest, a ZG cell has a strongly negative potential due to the concentration gradient of K^+ across its membrane. AngII can induce depolarisation by inhibiting the function of potassium channels, resulting in the opening of voltage-gated calcium channels and a flood of calcium into the cytosol. Similarly, DAG generated by AngII inhibits TWIK-related acid-sensitive K^+ (TASK) channels, causing depolarisation of the cell membrane and calcium influx.

The result of this rise in Ca^{2+} concentration in the cytosol of ZG cells is the activation of calcium/calmodulin-dependent protein kinases (CaMK); CamKII appears to be particularly important to the aldosterone response in the ZG. As calcium concentrations increase, so too does the phosphorylation of StAR, stimulating the supply of cholesterol to the mitochondria and the early stages of steroidogenesis. Greater delivery of calcium to the mitochondrion raises the levels of cofactors vital to mitochondrial cytochrome P450 enzyme function, thereby aiding the activities of P450scc and aldosterone synthase. The increased demand of ZG cells for cholesterol is addressed through other actions of AngII; it increases the uptake of high- and low-density lipoproteins (HDL and LDL) for this purpose, while, intracellularly, it activates hormone-sensitive lipase (HSL) to release esterified cholesterol from lipid droplets.

In the longer-term, expression of the various steroidogenic components can also be raised to boost aldosterone production. CaMKs activate various transcription factors capable of binding specific *cis*-acting responsive elements upstream of the *CYP11B2* and *StAR* genes to stimulate their transcription. The Ad-5 cis-element in *CYP11B2* binds steroidogenic factor 1 (SF-1), members of the NGFI-B family and chicken ovalbumin upstream promoter-transcription factor (COUP-TF). In addition, NURR1, a member of the NGFI-B family of orphan nuclear receptors, is found at high levels in ZG, and its levels are raised by AngII; it is also upregulated in aldosterone-secreting tumours.

10.3.2 Potassium

Aldosterone secretion is stimulated by $[K^+]_e$, thereby maintaining potassium homeostasis through its excretion. ZG cells are acutely sensitive to increased K^+, and many of its effects synergise with those of AngII. Increased $[K^+]_e$ raises the likelihood of ZG cell depolarisation, resulting in Ca^{2+} channel opening and CAMK activation, as described above. Therefore, stimulation of *CYP11B2* transcription, for example, by both AngII and K^+, occurs through a common pathway. In contrast with AngII, there is some evidence that potassium may also employ cyclic AMP (cAMP) as a second messenger, albeit at a low level. This suggests potassium may also share regulatory pathways with ACTH, which does stimulate aldosterone production via cAMP.

10.3.3 ACTH

As the major regulator of cortisol secretion from the adrenal cortex, ACTH is a component of the stress-responsive HPA. It is a 39 amino acid peptide synthesised as part of the larger precursor molecule pro-opiomelanocorticotrophin (POMC) and is secreted from the anterior pituitary under a diurnal rhythm, its levels being highest in the morning and lowest at night. Underlying this is a continuous pulsatility apparent from plasma cortisol concentrations. Acutely, ACTH stimulates adrenal blood flow and increases aldosterone production by binding its membrane-bound G protein-coupled receptor, the melanocortin type 2 receptor (MC2R), thereby generating higher levels of intracellular cAMP. This in turn activates protein kinase A (PKA), which can induce steroidogenesis through phosphorylation of proteins, such as StAR, and induce gene expression via promoters that possess a cAMP-responsive element (CRE). Upon ACTH-induced phosphorylation, CRE-binding (CREB) transcription factors can stimulate such genes, which include *CYP11B2*. The *StAR* promoter lacks a canonical CRE sequence in its promoter but is still capable of stimulation by cAMP due to its activator protein-1 (AP1)-like element, which can bind CREB. PKA may also stimulate the Ca^{2+} influx through phosphorylation of calcium channels. Early studies of ACTH and its effect on

aldosterone secretion showed that, while ACTH is clearly a significant acute regulator of aldosterone in human subjects, chronic administration of ACTH sees aldosterone fall back to basal levels within 72 h, following an initial rise. The mechanism by which aldosterone secretion and *CYP11B2* expression become suppressed under such conditions is not known but has led to a general perception that ACTH is of little importance in the long-term control of aldosterone, particularly in comparison to AngII and K^+. However, there is growing evidence to support its role as a key regulator of aldosterone [5]. Studies of the POMC knockout mouse show abnormal adrenocortical morphology and reduced but detectable levels of aldosterone, implying that ACTH is required for normal aldosterone synthesis. Similar circadian rhythms have been identified for ACTH and aldosterone, with both peaking in the morning and falling throughout the day. Furthermore, chronic administration of ACTH to human subjects in a pulsatile manner more reflective of in vivo ACTH secretion is found not to result in inhibition of aldosterone levels, which remains elevated throughout. Therefore, ACTH may have a physiological role in modulating the aldosterone response to the major regulatory factors AngII and potassium. This effect can become more apparent when regulation breaks down. A recent study of essential hypertension patients identified fully a quarter of these subjects to be 'hyperresponsive' to ultra-low doses of ACTH, their circulating aldosterone reaching levels four times higher than that of the remaining hypertensive subjects and normotensive control subjects; cortisol secretion response did not differ across these subjects [6]. Similar results were also obtained by subjecting study participants to low-level stress in the form of physical exercise. These findings suggest that ZG cells in a large subset of hypertensive individuals can be rendered sensitive to stress-induced ACTH secretion, possibly due to genetic mutations that predispose to ZG responsiveness. It is interesting to note that high levels of cortisol are also known to sensitise the adrenal gland to AngII by upregulating AT1R expression, further demonstrating the interplay between stress response and aldosterone secretion. The implication that stressful lifestyles might have a significant deleterious impact on cardiovascular health through such mechanisms clearly warrants further investigation.

In addition to adrenal production of aldosterone, many studies – including our own – have focused on the possibility of aldosterone biosynthesis from cholesterol occurring in extra-adrenal tissues, particularly the vasculature, CNS and adipose tissue, where proximity of aldosterone production to its receptors would imply a paracrine or autocrine mode of action. Although early studies were promising with, for example, expression of steroidogenic enzymes including *CYP11B2* being observed in rodent brains, studies of human tissue have either failed to identify consistent evidence of such expression or have found it to occur at such low levels that any physiological impact is questionable [7]. Definitive proof of significant extra-adrenal biosynthesis in human tissues is still awaited.

In recent years attention has also turned to the role that epigenetic mechanisms of regulation may play in controlling aldosterone secretion. While there has been some study of chromatin regulation and DNA methylation [8], most investigations have focused on noncoding RNA and, in particular, microRNA (miRNA). This is a class of small single-stranded RNA molecules, approximately 22 nucleotides in length, which is able to 'fine-tune' expression of specific target genes. This is achieved post-transcriptionally through partial complementary binding of miRNA to the 3′ untranslated regions of target mRNA, inducing either its degradation or repression of its translation, in order to downregulate expression. By inhibiting the production of all miRNAs within adrenocortical cells, our own studies have demonstrated a significant role for miRNAs in the regulation of aldosterone secretion [9]. Our subsequent work has focused on identifying individual adrenally expressed miRNAs that target specific components of the steroidogenic machinery. These include miR-24, mir-125a-5p and miR-125b-5p, which each target *CYP11B2* mRNA, as well as miR-320a-3p, which targets *CYP11A1*. Others have shown miRNA regulation of StAR, while miR-34 and miR-23 targeting of potassium channel expression has also been shown to affect levels of aldosterone and *CYP11B2* expression [10]. Therefore, there is great interest not only in which miRNAs target which mRNAs but also in how individual miRNA levels are themselves controlled, particularly under such physiologically relevant conditions as AngII stimulation. Although the impact of a miRNA on a single

target mRNA may be small when compared to more conventional genetic regulatory mechanisms, the ability of multiple miRNAs to target a single mRNA species – or of a single miRNA species to target many different mRNAs simultaneously – means these noncoding RNAs are likely to be highly important in aldosterone homeostasis and, therefore, a valuable diagnostic and therapeutic biomarker in disease. Indeed, dysregulation of miRNA levels in aldosterone-producing adenoma (APA; see below) relative to non-diseased adrenal tissue has been demonstrated [9], and such comparisons are likely to aid in identifying those miRNAs that are of importance in aldosterone secretion. Furthermore, the secretion by tissues of certain miRNAs into the plasma, where they circulate with relative stability, offers the prospect that certain miRNAs derived from diseased adrenal glands may be easily sampled and identified for the diagnosis of conditions that are currently hard to diagnose accurately, as in subtypes of primary aldosteronism (see below).

In summary, aldosterone secretion from the adrenal cortex is tightly controlled to preserve the circulating volume and potassium homeostasis. The amplification of AngII effects on aldosterone by potassium status illustrates the importance of guarding against hyperkalaemia, while there is emerging evidence that ACTH may be of importance when normal regulation of aldosterone breaks down. The consequences of such regulatory loss on cardiovascular function will be discussed later.

10.4 Aldosterone Action

Like other steroid hormones, following its synthesis aldosterone is capable of diffusing freely through target cell membranes to act on the intracellular receptors within. By definition, any cell that expresses the receptor is a target of the hormone. The classic effects of aldosterone are mediated via the mineralocorticoid receptor (MR), which is the product of the *NR3C2* gene. MR, like the glucocorticoid receptor (GR), progesterone receptor and androgen receptor, is a member of the nuclear receptor subfamily 3C, which is a large and diverse group of transcription factors [11]. MR and GR share a high degree of structural homology, which reflects the great similarity between their corticosteroid ligands. While GR is widely expressed, MR distribution is more limited, but it is still found in such diverse tissues as the kidney, colon, CNS, heart, sweat glands and adipose tissue.

In its unbound state, MR is located mainly in the cytoplasm of target cells where it is stabilised by various heat-shock proteins (HSPs). Binding of the MR by aldosterone (or another ligand with affinity for the receptor) induces a conformational change in the receptor, dissociation of the HSPs and translocation of the aldosterone-MR complex to the cell nucleus [12]. Here it acts as a transcription factor, binding the promoter regions of target genes at specific hormone response element (HRE) sequences to influence transcription (Fig. 10.2). These so-called genomic actions of aldosterone/MR lead primarily to the expression of proteins involved in sodium reabsorption. These include the Na^+/K^+-ATPase, a pump located in the basolateral membrane of the distal nephron, and the epithelial sodium channel (ENaC), which is present in the apical membrane of the same structure, as well as in other parts of the body involved in sodium and fluid transport. The Na^+/K^+-ATPase transports sodium from the cell into the interstitial space on the basolateral side of the cell; it is an antiporter, transporting 2 K^+ ions into the cell for every 3 Na^+ passed into the interstitium. MR activation also upregulates expression of the serum and glucocorticoid-induced kinase 1 (SGK1), a member of the serine/threonine protein kinase family that regulates the function of Na^+ channels, either directly or by targeting various other proteins. For example, SGK1 phosphorylates the ubiquitin-protein ligase neural precursor cell-expressed developmentally downregulated-4 (NEDD4), reducing its affinity for ENaC subunits, which results in post-translational activation of these sodium channels and increased sodium transport. The result of increased ENaC expression and activity on the apical membrane is the promotion of sodium reabsorption. In summary, therefore, the main actions of aldosterone include promoting the permeability of the apical membrane of the distal nephron to potassium and sodium through activation of the basolateral Na^+/K^+-ATPase pumps, promoting Na+ reabsorption into the blood and K+ secretion into the urine. Aldosterone also stimulates H^+ secretion by intercalated cells in the collecting duct, regulating plasma bicarbonate levels and acid/base balance. Aldosterone may

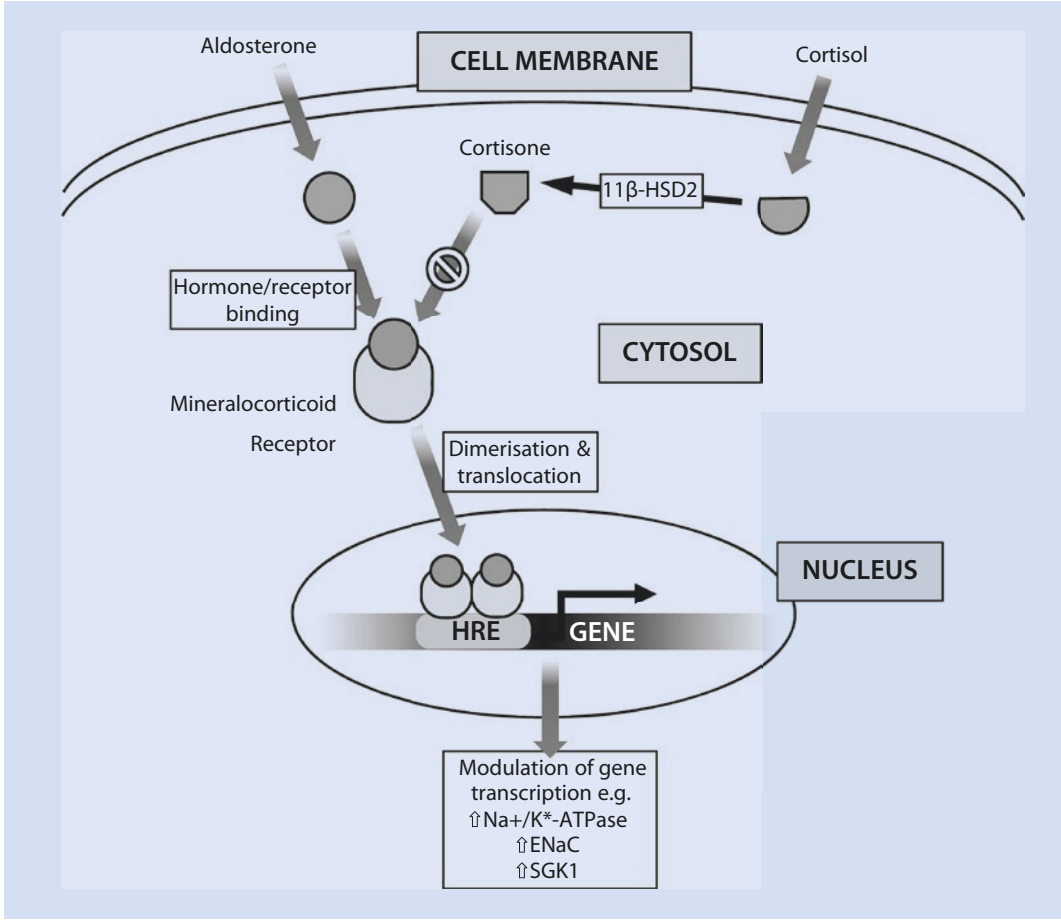

Fig. 10.2 Summary of aldosterone action in target tissues via the genomic mechanism. Aldosterone can pass freely through the cell membrane to bind free mineralocorticoid receptor in the cytosol. The hormone/receptor complex then dimerises and passes to the nucleus where it binds hormone response elements (HRE) present in selected genes, thereby stimulating or repressing their expression. Cortisol is also capable of binding mineralocorticoid receptor but, in aldosterone-selective tissues, is prevented from doing so by the action of 11β-HSD2, which converts it to inactive cortisone

also act on the CNS via the posterior pituitary gland to release vasopressin, thereby conserving water through its effects on renal tubular reabsorption.

Control of MR action is significantly complicated by the fact that, in vitro, cortisol and aldosterone have similar affinity for the receptor, while in humans cortisol circulates at levels approximately 100–1000 times more abundant than aldosterone. All things being equal, this means that the majority of MR would be bound by cortisol at any given time, and, in many tissues that express MR, this appears to be the case. However, certain key tissues, including the kidney, have a system in place that protects the MR from cortisol occupation leaving entry clear for aldosterone. This is made possible in such mineralocorticoid-sensitive cells by the presence of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts active cortisol to the biologically inactive cortisone. As cortisone is unable to bind MR, receptor selectivity is therefore conferred upon cells expressing 11β-HSD2 [13]. Therefore, renal MR is selective due to the expression of 11β-HSD2, whereas in the hippocampus, where MR is highly expressed but 11β-HSD2 is not, the majority of receptors will be bound by cortisol. (Note that 11β-HSD2 should not be confused with the 11β-HSD1 enzyme, which is widely expressed and generally results in the conversion of cortisone back to active cortisol, thereby amplifying the levels of cortisol in certain tissues.)

In addition to the genomic effects mediated by aldosterone-bound MR, other actions of aldosterone have been observed that occur too rapidly to fit with the 'classical' or 'genomic' mechanism of aldosterone action described above, which requires the actions of newly created proteins only after transcription and translation have had time to occur. For this reason, phenotypic effects of aldosterone detectable within 30 min of aldosterone administration are attributed to a 'nongenomic' mechanism, resulting in the rapid activation of various signalling molecules including intermediate tyrosine kinases (IPYKs), PLC, IP3, DAG, PKC and increased intracellular calcium [14]. Originally such effects were hypothesised to be mediated by a specific membrane-bound aldosterone receptor quite separate from the MR, but despite much work over the last 30 years, no such receptor has been identified. Meanwhile, studies of MR-knockout models and MR antagonists support the theory that MR itself is capable of nongenomic actions. However, the mechanisms underlying rapid, nongenomic aldosterone actions remain poorly understood.

10.5 Primary Aldosteronism

Primary aldosteronism (PA) is the excessive secretion of aldosterone by the adrenal glands, which leads to severe hypertension with a markedly increased risk of myocardial infarction, stroke and left ventricular hypertrophy. While gross examples of these conditions were once thought rare, the frequency of PA in hypertensives is now commonly accepted to be 10–15%. The condition is characterised by an elevated ARR owing to elevated aldosterone and suppressed renin; low potassium (hypokalaemia) is apparent in approximately one quarter of PA patients. Almost all cases result from either a unilateral aldosterone-producing adenoma (APA) or from hyperplasia of both adrenal glands (i.e. bilateral adrenal hyperplasia or BAH), and the two occur with approximately equal frequency. Surgical removal of unilateral APA can restore aldosterone levels and blood pressure to normal levels. In the case of BAH, such surgery would involve the removal of both adrenal glands, so is clearly not a viable option. For this reason, the bilateral form of the disease is treated with mineralocorticoid receptor antagonists (MRAs), such as spironolactone or eplerenone, and with other antihypertensive agents. Given these radically contrasting approaches to management, accurate differential diagnosis of APA and BAH is clearly highly desirable but, in practice, is complex, invasive, time-consuming and expensive. Therefore, better diagnostic methods involving improved imaging techniques or omics-based strategies (possibly involving circulating microRNA 'signatures') are a highly active area of current research [4, 15].

Significant advances have been made recently in identifying the underlying cause of APA, and at least half of all cases are now recognised to result from somatic mutation to ion channels and ATPases, which disrupt the cell membrane potential and cause dysregulated aldosterone secretion [16]. Mutation of the *KCNJ5* gene encoding the G protein-activated inward rectifier potassium channel GIRK4 (or KIR3.4) is the most common identifiable cause of APA, being present in ~38% of cases (although this figure appears subject to great variation among different populations, with frequencies of 70% reported in Japanese patients). Other commonly mutated genes include *CACNA1D*, which encodes a subunit of the voltage-dependent Cav1.3 calcium channel, and *ATP1A1*, which encodes a Na^+/K^+ ATPase subunit.

Other causes of PA include adrenal cancer and the genetic disorder familial hyperaldosteronism type I (FH-I), although these are much less frequent than those described above. In the case of FH-I, fusion of the regulatory region of *CYP11B1* to the coding region of *CYP11B2* leads to expression of aldosterone synthase throughout the ZF and consequent aldosterone hypersecretion [2]. While infrequent, this condition establishes the principle that mutation of *CYP11B2* alone can result in dramatic changes to aldosterone biosynthesis with significant cardiovascular consequences.

10.6 Genetic Polymorphism Influences on Aldosterone Secretion

Mutations with severe impact, such as GSH, can occur at any of the steroidogenic genes, but these are rare in the general population. However, it is apparent that some of the numerous common

polymorphisms that also exist at these loci have subtle functional effects which, in certain cases, have been proven to influence steroid biosynthesis and blood pressure. Aldosterone secretion rate and its plasma concentration are heritable traits, so particular attention has fallen on polymorphisms at the *CYP11B2* gene, which is unique to the biosynthesis of aldosterone alone. The most studied polymorphism at this locus is a C/T single nucleotide polymorphism (SNP) rs1799998, which is located 344 bases upstream of the *CYP11B2* transcription start site (TSS). This is a common SNP; its minor T allele has a frequency of ~0.43 in Western European populations and associates significantly with increased excretion of tetrahydroaldosterone (THaldo), the major urinary metabolite of aldosterone. However, functional studies of this SNP could not identify a significant effect on *CYP11B2* gene function. Subsequently, our studies showed that the correlations of rs1799998 with gene function were probably due to it being in tight linkage disequilibrium with another functional SNP, which we identified. This C/T SNP, rs13268025, is positioned 1651 bases upstream of the TSS and, in its minor C form, significantly increases *CYP11B2* transcription by disrupting binding of the transcriptional repressor APEX1 [17]. Individuals homozygous for this minor C allele (which has a mean frequency of 0.47) excrete significantly more THaldo than their major T allele counterparts. This example, now joined by several others, demonstrates the principle that certain common polymorphisms at steroidogenic loci can subtly but significantly affect hormone secretion and that this has a measurable impact on blood pressure [5]. Identification of further such polymorphisms that disrupt gene function through changes in transcriptional or microRNA regulation, for example, is ongoing and has expanded to include genes that regulate steroidogenesis in addition to those that produce enzymes directly involved with biosynthesis. When one notes that almost half the population carry at least one copy of the disruptive C rs13268025 allele, the potential to identify and target antihypertensive treatments at potentially huge numbers of people becomes apparent. The possible impact of such a 'personalised' approach will only increase as more polymorphisms, cumulatively accounting for ever greater shares of blood pressure heritability, come to light.

10.7 Direct Cardiovascular Effects of Aldosterone

Aside from its blood pressure-modulating actions in the kidney, aldosterone is also capable of exerting effects directly on numerous non-epithelial tissues that happen to express MR, including the heart and vascular tissue. Although the role these cardiovascular MR play in normal physiology is not known, it is apparent that – in the presence of high salt – their activation contributes to pathological changes to cardiac tissue in heart failure and, in particular, cardiac fibrosis. The deposition and crosslinking of extracellular matrix protein that occur in cardiac fibrosis stiffen the tissue and reduce its contractile force. Ultimately, this contributes to reduced cardiac output and heart failure [18].

The influence of MR on this process was originally observed in rats fed a high-salt diet and were found to develop cardiac fibrosis following aldosterone infusion. Such animal work led, ultimately, to several clinical studies – most notably the RALES trial [19] – that demonstrated markedly reduced mortality in heart failure patients who were administered MRAs such as spironolactone. This effect was largely attributed to a reduction in cardiac fibrosis. Indeed, a recent study has shown that administration of eplerenone to patients with acute myocardial infarction significantly reduces circulating levels of various biomarkers of cardiac extracellular matrix formation. Although the greatest benefit of such MRA therapy derives from decreased fibrosis, other effects include regression of hypertrophy, improved endothelial function and reduction in arrhythmia [20].

The reduction in fibrosis following MR antagonism is independent of any blood pressure effects and was therefore attributed to the blockade of cardiac and vascular MR. These are expressed in numerous cardiovascular cell types including endothelial cells, vascular smooth muscle cells (VSMCs) and cardiomyocytes. The picture is complicated by the fact that 11β-HSD2 expression, which confers aldosterone selectivity on the MR (see above), is not so widespread; in the vasculature it is found mainly in the endothelium, while its overall cardiac expression is low and probably confined to coronary vessels [13]. Nevertheless, it appears that activation of these MRs contributes to vascular stiffness and cardiac

fibrosis. One mechanism involves activated endothelial cell MRs raising expression of the endothelial form of ENaC (EnNaC). This enhances Na^+ entry to the cell which, in turn, reduces nitric oxide production and increases endothelial stiffness [21]. Vascular smooth muscle also has a role: tissue-specific knockout of the MR in rodents was shown to reduce the aldosterone and salt-induced stiffening of elastic arteries. Blockade of such MR is therefore likely to reduce the vascular remodelling that results from hypertension and ageing and which contributes to cardiovascular disease [22].

10.8 Therapeutic Reduction of Aldosterone Action in Hypertension

Although originally intended for the treatment of certain specific conditions including heart failure and PA, interest in expanding the use of MRAs to the treatment of hypertension has grown over the years. This arises from a recognition that aldosterone may be of particular relevance to patients with resistant hypertension (where simultaneous use of three or more different classes of antihypertensive agents has failed to control elevated blood pressure) and that MR blockade may therefore be a particularly effective therapeutic approach. (Indeed, blood pressure in the subset of essential hypertensive patients mentioned previously, whose aldosterone secretion is 'hyperresponsive' to ACTH, responds much better to MRA therapy than those with a 'normal' response [6].) Furthermore, the results of the RALES and EPHESUS trials proved that significant improvements to morbidity and mortality accrue from MRA therapy, and this is likely to arise, at least in part, from significant benefits to endothelial function conferred by MR blockade [23]. In spite of these positive outcomes, widespread use of MRAs is restricted due to their side effects, which include hyperkalaemia and, in the case of spironolactone, gynaecomastia. The development of newer agents capable of specifically blocking MR activation without accompanying rises in serum potassium would therefore be highly desirable, and the search is ongoing [24].

Finally, an alternative therapeutic approach, which is also the subject of some study, involves the prevention of aldosterone synthesis. As noted previously, the aldosterone synthase enzyme is uniquely required for aldosterone biosynthesis but not for the production of any other corticosteroid. Therefore, an inhibitor of this enzyme could in theory moderate the level of aldosterone production, alleviating many of the undesirable consequences of its excess while causing limited side effects. To date, the main barrier to this strategy has been the high degree of similarity between the aldosterone synthase (CYP11B2) and the 11β-hydroxylase (CYP11B1) enzymes. The development of a specific inhibitor that limits production of aldosterone while leaving cortisol unaffected is therefore difficult but not impossible [25]. The major question is whether lowering aldosterone in this manner is sufficient to prevent the cardiovascular damage associated with MR activation.

Conclusions and Clinical Perspectives

In a clinical setting, the importance of aldosterone is most apparent in the disorder collectively termed PA (or Conn's syndrome), where aldosterone secretion is inappropriately high relative to sodium status, is not adequately controlled by major regulators such as AngII and plasma potassium and is not suppressed by sodium loading. The resulting hypertension and cardiovascular damage require accurate diagnosis and effective treatment. The most recent clinical practice guidelines issued by the Endocrine Society recommend screening for PA in patients with blood pressure measuring >150/100 mm Hg on three separate days or in those resistant to blood pressure control using conventional antihypertensive drugs; hypokalaemia, early-onset hypertension, sleep apnoea and first-degree relatives with PA are also strong indicators [26]. Such patients are recommended to undergo assessment of plasma ARR. However, ARR is an imperfect test owing to various factors including assay reliability, time of sampling and the confounding effects of some (but by no means all) antihypertensive medications. There is also disagreement over the optimal 'cutoff' value for ARR in diagnosing PA. Therefore, further confirmatory tests are important as these will spare patients with false-positive ARRs the ordeal of subsequent intrusive lateralisation procedures. Such procedures include costly imaging tests and adrenal vein sampling (AVS) designed to distinguish unilateral disease – which can be cured surgically by

removal of the affected gland – and bilateral disease, where medical therapy is preferred. However, AVS is a highly specialised procedure requiring experienced radiologists, while medical treatment is heavily reliant on the MR antagonists (MRAs) spironolactone and eplerenone, which are problematic owing to side effects and expense, respectively. Therefore, there is need and potential to improve every aspect of PA diagnosis and treatment. Furthermore, it is becoming apparent that we will need to broaden our definitions of what constitutes PA, so as to include patients with aldosterone dysregulation (e.g. ACTH 'hyperresponders') who are likely to gain significant benefit from medical treatment such as MRAs [27].

In conclusion, the last 60 years has seen considerable progress in our understanding of aldosterone production and action but has inevitably led to ever more questions being raised. In the preceding article, we have attempted to summarise the major points of aldosterone biosynthesis, regulation and action while also highlighting the gaps in current knowledge that we judge to be the most urgent, interesting or of greatest potential therapeutic impact. However, there exist many more, and other prominent researchers in the field have highlighted their own priorities for future study [27, 28]. These numerous and diverse areas of current research serve to underline the complexity of this system, but it is undeniable that better understanding of aldosterone has already yielded huge benefits in healthcare and has the capacity to achieve far more. As mentioned previously, the recognised level of PA in the population has been rising steadily due to improved diagnosis. This has enabled ever more people to benefit from the highly effective treatments that are available in order to combat what is the most common curable cause of hypertension. Through improving our knowledge of aldosterone action, regulation and dysregulation, we can improve diagnostic tests still further and identify the many more people who currently still slip through the net. Emerging biomarkers, such as circulating microRNAs, may be of great value in diagnosing such individuals and may also provide new mechanistic insights that lead to novel therapies. It may be that such medicines have potentially universal application, as with MR blockade, or – if the promise of personalised medicine is realised – we may be able to match patients' treatment more closely to their particular form of disease. Genetic analysis has already broken down APA into several major subtypes, and it will be fascinating to see how this more precise categorisation of PA develops in years to come, potentially stratifying patients and personalising treatments.

Gaps in Knowledge

- What is the significance of epigenetic forms of regulation (e.g. microRNA) in aldosterone secretion?
- What are the mechanisms of nongenomic aldosterone action?
- How can diagnosis of PA be improved to capture those patients currently evading detection, better distinguish its various subtypes and before significant end organ damage has occurred?
- Many mutations driving APA formation have now been identified, but what are the equivalent drivers for BAH?
- Besides PA (as currently defined), what other forms of aldosterone dysregulation exist on the hypertensive population that might be readily treatable through MR blockade?

Acknowledgements S.M.M., J.C.v.K. and E.D. are members of the ENS@T-HT EU-funded Horizon 2020 research and innovation project into hypertension and personalised treatments (▶ http://www.ensat-ht.eu).

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Nitric Oxide

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_11

Key Concepts

- Nitric oxide is a signalling molecule with complex functions in many organ systems.
- Nitric oxide is a highly reactive, redox sensitive molecule with a very short biological half-life.
- Tight spatial and temporal regulation of nitric oxide synthesis is necessary for cardiovascular homeostasis and is achieved by the integration of signals via multiple regulatory mechanisms.
- Therapeutic manipulation of nitric oxide signalling has been hampered by a narrow therapeutic window and significant side effects of both over and under dosing.

11.1 Synthesis of NO

Nitric oxide was identified as the first endogenously produced gaseous signalling molecule in the 1980s [1]. The pioneering research for Robert Furchgott described the endothelial-dependent relaxing factor (EDRF) as a critical determinant of vascular reactivity. Studies in Furchgott's laboratory identified a highly labile factor released from vascular endothelial cells that could induce rapid relaxation of smooth muscle cells (SMC). In organ culture experiments, removal of the endothelium abolished relaxation in response to mediators such as acetylcholine. Treatment of endothelium-denuded vascular segments with culture medium from endothelial cells resulted in rapid relaxation; however it was observed that this effect was extremely labile with a half-life measured in seconds and was quenched in the presence of haemoglobin. Concurrent studies of the enzyme soluble guanylate cyclase (sGC) in Ferid Murad's laboratory identified NO as a potent activator of sGC activity in vascular smooth muscle cells. Elevation of cGMP in vascular SMCs activated a signalling cascade that resulted in vascular relaxation. Further studies by Furchgott, Ignarro and Murad identified NO as EDRF, and this work was recognised by the award of the Nobel Prize in Physiology or Medicine in 1998 [2]. NO is synthesised from L-arginine in a reaction that is catalysed by a family of enzymes, the NO synthases (NOS). Three NOS isoforms have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible or inflammatory NOS (iNOS) (Fig. 11.1). These enzymes are evolutionarily conserved at the amino acid sequence level and catalyse the NADPH- and O_2-dependent oxidation of L-arginine to NO and citrulline, with N^{ω}-hydroxy-L-arginine formed as an intermediate. NOSs are flavohaem enzymes that are active only as dimers. Each monomer has a carboxy-terminal diflavin-reductase domain and an amino-terminal oxygenase domain. Dimerization activates the enzyme by sequestering iron, generating high-affinity binding sites for arginine and the essential cofactor tetrahydrobiopterin (BH_4) and allowing electron transfer from the reductase-domain flavins to the oxygenase-domain haem. Activity is also dependent on bound calmodulin. In iNOS, calmodulin is tightly bound, whereas in eNOS and nNOS, calmodulin binding is dependent on calcium, and enzyme activity is therefore calcium dependent. Calmodulin binding enhances the rates of electron transfer through the reductase to the oxygenase domain.

For enzymatic activity, nitric oxide synthase (NOS) enzymes must dimerize and bind the cofactors tetrahydrobiopterin (BH_4), haem, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). On binding calmodulin (CAL), the active enzyme catalyses the oxidation of L-arginine to citrulline and nitric oxide (NO) and requires molecular oxygen and NADPH as co-substrates. Each NOS dimer coordinates a single zinc (Zn) atom.

In addition to regulation of NO synthesis by cofactors and calcium, the activities of NOSs can be altered by post-translational modifications and by protein–protein interactions. For example, eNOS activity is increased by phosphorylation of Ser1179 and is inhibited by interaction with the scaffolding domain of the membrane protein caveolin-1.

11.2 Targets for NO

The first described physiological target for NO was soluble guanylyl cyclase (sGC) [3]. Binding of NO to the iron within the haem moiety of sGC produces a conformational change that leads to enzyme activation (Fig. 11.2). The subsequent rise in cyclic GMP accounts for many of the

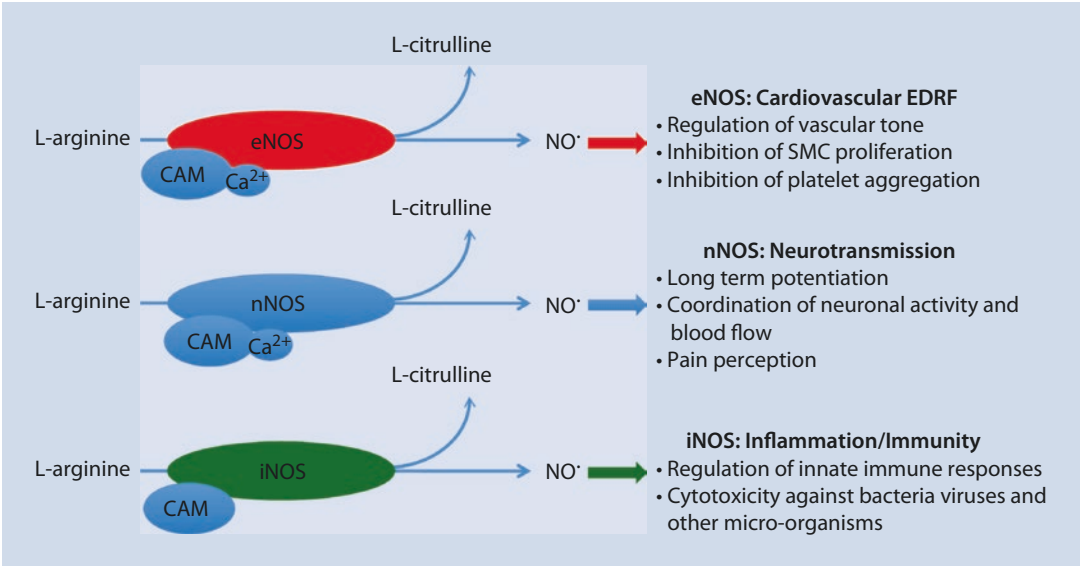

Fig. 11.1 Mammalian nitric oxide synthase enzymes. The nitric oxide synthases isoforms, encoded by separate genes, are found in mammals. The enzymes have been named according to the cell type or conditions under which they were first discovered. Endothelial and neuronal NOS enzymes (eNOS and nNOS, respectively) are constitutively expressed and calcium-calmodulin-dependent producing relatively low levels (pmol-nmol) of NO. In contrast, inducible NOS (iNOS) is only expressed following activation of certain cell types such as monocytes and macrophages. iNOS constitutively binds calmodulin, and therefore iNOS activity is calcium independent. Following induction iNOS generates relatively high levels (μmol) of NO. All mammalian NOS enzymes produce NO by the sequential oxidation and reduction of the semi-essential amino acid L-arginine and produce citrulline as a side product

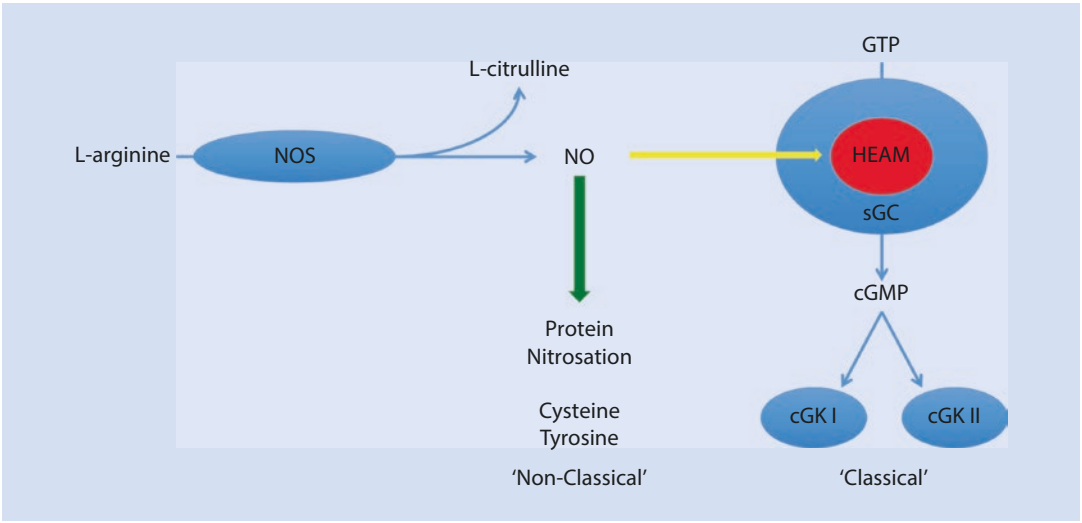

Fig. 11.2 Nitric oxide signalling. NO is a free radical gas that rapidly diffuses throughout generator cells and into neighbouring target cells to exert autocrine and paracrine effects. Two major signalling pathways for NO have been described. Classical NO signalling involves the binding of NO to the haem moiety of soluble guanylate cyclase (sGC) causing a conformational change of the enzyme resulting in activation of cyclic GMP (cGMP) synthesis. Increased concentrations of cGMP activate numerous downstream signalling molecules of which cGMP-dependent protein kinases are prominent targets. Nonclassical NO signalling in which NO directly reacts with certain cysteine or tyrosine residues in target proteins to modify protein function has been more recently described. The contribution of classical versus nonclassical signalling to NO-mediated effects is dependent on cell type and the enzymatic source of NO

physiological effects of NO and has been described as 'classical' NO signalling (Fig. 11.2). However, NO has the potential to interact directly or indirectly with metals, thiols and oxides and affect proteins, nucleic acids, lipids and sugars. The ability of NO to react with cysteine and tyrosine residues within certain proteins and thereby modify protein function has been termed 'non-classical' NO signalling and has been the focus of significant research activity. The reversible modification of thiol residues within proteins by NO constitutes a novel post-translational modification that appears to be widespread and play significant roles in both physiological and pathophysiological states [4]. Indeed, because NO is a free radical (it has an unpaired electron) and the nitrogen can exist in various oxidation states to generate nitroxyl ions (NO^-), NO free radicals ($NO^{\cdot}$), nitrosonium cations (NO^+), nitrite ions (NO_2^-) or nitrate ions (NO_3^-), the biological chemistry of NO is complex, and its potential effects within biological systems are many.

The fastest reaction rates of NO are with superoxide ions to form peroxynitrite ($ONOO^-$), a powerful oxidant that can modify proteins and lipids by nitration. Reaction rates with metals are also high, but except for certain haem proteins, such as soluble guanylyl cyclase, the metal centres are generally not available for reaction with NO. Reactions with thiols such as *S*-nitrosation of cysteine residues can affect protein function in a reversible manner. Ion channels, enzymes, transcription factors and G proteins can all be modified in this way although the determination of consensus sequences for *S*-nitrosation that might allow identification of potential targets based on amino acid sequences is incomplete. These observations indicate that NO signalling is influenced by the redox state of the cell and has the potential to interact with many signalling pathways. These multiple actions of NO and dependence on thiol concentration and redox state probably explain why NO can have both protective and harmful effects. The interaction of NO with respiratory chain enzymes illustrates many of these mechanism; NO can bind reversibly to the haem moiety of cytochrome *c* oxidase, NO^+-like reactions lead to reversible *S*-nitrosation of mitochondrial complex I, and generation of $ONOO^-$ can irreversibly inhibit multiple complexes, as well as aconitase. These effects activate proton leak and the permeability transition pore, leading to cell death [5].

11.3 NO in Physiology

The physiological roles of NO described below have been categorised according to the NOS isoform responsible for NO synthesis. Whilst this is a useful way to differentiate effects of NO, it should be noted that in many tissues, multiple NOS isoforms are concurrently expressed, and therefore some degree of overlap in function is likely.

11.3.1 eNOS

NO generation from the endothelium is critical to maintain the vasculature in a relaxed state, prevent the adhesion of platelets and white cells and inhibit smooth muscle cell proliferation [6]. Consistent with these roles, pharmacological inhibition of NOS causes vasoconstriction, hypertension and enhanced platelet activation and increases atherogenesis in experimental animals. Genetic approaches, principally the production of *eNos* knockout mice, confirm the pharmacological studies but also demonstrate that eNOS also regulates vascular endothelial growth factor (VEGF) expression and promotes angiogenesis.

11.3.2 nNOS

nNOS synthesised NO generated in peripheral nerves is also important in the relaxation of vascular and non-vascular smooth muscle [7]. It relaxes the corpus cavernosum and thereby causes penile erection; relaxes the bladder, urethra and sphincters in the gut; and alters responses in airways. *nNos* knockout mice have dilated bladders and increased urinary frequency. Pyloric stenosis and grossly dilated stomachs are a prominent phenotype of nNOS deficient mice. However, strips of corpus cavernosum from nNOS deficient relax on electrical field stimulation and mice achieve penile erection. The apparent insensitivity of erectile tissue to nNOS deletion may be explained by the incomplete deletion of nNOS in some strains of genetically modified mice. A prominent phenotype of *nNos* knockout mice is an increase in aggression and inappropriate mounting behaviour, suggesting that nNOS-derived NO in the CNS may be a mediator of behavioural inhibition. Consistent with high

levels of nNOS expression in the cerebellum, nNOS-deficient mice show defects in coordination and balance.

In both skeletal and cardiac muscles, high-level expression of nNOS has prompted a number of genetic and pharmacological studies aimed at identifying physiological roles. Pharmacological studies using selective inhibitors suggest that nNOS-derived NO has roles in maintenance of arteriolar tone, regulation muscle contraction and glucose uptake and within the muscle. Histological studies have indicated that nNOS protein localises to the dystrophin complex suggesting a role in muscular dystrophy. In cardiac tissue nNOS and eNOS might have opposing effects with nNOS-derived NO acting as a positive inotropic response, whereas eNOS-derived NO decreases β-adrenoceptor-induced contractility via L-type calcium channels. In airways, nNOS-derived NO appears to provide significant protection against experimentally induced airway hyperresponsiveness.

11.3.3 iNOS

iNOS was initially identified in macrophages as a mechanism of macrophage cytotoxicity [8]. iNOS is rapidly transcriptionally induced in multiple cell types in response to stimulation with bacterial endotoxins or pro-inflammatory cytokines. Whilst iNOS expression was originally thought to be absent in non-stimulated cells, it appears that this isoform might be constitutively express in certain cell types although the physiological significance of these observations remains unclear. Once expressed, it generates large amounts of NO, and its activity is not dependent on intracellular calcium (Fig. 11.1). It is becoming clear that there are species-, tissue- and cell-specific conditions for inducing the expression of active iNOS. In contrast to a relatively high degree of conservation across species at the amino acid level, sequence analysis of the DNA upstream of the transcription start site indicates that the promoter region varies greatly between species. In addition to promoter variation, five copies of the iNOS gene have been described in humans whilst only one is present in rodents. It appears that only one of the human iNOS gene copies can produce an active enzyme. These differences necessitate caution when extrapolating the roles of iNOS from rodents to humans. Despite these differences it is clear that iNOS activity is important for killing or host defence against certain protozoa, bacteria, fungi and viruses. It is also clear that iNOS is induced in cells that are not obviously involved in host defence and in situations in which no live organism is present. Overproduction of NO in response to infection appears to significantly contribute to cell death and tissue damage caused by infection. Similarly chronic induction of iNOS in inflammatory disease states appears to play a significant role in in tissue damage seen in these diseases.

Taken together the observations described above indicate key roles for NO in the maintenance of homeostasis in multiple organ systems. Nowhere are the effects of NO more prominent that in the cardiovascular system. Since the discovery of NO signalling in the 1980s, significant academic and pharmaceutical research activity has focussed on manipulation of NO signalling to maintain the cardiovascular protective effects of low-level constitutive NO production and prevent the deleterious effects of NO overproduction. In the remainder of this chapter, we will summarise current therapeutic strategies for manipulation of cardiovascular NO.

11.4 Regulation of Cardiovascular NO Synthesis

In light of the numerous physiological signalling roles of NO, it is perhaps not surprising that NO synthesis is very tightly regulated. The mechanisms that regulate NO synthesis have been reviewed in detail elsewhere, and an extensive review of this literature is outside the scope of this chapter. Instead we will provide an overview of the mechanisms that acutely regulate eNOS activity in the cardiovascular system and highlight mechanisms that play a role in in the development of cardiovascular disease.

11.4.1 Intracellular Localisation

Within endothelial cells eNOS localises to membrane-limited structures. Membrane association is mediated by post-translational palmitoylation and myristoylation of the protein resulting in accumulation of eNOS at the plasma

membrane, Golgi apparatus, mitochondria and nuclear envelope. At the plasma membrane, eNOS localises to cholesterol- and sphingolipid-rich regions termed caveolae. Exposure of endothelial cells to oxidised lipoprotein (oxLDL) depletes the plasma membrane of cholesterol and resulting in loss of eNOS from the plasma membrane and reduced NO synthesis.

11.4.2 Phosphorylation

eNOS activity is dynamically regulated by phosphorylation. A number of studies have identified phosphorylation sites that either increase or decrease eNOS activity, and these residues are phosphorylated by kinases including protein kinase B (Akt), CaM protein kinase II, adenosine monophosphate-activated kinase (AMPK) and protein kinase C (PKC), respectively. Thus stimulation of endothelial cells by shear stress leads to phosphorylation of eNOS by Akt resulting in increased electron flow through the reductase domain of the enzyme and activation of enzyme activity.

11.4.3 Substrate, Cofactor and Inhibitor Availability

All NOS enzymes utilise the semi-essential amino acid L-arginine as a substrate, require the presence of cofactors including NADPH and tetrahydrobiopterin (BH4) and are competitively inhibited by endogenously produced inhibitors such as asymmetric dimethylarginine (ADMA). In certain disease states such as hypercholesterolemia and chronic inflammation, L-arginine levels have been shown to fall to levels that might limit NO production. Several preclinical and clinical trials have tested the therapeutic utility of L-arginine supplementation with promising results in preclinical models that to date have not been replicated in humans. BH4 is a redox sensitive cofactor that is essential for optimal electron flow within the NOS enzyme. Reduced levels of BH4 or oxidation of BH4 to dihydrobiopterin (BH2) impair NOS activity and can lead to enzyme 'uncoupling' where NOS preferentially produces superoxide rather than NO. Overproduction of superoxide and reduced NO synthesis have been reported in several cardiovascular disease states prompting trials of BH4 supplementation. Once again promising results in preclinical models have not been replicated in humans. Levels of the endogenous NOS inhibitor ADMA have been reported in a number of common cardiovascular disease states, and preclinical models have indicated that reduction of endogenous ADMA levels may improve vascular reactivity. To date no pharmacological interventions that reduce ADMA levels have been identified, but small drug-like molecules that elevate ADMA have considerable therapeutic potential in conditions such as sepsis and cancer where overproduction of NO contributes to disease [9].

11.4.4 Protein–Protein Interactions

All three NOS enzymes bind to the small calcium-binding protein calmodulin. NOS binding to calmodulin displaces an autoinhibitory loop in the NOS protein and promotes enzyme activity by enhancing electron flow within the NOS enzyme. eNOS and nNOS only bind to calmodulin in the presence of calcium, whereas iNOS binds tightly to calmodulin in the absence of calcium rendering iNOS activity calcium insensitive. eNOS has been shown to bind directly to caveolin, a membrane-bound protein that is concentrated in caveolae. eNOS-caveolin binding inhibits eNOS activity, but caveolin can be displaced from eNOS by calcium-bound calmodulin leading to derepression and activation of eNOS activity. eNOS has also been shown to bind to several other proteins including Hsp90, NOSIP and NOSTRIN that either regulate enzyme activity or intracellular localisation. The emerging picture of eNOS protein–protein interactions is one of eNOS as the catalytic core of a larger protein complex that integrates intra- and extracellular signalling pathways to determine rates of NO synthesis in a highly dynamic and spatially defined pattern.

11.5 Pharmacological Approaches to Increase or Potentiate NO Signalling

Long before the elegant studies that elucidated the physiological and pathophysiological roles of NO were performed drugs that elevated NO signalling were in clinical use. Thus glyceroltrinitrate (GTN) was first developed by Ascanio Sobrero in 1847

and used to treat patients with angina pectoris by William Murrell as early as 1876. Unbeknown to both Sobrero and Murrell, GTN is metabolised by the body to release NO that dilates blood vessels and relieves the symptoms of angina pectoris. Following the identification of NO as EDRF and the elucidation of NO synthesis and signalling, new approaches to elevate NO signalling have been developed. Broadly these can be divided into three categories, drugs that promote NO, compounds that prevent the breakdown of NO and interventions that directly stimulate pathways downstream of NO.

11.5.1 NO Promoting Compounds

The development of these compounds follows the work of Sobrero and Murrell and aims to increase the amount of NO by either directly donating free NO, boosting the activity of NOS enzymes or providing compounds that are metabolised within the body to finally release NO. Direct delivery of NO has been achieved in the pulmonary system by inhalation of NO gas [10]. This strategy has been successfully employed to treat persistent pulmonary hypertension of the newborn and may provide some systemic benefits due to endocrine effects of NO. Recently a new strategy that aims to couple the beneficial effects of NO to a currently used drug to either enhance effects or mitigate known side effects has been perused. An example of this is non-steroidal anti-inflammatory drugs (NSAIDs) that are known to increase blood pressure, and therefore NO-NSAIDs have been developed to offset the blood pressure effects of NSAID use. Unfortunately these compounds have not shown significant improvements in NSAID profile and are limited by the 1:1 ratio of NO to NSAID that is not reflective of the biological potency of these compounds in vivo.

An alternative approach has been to increase the activity of NOS enzymes. This can be achieved by increasing the availability of substrates and cofactors or by preventing the accumulation of NOS inhibitors. Dietary supplementation of the NOS substrate L-arginine has shown promise in small studies of vascular function in hypercholesterolaemic individuals, but in a large randomised trial in patients with peripheral vascular disease, no evidence of benefit was apparent [11]. It has been suggested that this lack of efficacy might be related to the many roles of arginine in vivo that, in addition to NO formation, include polyamine synthesis. Similarly, increasing levels of the NOS cofactor BH4 improves vascular function in experimental animals but did not show efficacy in man. In these studies oral administration of BH4 was not sufficient to elevate levels in the vasculature due to redox inactivation of BH4 to produce BH2. An alternative approach to boost NOS activity is to reduce the level of endogenously produced NOS inhibitors (such as ADMA) that are produced by all cells [12]. ADMA is actively metabolised by dimethylarginine dimethylaminohydrolase (DDAH) enzymes, and genetic approaches to elevate DDAH activity reduce ADMA and improve cardiovascular function in experimental animals. Currently small molecule activators of DDAH have not been identified, and therefore therapeutic manipulation of DDAH would require a gene therapy approach.

Recently a novel pathway for the production of NO in vivo has been described. In this pathway dietary nitrate is converted to nitrite by enterosalivary bacteria in the mouth. Nitrite is then absorbed through the gut and reduced to NO by the action of metalloprotein oxidoreductases such as xanthine oxidase [13]. Dietary nitrate has been demonstrated to reduce blood pressure and protect against cardiovascular disease in experimental animals and to reduce blood pressure in humans. Currently several clinical trials are underway to evaluate the efficacy of dietary nitrate in a range of cardiovascular disease states.

11.5.2 Increasing NO Bioavailability

The chemical nature of NO, a free radical gas with an unpaired electron, makes it highly reactive and susceptible to quenching by reactive molecules such as ROS. In mammals ROS are generated by as side products by numerous pathways. In contrast, the primary function of the NOX family of enzymes is to generate ROS and H_2O_2, and therefore inhibition of NOX activity is an attractive strategy to boost NO bioavailability [14]. Two approaches have been taken to reduce NOX-mediated ROS production in the cardiovascular system. In the first approach, the induction of NOX gene expression by angiotensin II acting via the AT1 receptor has been targeted by ACE inhibitors and AT1 antagonists to increase NO

bioavailability. An alternative approach has been to develop small molecule inhibitors of NOX isoforms (principally NOX1, 2 and 4). A range of molecules have been identified in screens, but none have so far reached the clinic due to lack of potency/selectivity or concerns relating to immune suppression in chronic disease settings.

11.5.3 Activating Downstream Signalling

This approach is exemplified by the development of inhibitors of cGMP-specific phosphodiesterases such as sildenafil [15]. These compounds prevent the breakdown of cGMP that is generated by NO-activated sGC (Fig. 11.2). In this way phosphodiesterase inhibitors potentiate and amplify endogenous NO signalling and thereby retain important temporal and spatial patterns of activity. Sildenafil was initially registered for the treatment of erectile dysfunction but has shown promise in treatment of a wide range of cardiovascular disorders including PAH, peripheral arterial disease, ischaemic heart failure and cardiomyopathy.

A second downstream target of NO that has attracted significant pharmaceutical development is sGC. Agonists of sGC mimic the action of NO and have been particularly beneficial in the treatment of PAH [16]. Significant benefits in addition to vasodilation have been reported in experimental models of heart failure, cardiac fibrosis and hypertrophy. In contrast to phosphodiesterase inhibitors that potentiate NO signalling, sGC agonists activate downstream effectors in the absence of NO. Therefore hypotension is a significant side effect of the use of sGC agonists that might limit the range of use.

11.6 Overproduction of NO in Pathology

Increased generation of NO, either alone or in the presence of other free radicals, such as superoxide, has been implicated in pathophysiological changes in virtually every organ system [17]. Evidence comes from studies of NO generation, NOS isoform expression and effects of NOS inhibitors (usually isoform nonselective). In the vast majority of cases, pathology related to overproduction of NO is associated with expression of iNOS. Induction of iNOS is seen in models of septic shock, inflammatory and non-inflammatory pain, arthritis, inflammatory bowel disease, asthma and in the brain after ischaemia or trauma, as well as in various models of neurodegeneration or cerebral inflammation. There is also evidence from human samples that iNOS is expressed in various pathological tissues. The potential importance of iNOS in contributing to pathophysiology is exemplified by the findings from *iNos* knockout mice.

11.6.1 Inhibition of iNOS

It seems clear that blocking iNOS has the potential to produce therapeutic benefit. The difficulties lie in achieving isoform specificity, in targeting to specific cells or tissues and in ensuring that the correct degree of inhibition is achieved. A key issue for any inhibitor is the degree of selectivity over other NOS isoforms and specificity for NOS over other potential targets. Several arginine metabolising or transporter proteins might be affected by substrate-based NOS inhibitors, and there is little information available at present on the effects on enzymes such as dimethylarginine dimethylaminohydrolase (DDAH), arginine glycine amidinotransferase, arginase, argininosuccinate synthase or peptide arginine deiminases.

A range of approaches including small molecule substrate analogues, dimerization inhibitors, cofactor inhibitors and transcriptional regulators have been developed to reduce iNOS expression/activity. This literature has been extensively reviewed elsewhere and is outside the scope of this article. For reasons including selectivity, potency and toxicity, none of these molecules have successfully progressed into clinical use [17]. The development of iNOS inhibitors for the treatment of septic shock illustrates some of the challenges and potential new approaches to NO-focussed drug development.

In patients with septic shock, the isoform nonspecific inhibitor N^G-monomethyl-L-arginine (L-NMMA) restores blood pressure and seems to improve haemodynamics. However, the largest study so far showed an adverse effect on outcome [18]. A post hoc analysis indicated that low doses might have been beneficial and that the harm occurred when larger doses were used (and very

high circulating concentrations of L-NMMA were achieved). Interestingly L-NMMA is a naturally occurring amino acid that is generated endogenously. Arginine residues on proteins are methylated by the action of protein arginine methyltransferases (PRMTs). Three methylarginines are generated: L-NMMA, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). The asymmetrically methylated arginine residues (ADMA and L-NMMA) inhibit all three isoforms of NOS, whereas SDMA is not an NOS inhibitor. Normally, the circulating concentration of ADMA and L-NMMA is kept ~0.5 μM by the action of DDAH, which catalyses their conversion to citrulline and dimethylamine or monomethylamine, respectively. This pathway represents an endogenous mechanism for the regulation of NO production by competitive inhibition. Pharmacological inhibition of DDAH causes a rise in ADMA levels sufficient to block NO generation providing a novel approach to blocking overproduction of NO [9].

Two isoforms of DDAH have been identified, one with a widespread distribution (DDAH1) and the other with high-level expression in immune cells and the heart (DDAH2). Isoform-selective inhibition of DDAH1 might provide a different profile of NOS inhibition to direct inhibition of NOS isozymes. Clearly, inhibition of DDAH1 would not provide isoform-selective inhibition of NOS, as ADMA and L-NMMA block all three isoforms. It is also unlikely that endogenous ADMA or L-NMMA would ever increase to levels sufficient to inhibit NOS by more than 30%. However, inhibition of DDAH might provide tissue-specific partial inhibition of NOS. Consistent with this proposition, selective small molecule inhibitors of DDAH1 attenuate iNOS-mediated vasodilatation in blood vessels ex vivo and block endotoxin-induced hypotension in vivo. In a rodent model of peritonitis, selective inhibition of DDAH1 improved hemodynamic performance and improved survival [19]. Consistent with the tissue distribution of DDAH isoforms, DDAH1 inhibition had no effect on immune function or cardiac performance in experimental sepsis. Whilst significant differences have been noted in the rodent and human response to sepsis, the beneficial effect of reduced DDAH1 activity appears to be conserved between species. Thus polymorphic variants in the human DDAH1 gene that are associated with lower DDAH1 expression and activity and higher ADMA levels are associated with increased survival in patients with septic shock. Small molecule inhibitors of DDAH1 will shortly enter clinical trials in sepsis and might also be beneficial in other indications where excessive NO generation drives pathology.

The notion of preferentially inhibiting excess NO generation whilst leaving physiological NO production untouched also underlies the approach of targeting enzymes that allow regeneration of arginine within cells. Argininosuccinate-synthase activity appears to be required for high-output NO synthesis, at least in some situations, and therefore might provide a target for drug action. An alternative approach that could emerge is to target individual proteins on which NO acts to inhibit specific adverse effects of NO whilst leaving other NO signalling pathways intact.

Conclusions and Clinical Perspectives

The identification of NO as a biological mediator has led to vast numbers of publications—in the order of 3000 per year at present. There can be little doubt that NOS activity is of considerable importance physiologically or that dysregulation of NOS activity or NO bioavailability has the potential to cause harm. However, a major problem in turning scientific discovery into useful therapeutics has been the sheer range of processes in which NO has been implicated and the opposing effects of NO even within a single disease. A good example is provided in the field of cancer biology. High-output NO generation from macrophages or from tumour cells themselves can be tumoricidal and prevent metastasis. However, iNOS is expressed constitutively in some tumour cells [20], where it promotes tumour growth, neovascularization and invasiveness through induction of mutations to the tumour-suppressor gene TP53 and upregulation of VEGF expression. Furthermore, in other tumours, it is eNOS expression that correlates with tumour malignancy and vascularity [21]. A major challenge for development of new therapies targeting NO is to achieve selective benefits without incurring a host of side effects. The identification of single therapeutic targets with profiles appropriate for specific indications will be key to progress in this field.

Gaps in Knowledge

- Despite a wealth of literature describing the roles of NO in health and disease, complete integration of this information to identify specific therapeutic targets for individual disease states is lacking.
- Current understanding of endogenous regulatory pathways that determine NO output from specific cells and tissues is fragmented.
- The extent to which 'regulating the regulators' can reverse or attenuate pathological changes in NO signalling has not been fully determined.

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Reactive Oxygen Species

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_12

Key Concepts

- ROS-induced oxidative post-translational modifications of proteins are important regulatory mechanisms of signalling pathways involved in key cellular functions in vascular cells.
- ROS production is tightly regulated by enzymatic sources and antioxidants to maintain redox status and to prevent accumulation of injurious ROS.
- In pathological conditions, high concentrations of ROS lead to oxidative stress by disruption of redox signalling circuits and aberrant signalling associated with vascular inflammation and dysfunction.
- Identification of molecular targets and specific sources of vascular ROS will contribute to development of new therapeutic strategies targeting oxidative stress in cardiovascular diseases.

12.1 Introduction

Redox signalling refers to modification of signalling molecules by reduction/oxidation reactions. The main mediators of redox signalling are reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). A major mechanism whereby ROS influence cell function is through post-translational oxidative modification of downstream protein targets. Cysteine residues in proteins are particularly susceptible to oxidation and function as a redox switch, activating or inhibiting protein function. Because of its reactive nature, ROS can also react with other cellular components such as DNA and lipids, causing damage. Therefore, ROS generation and scavenging is a tightly controlled process. Redox signalling is thought to occur in redox modules where the source of ROS is near the target protein and antioxidant systems. Several cellular events in the vasculature are regulated by redox-sensitive pathways. ROS are involved in vascular contraction and relaxation, cell growth, migration, differentiation, survival and apoptosis [1].

In pathological conditions increased ROS generation and/or impaired antioxidant capacity results in oxidative stress. Increased ROS bioavailability is associated with aberrant redox signalling leading to dysregulated endothelial cell and VSMC function and consequent vascular damage. High levels of ROS have been linked to vascular hypercontractility, endothelial dysfunction, inflammation, calcification and fibrosis, which are hallmarks of vascular dysfunction associated with cardiovascular diseases, such as hypertension [2].

12.2 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are small molecules generated by incomplete reduction of molecular oxygen. The major ROS produced by cells in the vasculature are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-) and nitric oxide (NO). NO is a potent vasodilator, produced primarily by endothelial cells.

Reduction of oxygen in the presence of one free electron results in generation of the free radical $O_2^{\bullet-}$. Generation of $O_2^{\bullet-}$ occurs as a by-product of mitochondrial respiration as well as by enzymatic sources such as the NADPH oxidases. Due to its highly reactive nature, its short half-life and its charge, $O_2^{\bullet-}$ is not able to freely cross biological membranes limiting its effects. However, most of the $O_2^{\bullet-}$ is converted to H_2O_2 spontaneously or enzymatically where it is catalysed by superoxide dismutase (SOD) [3].

Hydrogen peroxide is not a free radical by definition since it does not possess a free electron, but it is a small, uncharged reactive molecule capable of crossing cell membranes. These characteristics confer a higher half-life to H_2O_2 than its precursor and the ability to reach more signalling targets. The enzyme catalase scavenges H_2O_2, a reaction that results in formation of H_2O and O_2, controlling its effects. Therefore, H_2O_2 is thought to be more suitable as a signalling molecule and second messenger than $O_2^{\bullet-}$. In addition, it can react with cysteine (Cys) residues in proteins altering the conformation and function of downstream targets [4].

Reaction of H_2O_2 with metals, such as Fe and Cu, results in generation of hydroxyl anion ($OH^{\bullet-}$). This process is known as the Fenton reaction and can contribute to increased oxidant damage [5]. $OH^{\bullet-}$ is an extremely reactive radical able to promote oxidation of most cellular components such as proteins, DNA and lipids. Therefore, ROS generation in cells is an extremely

controlled process involving enzymatic sources of ROS as well as antioxidant systems including SOD, catalase and peroxidases.

12.2.1 Antioxidants

ROS effects are controlled in part by reaction with antioxidants. Superoxide anion dismutation is catalysed by SOD. There are three SOD isoforms (SOD1, SOD2 and SOD3) that differ in cellular localization and in the cofactor necessary for activity. SOD1 (Cu/Zn SOD) is mainly expressed in the cytosol but is also found in the mitochondrial intermembrane space. SOD2 (MnSOD) is present in the mitochondrial matrix. SOD3 is an extracellular Cu-/Zn-dependent SOD, also known as ecSOD. This isoform is the major SOD in the extracellular vascular space found in the extracellular matrix and on cell surfaces [3].

Hydrogen peroxide can be scavenged by catalase, glutathione peroxidase and peroxiredoxins. Catalase is mainly present in peroxisomes and reacts with H_2O_2 to generate H_2O and O_2 [4].

Glutathione peroxidases (GPx) possess selenocysteine in their catalytic site that react rapidly with H_2O_2. In addition, GPx also reduces peroxide radicals to H_2O and alcohols. GPx uses glutathione (GSH) as a cofactor in the reduction of hydrogen peroxide. GSH is a peptide that contains a critical thiol group (SH) prone to oxidation. Therefore, GSH acts as an antioxidant, ROS scavenger and provides reduction equivalents for many redox reactions. There are four GPx isoforms, GPx1-4, that are differentially expressed in the tissues. GPx1 is ubiquitously expressed, found in the cytosol, mitochondria and to some extent peroxisomes in all cell types. GPx2 is highly expressed in epithelial cells in the gastrointestinal tract. GPx3 is an extracellular isoform, while GPx4 is membrane bound [6]. GPx1 play an important role in the control of intracellular levels of hydrogen peroxide, and lack of GPx1 is involved in oxidative vascular damage [7].

An important H_2O_2 scavenging system is composed by peroxiredoxins (Prx), cysteine-dependent enzymes that do not require additional cofactors for their activity. The typical Prx has two cysteine (Cys) residues in the catalytic site, the peroxidatic Cys and the resolving Cys. Reaction with H_2O_2 results in direct oxidation of the peroxidatic Cys that in turn reacts with the resolving Cys to form a disulphide bridge stabilizing the Prx molecule. Enzymes such as thioredoxin or thioredoxin-like proteins can reduce Prx, restoring its function as antioxidants. Prx1-4 are typical 2-Cys Prxs distributed in different compartments within the cells. Prx1 and Prx2 are found in the cytosol and nucleus, Prx3 in the mitochondria and Prx4 in the endoplasmic reticulum. Prx5, an atypical 2-Cys Prx, is found in peroxisomes, mitochondria and in the cytosol. Prx6 is also found in the cytosol and is a 1-Cys Prx, as it lacks the resolving Cys in its structure [8]. Hydrogen peroxide can cause hyperoxidation of Prxs, inactivating the enzyme. This effect could be useful to allow oxidation of protein targets for signalling purposes. However, recent studies demonstrated that hyperoxidation of Prxs results in a switch to a redox sensor and chaperone molecule involved in cell signalling. Therefore, Prxs are important regulators of H_2O_2 signalling but can also have independent effects [9]. Prx2 is implicated in PDGF-induced cell proliferation in VSMCs [10].

Another important antioxidant system is the thioredoxin system, composed by thioredoxin (Trx), NADPH, thioredoxin reductase (TrxR) and the thioredoxin interaction protein TXNIP. The Trx system can reduce oxidize Cys residues in proteins. The Trx active site reacts with the oxidized Cys forming a disulphide bond, which is reduced by TrxR and NADPH. There are three Trx isoforms, cytosolic Trx1, mitochondrial Trx2 and Trx3, that is mainly expressed in spermatozoa. In addition to controlling the cellular redox environment in the cytosol, Trx1 can translocate to the nucleus and regulate transcription factors such as HIF-1α (hypoxia-induced factor 1α), NFκ-b (nuclear factor kappa b), AP-1, Nrf2 and p53, influencing gene expression. Trx is widely expressed in endothelial cells and VSMCs and has an important role in protection against H_2O_2-induced toxicity [11] (Fig. 12.1).

12.2.2 Sources of ROS

ROS are produced as by-products of metabolic and enzymatic activities within cells. Superoxide anion is a natural result of oxygen reduction in mitochondrial respiratory chain [12]. Hydrogen peroxide is produced in the process of oxidative protein folding in the endoplasmic reticulum [13]. Enzymatic sources of ROS include the NADPH

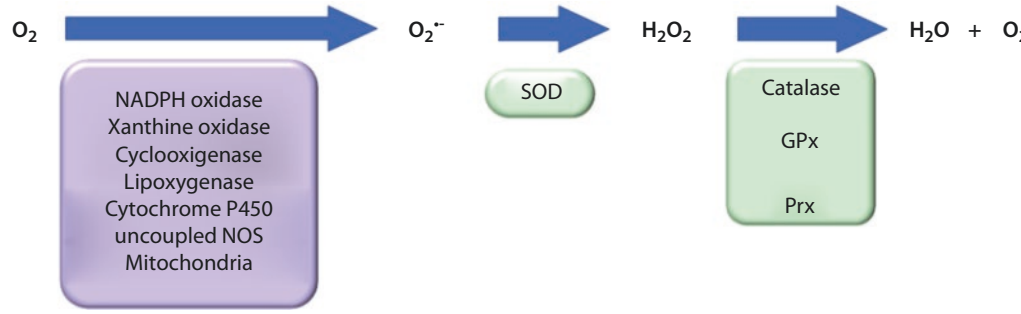

Fig. 12.1 ROS generation and antioxidant systems. Reduction of oxygen in the presence of one free electron results in generation of the free radical superoxide anion ($O_2^{\bullet-}$). Enzymatic sources of superoxide include the NADPH oxidases, xanthine oxidase, cyclooxygenases, lipoxygenases, cytochrome P450 enzymes, uncoupled nitric oxide synthase (NOS) and mitochondria. Superoxide anion is converted to hydrogen peroxide (H_2O_2) spontaneously or catalysed by the enzyme superoxide dismutase (SOD). Hydrogen peroxide is scavenged by catalase, glutathione peroxidase (GPx) and peroxiredoxin (Prx)

oxidases (Nox), xanthine oxidase, uncoupled nitric oxide synthase (NOS), cyclooxygenases, lipoxygenases and cytochrome P450 enzymes [14].

12.2.2.1 Mitochondrial ROS

Mitochondria are responsible for fundamental cellular processes such as ATP synthesis, oxygen sensing, biosynthetic pathways, intracellular Ca^{2+} homeostasis and regulation of programed cell death. In addition, mitochondria are one of the major sites of ROS production in most cell types. ATP synthesis involves transfer of electrons through enzymatic complexes (complex I to IV) that ultimately are transferred to molecular oxygen. During this process 1–4% of oxygen is incompletely reduced to $O_2^{\bullet-}$. Complex I and complex III are mainly involved in mitochondrial ROS generation in mitochondria inner membrane, releasing $O_2^{\bullet-}$ into the mitochondria matrix and intermembrane space. In addition other sources of mitochondrial ROS in vascular cells include the growth factor adaptor protein p66Shc and monoamine oxidases (MAO) [16].

In physiological conditions, normal respiration leads to constant low levels of ROS generation. Mitochondria are equipped with antioxidant enzymes, such as SOD that catalyses $O_2^{\bullet-}$ dismutase to H_2O_2. In addition, catalase, GPx and Prx can scavenge H_2O_2 produced by mitochondria. However, $O_2^{\bullet-}$ can be diffused to the cytosol via the mitochondrial permeability transition pore (MPTP), while H_2O_2 can cross the mitochondrial membrane. Additionally, in the presence of metal ions, H_2O_2 can be converted to $OH^{\bullet-}$ via the Fenton reaction. Therefore, ROS generated in the mitochondria can target molecules in the cytosol and other cellular compartments [17].

Vascular cells present relative low mitochondria content and low energy requirement compared to cardiomyocytes, for instance. Therefore, evidence indicates an important role for mitochondrial ROS in cellular signalling rather than energy production [18]. In endothelial cells mitochondria are involved in important functions such as shear stress-induced vasodilation and hypoxia signalling. Increased mitochondrial ROS can cause damage to mitochondrial components resulting in mitochondrial dysfunction. In turn, dysregulated mitochondria further increase ROS generation that can leak into other cellular compartments affecting cellular processes. Excess ROS from mitochondria is involved in endothelial dysfunction and vascular inflammation, common features of cardiovascular diseases including hypertension and atherosclerosis [17, 18].

12.2.2.2 NADPH Oxidases

The NADPH oxidase (Nox) family of enzymes are the only known enzymes with the exclusive function of ROS production. The Nox family is composed of seven isoforms named after the catalytic subunit, Nox 1–5, Duox1 and Duox2. All Nox isoforms are transmembrane proteins that transfer an electron from NADPH to oxygen, producing superoxide anion, that is rapidly converted to H_2O_2 [15]. Nox1, nox3, nox4 and Nox5 are present in all cell types in the vasculature and are the major enzymatic source of ROS in the cardiovascular system. Activation of Nox enzymes involves

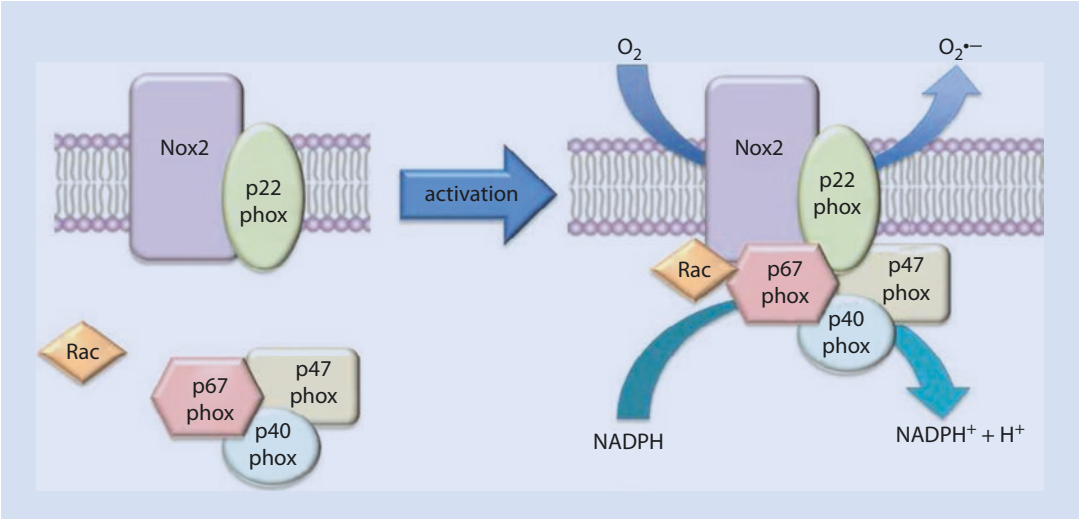

Fig. 12.2 Mechanism of activation of NADPH oxidases. In resting conditions Nox2 is associated with p22phox in the membrane, while p67phox, p47 phox, p40phox and Rac are found in the cytosol. Upon activation p47 phox is phosphorylated, and the cytosolic subunits translocate to the membrane assembling the NADPH oxidase complex

association with regulatory proteins and/or post-translational modifications that can be triggered by important vasoactive agents such as angiotensin II (Ang II) and endothelin-1 (ET-1) [14]. Several Nox isoforms are involved in different cellular functions in the vasculature.

Nox2

Nox2 was the first Nox isoform to be discovered in phagocytes, where it plays a fundamental role in phagocytosis [19]. Nox2 is an enzymatic complex composed of five subunits, and its activation requires complex assembly. Together with p22phox, Nox2 forms the membrane-bound enzymatic core, also known as flavocytochrome b558. Cytosolic subunits include p47phox, p67phox, p40phox and the small G-protein Rac1 that translocate to the membrane upon activation. In the vasculature, Nox 2 is present in endothelial cells, fibroblasts and VSMCs. Regarding subcellular localization, Nox2 displays a plasma membrane and perinuclear distribution and also associates with caveola/lipid rafts and the cytoskeleton [15]. Nox 2 genetic deficiency in phagocytes causes chronic granulomatous disease (CGD), leading to persistent and severe infections due to failure in $O_2^{\bullet-}$ production, which is critically involved in host-defence systems. Patients with CGD have high flow-mediated vasodilation suggesting a role for Nox2 in the regulation of vascular tone [20]. Indeed, Nox 2-derived ROS is involved in vasoconstriction responses to increase intraluminal pressure and disturbances in blood flow [21] (Fig. 12.2).

Nox1

Nox1 is expressed in all vascular cell types and seems to have an important role in growth factor signalling in VSMC. Nox1 produces $O_2^{\bullet-}$ in an induced manner, and activation can be triggered by stimuli such as growth factors, cytokines, LPS, oxidized LDL and shear stress. Similar to Nox2, it is also associated with p22 phox, and upon activation cytosolic subunits NoxO1 (Nox organizer 1, p47phox homologue), NoxA1 (Nox activator 1, p67 phox homologue), p40 phox and Rac1 translocate to the membrane forming the active enzyme complex. Nox1 is usually found in the plasma membrane, caveola/lipid rafts, endosomes and endoplasmic reticulum. In the vasculature Nox1 has been shown to be important in VSMC proliferation and vascular hypertrophy. Growth factors (PDGF, FGF and EGF), vasoactive agents (Ang II, ET-1), thrombin, IL-18 and extracellular ROS elicit a proliferative response mediated by Nox1 in VSMCs. Overexpression of Nox1 in VSMC in culture induces growth and proliferation [22].

Nox4

Nox4 is also expressed in all vascular cell types and seems to have a role in endothelial cell survival and VSMC differentiation. Nox4 differs

from the other isoforms as it only associates with p22 phox in the plasma membrane and doesn't seem to require the classical NADPH oxidase cytosolic subunits for its activation. Nox4 is associated mainly with H_2O_2 production due to its unique structure of the third extracytosolic loop that functions as a source of protons to facilitate dismutation of $O_2^{\bullet-}$. Nox4 is constitutively active and responsible for maintenance of basal levels of H_2O_2. Nox4 is found in the plasma membrane and perinuclear region, where it localizes in an endoplasmic reticulum location [15]. Nox4 plays a critical role in differentiation of VSMC. Nox4 deletion results in VSMC dedifferentiation [23]. Nox4 has been associated with both cardiovascular protective and injurious effects [24, 25].

Nox5

Humans and higher mammals express another NADPH oxidase isoform, Nox5 that is not present in rodents. This isoform differs from the others as it does not require any additional subunit for its activation and is activated by calcium (Ca^{2+}) binding to its N-terminal EF hands. Five splice variants are known as Nox5α, Nox5β, Nox5γ, Nox5δ and Nox5S, a short form without Ca^{2+}-binding domain. Multiple isoforms are expressed in endothelial cells and VSMC in the human vasculature and respond to agonists that contribute to cardiovascular diseases. Nox5 is activated by Ang II and ET-1 in endothelial cells, and its overexpression leads to increased proliferation and angiogenesis in endothelial cells [2]. Additionally, recent studies demonstrated that Nox5 is important in redox-sensitive contraction, regulating Ca^{2+} and ROS to activation of the pro-contractile molecular machinery in VSMCs [26].

12.2.3 Post-translational Oxidative Modifications

Once generated in cells, ROS influence signalling proteins through post-translational oxidative modification. Depending on the type of ROS and the structure of the target protein, numerous redox-sensitive processes can occur. Modifications of cysteine residues within proteins are key components of redox signalling to modulate protein function. Cysteine thiols (-SH) can be oxidized initially to sulfenic acid (Cys-SOH), also termed protein sulfenylation. ROS can further oxidize the resultant cysteine S-sulfenylation into reversible oxidative modifications (formation of disulphide bonds, glutathionylation, among others) [27]. Reversible cysteine oxidation is key to redox signalling providing a mechanism of redox switch for protein function and cell function. These processes are important for normal cellular signalling, and oxidation is usually reversible.

However, high levels of ROS due to uncontrolled production can result in irreversible protein oxidation by formation of sulfinic ($-SO_2H$) or sulfonic acid ($-SO_3H$) in cysteine thiols [27]. Irreversible oxidation leads to altered signalling. In VSMCs from hypertensive rats, Ang II-induced oxidation of the phosphatase SHP-2 is increased leading to sustained activation of AKT (protein kinase B) signalling [28]. Additionally, other types of irreversible oxidation can occur, such as protein carbonylation (modification of amino acid side chains to carbonyl derivatives), leading to protein damage, degradation and cell death [29] (Fig. 12.3).

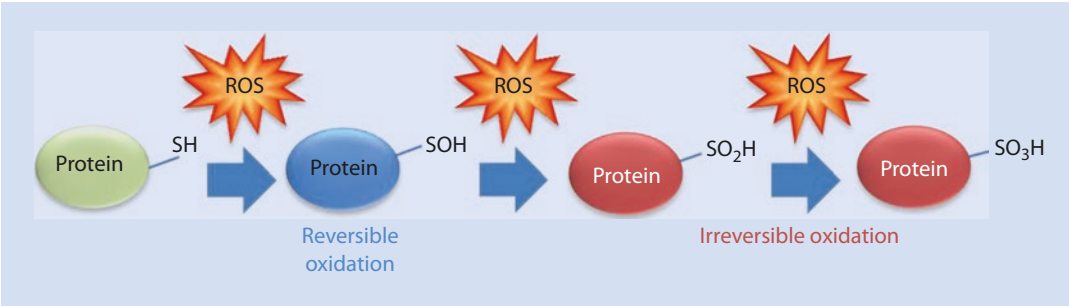

Fig. 12.3 Post-translational oxidative modifications in cysteine residues within proteins. ROS oxidize cysteine residues in proteins to influence protein function. Oxidation can be reversible, such as modification of cysteine to sulfenic acid (SOH), or irreversible, including formation of sulfinic and sulfonic acid on cysteine residues (SO_2H, SO_3H)

12.3 Redox Signalling in the Vasculature

Reactive oxygen species, which are now considered as second messengers, are an important component of signalling in vascular cells involved in diverse cellular functions. To achieve specificity, ROS generation is tightly controlled in discrete subcellular microdomains and organelles. Differential subcellular localization of Noxs as well as antioxidant enzymes contributes to activation of specific signalling molecules and maintenance of low ROS levels. In the vasculature, ROS are mediators of important endothelial and VSMC physiological functions.

Controlled ROS generation is critical to normal endothelial cell function. ROS are involved in the formation of new blood vessels during development and in response to vascular injury, a process called angiogenesis. Proliferation, adhesion and migration of endothelial cells are also mediated by ROS. Additionally, as endothelial cells are the barrier between blood and the other tissues, they are exposed to different biochemical and mechanical stimuli that induce ROS generation, such as growth factors (PDGF, FGF and EGF), vasoactive agents (Ang II, ET-1), cytokines and mechanical stimuli (shear stress, pressure) [30]. Similarly, in VSMC ROS participates in diverse physiological processes such as cell growth, migration, contraction, differentiation and regulation of extracellular matrix [14].

ROS can mediate such diverse effects through reversible oxidative post-translational modifications in proteins, which modulate the function of receptor and non-receptor tyrosine kinases, mitogen-activated protein kinases (MAPKs), protein tyrosine phosphatases (PTPs), ion channels and transcription factors. Some examples are the tyrosine kinase Src, protein kinase C (PKC), protein kinase B (PKB/AKT) and p38 MAPK, which are directly oxidized by ROS and are involved in growth factor-induced redox signalling in vascular cells. In this context, oxidation of PTPs is especially important because oxidation inactivates the enzymes, leading to phosphorylation of downstream kinases critically involved in the regulation of vascular function.

An example of redox-dependent mechanism is observed in the control of vascular tone. Superoxide anion acts as a vasoconstrictor, while H_2O_2 acts as a vasodilator in some vascular beds. Reactive oxygen species influence Ca^{2+} channels, such as second messenger-operated Ca^{2+} channel (SMOC), receptor-operated Ca^{2+} channel (ROC), voltage-gated Ca^{2+} channel (VOC), Na^{+}–Ca^{2+} exchanger (NCX) and transient receptor cation channels (TRPs), that increase transmembrane Ca^{2+} influx leading to increased intracellular Ca^{2+} levels, important in the initiation of vascular contraction. Vasoactive agents, through ROS, also promote release of Ca^{2+} from the sarcoplasmic reticulum via activation of IP receptors (IP_3R) and ryanodine receptors (RyR). Oxidation is a mechanism of activation of these Ca^{2+} channels. In addition, the sarcoplasmic reticulum Ca^{2+} pump (SERCA) that pumps Ca^{2+} back into the sarcoplasmic reticulum is inactivated by oxidation, further contributing to the increase in intracellular Ca^{2+} in conditions of oxidative stress. ROS is also involved in Ca^{2+}-independent pathways related to vascular contraction. Oxidation of the small GTPase RhoA, activated by vasoconstrictor agents, leads to activation of Rho kinase and consequent inhibition of myosin light chain phosphatase, allowing myosin-actin interaction and consequent contraction [1].

Targets of oxidative post-translational modifications also include transcription factors, placing ROS as modulators of gene expression in vascular cells. One example is the activation of Nrf2, involved in transcription of antioxidant genes. Under normal conditions, Nrf2 is suppressed by the inhibitor Keap1, which targets Nrf2 for proteasomal degradation. Oxidation of Keap1 leads to disruption of the Keap1-Nrf2 complex and nuclear translocation of Nrf2 [31].

12.4 ROS in Vascular Pathophysiology

Although diverse regulatory mechanisms control ROS generation and redox signalling, increased ROS bioavailability can occur resulting in oxidative stress. Increased ROS production and/or deficiency in antioxidant capacity is related to oxidative stress in pathological conditions. High levels of ROS cause disruption of redox signalling pathways, resulting in altered vascular function. Increased ROS generation, particularly via Noxs, is associated with several

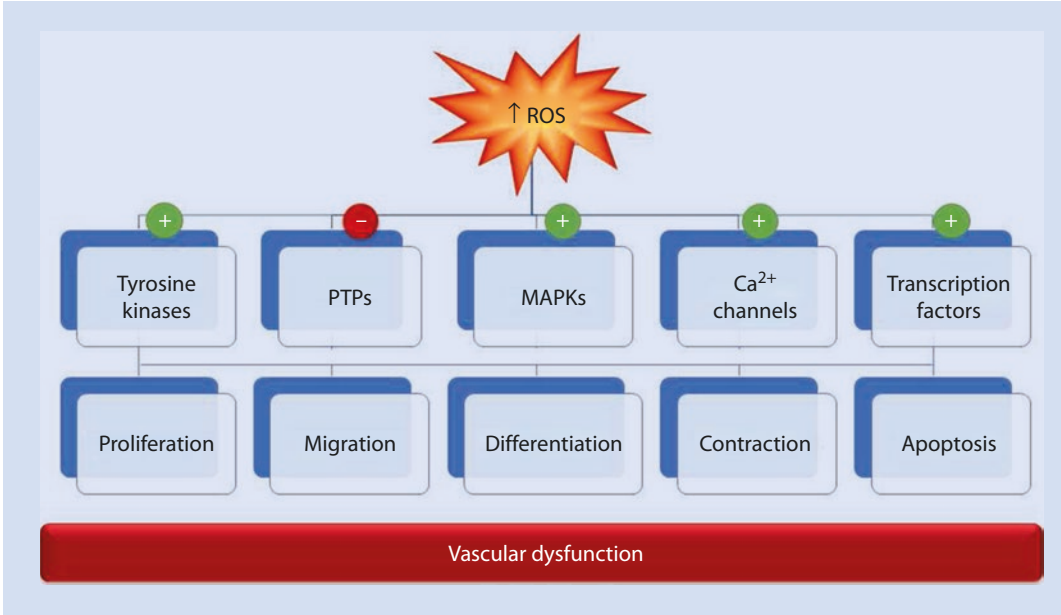

Fig. 12.4 Increased ROS generation plays a key role in vascular dysfunction. Through oxidative post-translational modifications, ROS modulates the function of receptor and non-receptor tyrosine kinases, mitogen-activated protein kinases (MAPKs), protein tyrosine phosphatases (PTPs), Ca^{2+} channels and transcription factors. In pathological conditions, high levels of ROS disrupt redox signalling pathways, resulting in aberrant signalling and consequent dysregulation of VSMC function leading to vascular dysfunction

cardiovascular diseases, such as hypertension, atherosclerosis, ischemic heart disease, myocardial infarction and stroke [14].

Increased or dysregulated ROS generation has an important impact in the vasculature. Endothelial cells produce nitric oxide (NO), an important endothelial-derived vasodilator that can be quenched by excess $O_2^{\bullet-}$ resulting in impaired vasorelaxation. Furthermore, this reaction results in formation of peroxynitrite (ONOO-), a highly reactive nitrogen species that also causes protein oxidation. Tetrahydrobiopterin (BH_4), an important cofactor for eNOS, is inactivated by peroxynitrite aggravating vascular dysfunction [32]. These phenomena characterize endothelial dysfunction, an underlying mechanism involved in vascular damage associated with several cardiovascular diseases.

Vascular dysfunction also involves alterations in VSMCs resulting in functional, structural and mechanical alterations in blood vessels. VSMCs are contractile cells critical in the control of blood flow and pressure. However, in pathological conditions, they become dedifferentiated, showing increased contraction, proliferation, migration and apoptosis. High levels of ROS are closely associated with these processes collectively termed vascular remodelling. Uncontrolled ROS generation disrupts redox signalling pathways, resulting in aberrant signalling and consequent dysregulation of VSMC function [2] (Fig. 12.4).

In pathological conditions, high concentrations of ROS promote irreversible oxidation of protein targets leading to protein damage and degradation [25]. Particularly, irreversible PTP oxidation could result in sustained kinase activity, enhancing growth factor signalling. Increased oxidation of redox-sensitive kinases such as Src and p38 MAPK is also involved in hyperactivation of growth signalling pathways in VSMCs. In addition, dysregulated ROS generation affects the function of Ca^{2+} channels, transcription factors and cytoskeletal proteins leading to altered contraction, migration, apoptosis and rearrangement and disorganization of the cytoskeleton [14]. Furthermore, Noxs play an important role in regulation of VSMC function. Nox4 is required for maintenance of VSMC in the differentiated contractile phenotype [23]. On the other hand, increased Nox1-derived ROS mediates VSMC switch from a contractile to a proliferative phenotype [22]. Recent evidence indicates that Nox5 is an important pro-contractile Nox isoform [26].

Conclusions and Clinical Perspectives

ROS are key mediators of signalling in vascular cells. Several cellular events involved in physiological vascular function, such as contraction, relaxation, proliferation, migration and differentiation are redox-sensitive. However, increased ROS generation can alter important signalling pathways and induce vascular dysfunction. Altered ROS production and scavenging are underlying mechanisms of cardiovascular diseases, making oxidative stress an interesting target for therapeutic intervention. In animal models of cardiovascular diseases, decreasing ROS bioavailability improves endothelial dysfunction and vascular remodelling and normalizes blood pressure in hypertensive models. Clinical studies with specific Nox inhibitors and other ROS modulators may improve treatment of cardiovascular diseases. Identification of disease-specific molecular targets and sources of ROS involved in pathological redox signalling will contribute to development of new therapeutic strategies to reduce oxidative stress and vascular injury.

Gaps in Knowledge

- Molecular mechanisms underlying differential ROS effects in the vasculature are still unclear.
- Further studies are needed to understand ROS generation in a spatial-temporal manner as well as investigation of how oxidative modifications influence vascular function in health and disease.
- Identification of specific ROS targets and specific ROS sources will contribute to development of new therapeutic strategies targeting oxidative stress in cardiovascular diseases.
- It is not yet possible to accurately assess ROS in vivo or in the clinic.

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TGF-β in Vascular Pathobiology

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_13

Key Concepts

- The TGF-β superfamily encompasses 32 cytokines which tightly control vascular cell proliferation, migration, apoptosis, differentiation and ECM maintenance, production and remodelling.
- TGF-β superfamily members signal via distinct type I and type II transmembrane serine/threonine kinase receptors activating canonical SMAD signalling and multiple non-canonical signalling pathways.
- Defects in genes encoding TGF-β superfamily signalling components cause impaired vascular morphogenesis/homeostasis and HVDs.
- Introduction of the novel anti-angiogenic drugs dalantercept, PF-03446962 and carotuximab for patients with advanced solid tumours and the introduction of bevacizumab treatment for HHT patients are promising new therapeutic strategies.
- Continued research on TGF-β superfamily signalling in tumour angiogenesis, HVDs and acquired cardiovascular disease will give rise to novel therapeutics specifically targeting these pathways.

13.1 Introduction

The TGF-β superfamily comprises 32 pleiotropic growth factors including three TGF-β isoforms (TGF-β_1, TGF-β_2 and TGF-β_3), several bone morphogenetic proteins (BMPs), activins, inhibins and myostatins [1]. Members of this growth factor superfamily regulate many distinct cellular functions crucial for normal embryonic development and for maintaining whole body homeostasis in adulthood [1].

13.2 TGF-β Superfamily Signalling

All TGF-β isoforms are synthesised as large monomeric proteins. Following removal of the signal peptide during translocation across the rough endoplasmic reticulum, three disulphide bonds are formed between two monomers forming the pro-TGF-β homodimer [2]. Endoprotease furin convertase then cleaves the pro-TGF-β homodimer within the Golgi apparatus resulting in latency-associated protein (LAP) homodimer and mature TGF-β homodimer separation [2]. However, these two homodimers remain bound by non-covalent bonds and form the small latent complex (SLC) [2]. The SLC then binds covalently to the large latent TGF-β-binding protein (LTBP) to form the large latent complex (LLC), which is secreted from the cell where it can be sequestered in the ECM in its inactive form [2]. TGF-β isoforms are activated following proteolytic cleavage of LTBP and release from LAP [2].

To date seven transmembrane serine/threonine kinase TGF-β superfamily type I receptors and five transmembrane serine/threonine kinase type II receptors have been identified [1]. Members of the TGF-β superfamily bind to extracellular domains of TGF-β type I (TβRI) and type II (TβRII) receptors (Fig. 13.1). Additionally, the membrane-bound TGF-β type III co-receptors (TβRIIIs) betaglycan and ENG are also capable of binding TGF-β superfamily ligands and forming complexes with TβRI or TβRIIs [1, 3].

Although the highly conserved canonical signalling pathway described in Fig. 13.1 is a fairly simple and linear cascade, TGF-β superfamily signalling, in reality, is much more complicated and depends on many variables. Different ligand-receptor affinities, different TGF-β superfamily expression levels and different TβRI/TβRII and TβRI/TβRII/TβRIII interactions as well as tightly regulated auto-inhibitory feedback mechanisms enable transduction of complex signals in distinct cell types in a temporal- and spatial-dependent manner. A brief overview of TGF-β superfamily member signalling pathways involved in normal vascular morphogenesis and homeostasis is given in Fig. 13.2.

Although ligand-induced SMAD2/3 signalling outweighs SMAD1/5/8 signalling in SMCs and ligand-induced SMAD1/5/8 signalling outweighs SMAD2/3 signalling in ECs in homeostatic conditions, both canonical pathways and multiple non-canonical pathways are present in each above-mentioned cell types and maintain a continuous physiological balance. The following sections will discuss the importance of intact TGF-β signalling during vascular morphogenesis and how genetic defects in TGF-β signalling components cause HVDs.

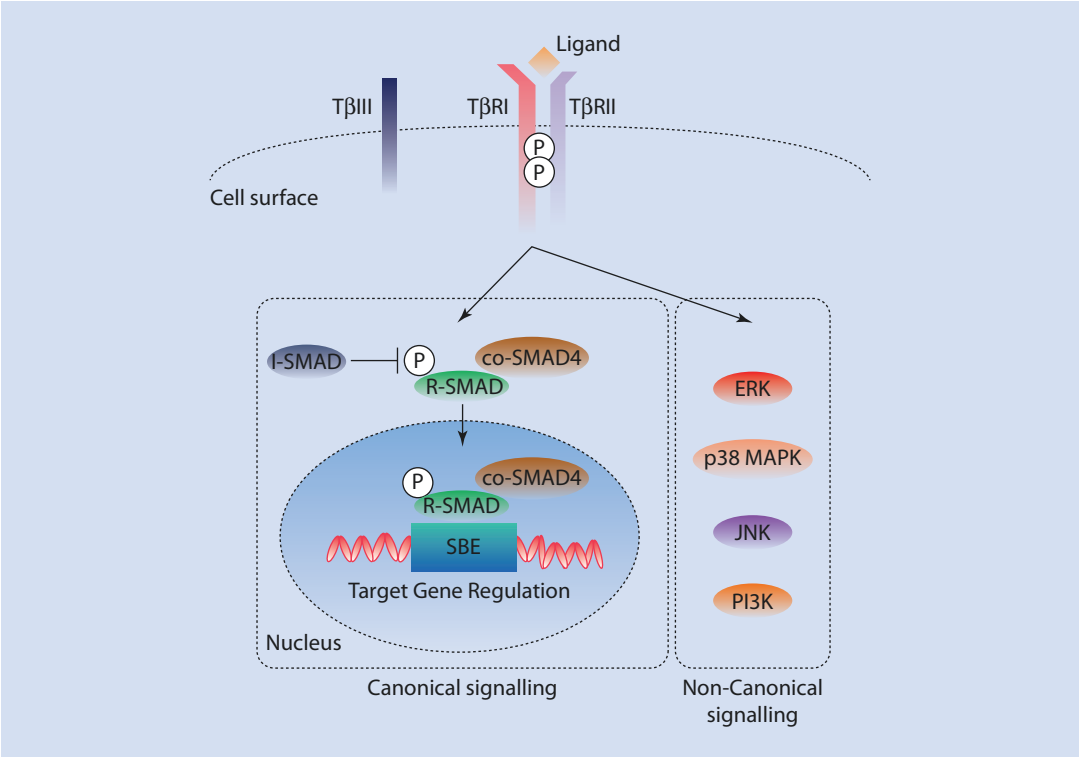

Fig. 13.1 Schematic representation of TGF-β superfamily signalling pathways. Receptor-ligand interactions trigger receptor transphosphorylation between TβRI and TβRII resulting in activation of the canonical SMAD signalling pathway or non-canonical pathways such as extracellular regulatory kinase (ERK), c-Jun N-terminal kinase (JNK), p38 microtubule-associated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) signalling [1, 3]. Phosphorylated R-SMADs (SMAD1, SMAD2, SMAD3, SMAD5, SMAD8) form a heteromeric complex with the common (co)-SMAD4 in order to translocate to the nucleus [1, 3]. Within the nucleus this complex directly binds to SMAD-binding elements (SBE) or interacts with other transcription factors in order to regulate target gene expression. Enhanced canonical R-SMAD signalling also results in auto-inhibition achieved by (i) increased production of inhibitory (I)-SMADs (SMAD6 and SMAD7) preventing R-SMAD phosphorylation or (ii) by internalisation and degradation of TβRI or TβRIIs [1, 3]. Membrane-bound TβRIIIs are also able to bind ligands and interact with TβRI or TβRIIs [1, 3]. (Figure adapted from David et al. [1] and Bobik et al. [3])

13.3 Vascular Morphogenesis

Vascular morphogenesis involves vasculogenesis and angiogenesis [6]. Vasculogenesis means de novo formation of blood vessels, while angiogenesis refers to the formation of new blood vessels from pre-existing ones. Angiogenesis also occurs in adulthood enabling physiological vascular homeostasis as well as tissue regeneration following injury [6]. Importantly, in certain tumour types, excessive vasculogenesis and angiogenesis contribute to tumour growth and also facilitate spreading of tumour cells to distant cites, thereby promoting metastasis [7].

Unimpaired vascular morphogenesis during embryogenesis heavily relies on a finely regulated sequence of complex events leading to the formation of a functional and healthy circulatory system [6]. Multiple animal gene deletion studies demonstrate the importance of intact and balanced TGF-β superfamily signalling during all stages of vascular morphogenesis.

Mesodermal progenitor-to-EC differentiation initiates vasculogenesis resulting in the formation of the primary capillary plexus (PCP) [6]. End-to-end EC sprouting and intussusceptive vascular growth then transform the PCP into new vessels [6]. Homozygous TGF-β_1-deficient C57BL/6 mice die of lethal impaired endothelial differentiation resulting in inadequate capillary tube formation highlighting the importance of this ligand during vasculogenesis [6, 8]. Similarly, *tgfbr1* (encodes ALK5)- and *tgfbr2* (encodes TGFBR2)-deficient mice display extensive vascular defects causing prenatal death [6].

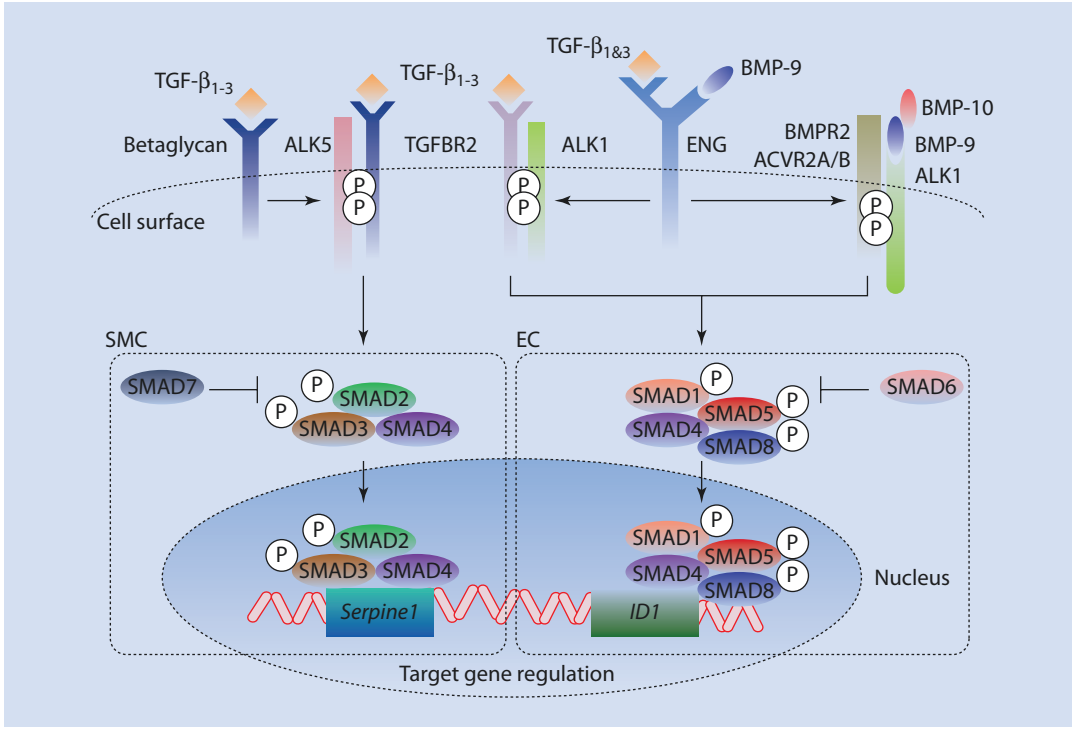

Fig. 13.2 Schematic representation of ALK5 and ALK1 signalling pathways. All three active TGF-β isoforms bind to the extracellular domain of the TGF-β receptor 2 (TGFBR2) which belongs to the TβRII family [1, 3]. Ligand-binding on vascular smooth muscles cells (SMCs) predominantly triggers complex formation with the TβRI activin-like kinase (ALK)5 receptor resulting in SMAD2/3 phosphorylation which subsequently form of heteromeric complex with co-SMAD4 in order to access the nucleus [3]. The *SERPINE1* gene (encodes plasminogen activator inhibitor-1) is a well-known target of this route [3]. The TβRIII betaglycan binds all three TGF-β isoforms and promotes TGFBR2/ALK5 complex formation [1]. Efficient TGF-β_2 signalling especially relies on the presence of betaglycan since this isoform alone has a low affinity for TGFBR2 [4]. In contrast, TGF-β_1/TGFBR2 complex formation on endothelial cells (ECs) can also recruit ALK1, another class of TβRIs, triggering SMAD1/5/8 phosphorylation [3]. Following heteromeric complex formation with the co-SMAD4, this complex also translocates to the nucleus where it induces *ID1* gene (encodes inhibitor of differentiation 1) expression. Additionally, ALK1 can also bind BMP-9 and BMP-10 in complex with the type II receptors BMP receptor 2 (BMPR2) or activin A receptor type II (ACVR2)A/B and subsequently triggering SMAD1/5/8 phosphorylation [1, 5]. Endoglin is predominantly expressed in ECs and can also bind BMP-9 [5]. Activation of endoglin promotes complex formation between ALK1 and its type II receptors [1]. (Figure adapted from David et al. [1], Bobik et al. [3] and Roman et al. [5])

Intact vessel wall muscularisation driven by progenitor-to-SMC/pericyte differentiation, SMC/pericyte recruitment and increased ECM deposition further relies on functional TGF-β signalling [6]. In line with in vitro findings demonstrating that TGF-β induces progenitor-to-SMC differentiation, TGF-β_1-deficient mice display impaired SMC differentiation in addition to weak vessels with reduced cellular adhesiveness [4, 8, 9]. Furthermore, SMC recruitment to the vessel wall depends on direct EC-to-SMC contact formation which requires the presence of eng since *eng*-deficiency in mice causes reduced SMC vessel coverage and subsequently dilated and fragile vessel walls [6]. Muscularisation is followed by vascular specification, a process whereby premature vessels develop into arterial, venous or lymphatic vessels. Loss of ALK1 in mice causes impaired arterial specification characterised by decreased arterial expression of the arterial marker ephrin-B2 [6]. In addition, *eng*-deficient mice demonstrate arterial expression of the venous marker COUPTF (chicken ovalbumin upstream promoter transcription factor) II, demonstrating the importance of functional ALK1 and ENG during vascular specification [6].

Following vascular specification further angiogenesis occurs [6]. This stage can roughly be

separated into the activation and the resolution phase [6]. Briefly, these two phases are characterised by EC-to-tip cell or stalk cell differentiation which dynamically proliferate and migrate resulting in the formation of new blood vessel branches [6]. Research suggests that impaired vascular specification disrupts normal tip and stalk cell determination causing abnormal vessel branching [6]. In addition to the importance of intact hypoxic-inducible factor (HIF)1-α and NOTCH signalling during angiogenesis, in vitro and in vivo studies have demonstrated that functional ALK1 is also required to regulate this complex sequence of cellular events [5, 6, 10].

Overall, intact vascular TGF-β superfamily signalling is critical for normal vascular morphogenesis and homeostasis. Genetic defects in genes encoding members of this family cause widespread vascular defects highlighting the importance of the TGF-β superfamily during all stages of embryonic vascular development (■ Table 13.1).

■ **Table 13.1** Phenotypes of mice with gene deletions of TGF-β pathway components

Gene	Animal model/clinical symptoms
tgfb1	KO: embryonic lethal with vascular defects or postnatal lethality from autoimmune disease
tgfb2	KO: aortic arch and cardiac septal defects, perinatal lethality
tgfb3	KO: cleft palate, delayed lung maturation, die shortly after birth
gdf2	KO: incomplete closure of the ductus arteriosus
bmp10	KO: embryonic lethal, impaired cardiac development and function
tgfbr1	KO: embryonic lethal, angiogenesis defects
tgfbr2	KO: embryonic lethal, vascular defects
tgfbr3	KO: poorly formed cardiac septa, incomplete compaction of ventricular walls
acvrl1	KO: embryonic lethal, AVMs, disrupted arterial identity, dilated vessels, impaired plexus remodelling, reduced vSMC coverage
bmpr2	KO: pre-angiogenesis lethality
	Transgenic *bmpr2*-mutant allele: pulmonary hypertension
eng	KO: embryonic lethal, no or only small AVMs, dilated vessels, impaired plexus remodelling, reduced vSMC coverage, heart defects
	HET: vascular lesions similar to HHT
madh1	KO: embryonic lethal due to defects in chorion-allantoic circulation
madh3	KO: die between 1 and 8 months of age due to metastatic colorectal cancer
madh4	KO: embryonic lethal
madh5	KO: embryonic lethal due to angiogenesis defects
madh6	KO: heart abnormalities, aortic ossification and elevated blood pressure
madh7	KO: embryonic lethal due to cardiovascular defects
madh9	Mutant allele: defective pulmonary vascular remodelling

Table adapted from Pardali et al. [6, 11], Levet et al. [12] and Chen et al. [13]
KO knockout, *vSMC* vascular smooth muscle cell, *AVM* arteriovenous malformation, *HET* heterozygous, *gdf2*, gene encoding BMP-9, *tgfbr3* gene encoding betaglycan, *acvrl1* gene encoding ALK1, *madh1* gene encoding SMAD1, *madh3* gene encoding SMAD3, *madh4* gene encoding SMAD4, *madh5* gene encoding SMAD5, *madh6* gene encoding SMAD6, *madh7* gene encoding SMAD7, *madh9* gene encoding SMAD8

13.4 Hereditary Vascular Disorders Caused by Defective TGF-β Superfamily Signalling

The above-mentioned gene deletion studies in mice stress the importance of intact TGF-β superfamily signalling during all stages of vascular morphogenesis. Unsurprisingly, defects in TGF-β superfamily signalling pathway genes are associated with HVDs. Although HVDs are rare, they are often associated with a poor prognosis, pose life-threatening complications and severely impair the quality of life of affected patients. A detailed summary of genetic defects in TGF-β superfamily signalling components causing HVDs in humans is presented in Table 13.2.

13.4.1 Hereditary Haemorrhagic Telangiectasia

Definition and Epidemiology HHT also known as Osler-Weber-Rendu disease is an autosomal dominant genetic syndrome that is mainly characterised by mucocutaneous telangiectasias (small blood vessel dilation) and visceral AVMs (abnormal arteriovenous connection without a capillary bed) [14]. This syndrome affects approximately 1 in 5000 individuals [14].

Clinical Symptoms Vessel walls within AVMs and telangiectasias are fragile and prone to recurrent haemorrhaging [14]. Depending on the AVM/telangiectasia localisation and size, this can

Table 13.2 List of genetic defects in TGF-β superfamily signalling components

Gene	Clinical symptoms	Human disease
FBN1	Aortic root aneurysm, ectopia lentis, skeletal overgrowth	MFS
TGFB2	Pectus deformities, joint hypermobility, cervical spine instability, aortic aneurysm/dissection	LDS type IV
TGFB3	Striae, velvety skin, translucent skin, easy bruising, club foot, aortic aneurysm/dissection	LDS type V
GDF2	AVM, telangiectasia	HHT5
ENG	AVM, telangiectasia, PAH	HHT1, HHT-associated PAH
TGFBR1	Arterial tortuosity, aortic/arterial aneurysms	LDS type I
ACVRL1	AVM, telangiectasia, PAH	HHT2, HHT-associated PAH
TGFBR2	Mitral valve abnormalities, hypertelorism, bifid uvula, aortic aneurysm/dissection	LDS type II
BMPR2	PAH	HPAH
BMPR1B	PAH	HPAH
MADH1	PAH	HPAH
MADH3	Cleft palate, retrognathia, early-onset osteoarthritis, aortic aneurysm/dissection	LDS type III
MADH4	AVM, telangiectasia, colonic polyps, colorectal cancer	JP-HHT syndrome
MADH5	PAH	HPAH
MADH9	PAH	HPAH
SKI	Infantile hypotonia, mental retardation, craniosynostosis, proptosis, downslating palpebral fissures, micrognathia, mitral valve prolapse, aortic root dilation	SGS

Table adapted from Kritharis et al. [14], Ruiz-Llorente et al. [15], Girerd et al. [23], Soubrier et al. [24] and Cannaerts et al. [27]
FBN1 gene encoding fibrillin-1, *JP-HHT* juvenile polyposis hereditary haemorrhagic telangiectasia, *SKI* gene encoding v-ski avian sarcoma viral oncogene homologue, *SGS* Shprintzen-Goldberg syndrome

potentially cause lethal intracranial, intrapulmonary, intrahepatic, gastrointestinal (GI) as well as recurrent nose bleeding (epistaxis). Chronic anaemia and liver and heart failure as a result of recurrent haemorrhaging and haemodynamically relevant AV shunts are common conditions found in HHT patients [14].

Diagnosis The Curaçao criteria are applied for diagnosing HHT and include the presence of spontaneous recurrent nose bleeding, telangiectasias at characteristic sites, visceral AVMs and a first-degree relative with HHT [14].

Pathogenesis To date, defects in four distinct genes encoding TGF-β superfamily signalling components have been identified and form the underlying mechanism for disease development and progression [15]. Mutations in the *ENG* gene (HHT1) are the most common cause of HHT cases followed by mutations in the *ACVRL1* gene (HHT2) [14, 15]. Mutations in the *GDF2* (HHT5) and the *MADH4* (juvenile polyposis-HHT syndrome) genes account for a small number of HHT patients [14, 15]. Mutations in the *ENG* and *ACVRL1* genes most likely result in haploinsufficiency which means that any affected cell only carries one fully functional *ENG*/*ACVRL1* gene copy [5, 15]. Transcription of mutated *ENG* and *ACVRL1* genes provokes the formation of dysfunctional ENG and ALK1 receptors resulting in imbalanced TGF-β/BMP signalling [5, 15].

Pathological AVM development most likely occurs as a combination of genetic defects and environmental insults, leading to impaired vascular morphogenesis and dysfunctional vascular responses to shear stress and injury during embryogenesis and adulthood [5, 15]. Homozygous deletion of the *acvrl1* and *eng* genes in mice is embryonic lethal leading to failed plexus remodelling, dilated vessels and decreased vSMC coverage [6]. However, *acvrl1* gene deletion, in contrast to *eng* gene deletion, results in AVM formation and disrupted arterial identity, whereas *eng*-deficient mice develop no or only small AVMs [5]. In support of the environmental vascular insult hypothesis resulting in a dysfunctional vascular response, one in vivo study demonstrated that AVMs developed when angiogenic stimuli were combined with eng depletion in tamoxifen-inducible (TI) endothelial-specific *eng* adult knockout mice [16]. Similarly, mechanical wounding of the skin in TI endothelial-specific *acvrl1* adult knockout mice resulted in AVM formation in subdermal blood vessels [17]. These studies highlight that normal vascular morphogenesis and repair depend on fully functional ENG and ALK1 receptors. In contrast, heterozygous *acvrl1* and *eng* gene deletion in mice, mimicking assumed haploinsufficiency in humans, are not lethal and demonstrate a milder vascular phenotype with incomplete penetrance [5].

Considering all of these in vivo findings and the fact that HHT severity and age of disease onset vary between individual patients, it is likely that a second hit (such as increased arterial shear stress or vascular injury) is required to trigger AVMs [15]. Previous studies have demonstrated that vascular injury in form of increased shear stress, hypoxia and inflammation induces up-regulation of endothelial ENG and ALK1 expression highlighting the importance of these two receptors in response to injury [15]. The second hit hypothesis postulates that pro-angiogenic stimuli in response to injury lead to a worsening of already imbalanced TGF-β/BMP signalling resulting in a dysfunctional vascular response. In line with this hypothesis, in vitro studies have shown that enhanced ALK1 pathway activity inhibits EC proliferation and migration and pathway inhibition promotes EC sprouting in 3D angiogenic sprouting assays [5]. Further in vivo studies have demonstrated that loss of endothelial ALK1 expression impairs EC polarisation and enhances EC migration, resulting in distal vessel thickening and AV shunt formation [5]. These studies indicate that the ability of endothelial ALK1 to promote EC polarisation and migration against arterial blood flow is crucial for normal arterial maturation.

One clinically relevant proposed mechanism of abnormal EC proliferation is explained by increased endothelial VEGF pathway activity and loss of anti-proliferative endothelial ALK1 signalling [15]. Clinically, HHT patients demonstrate increased circulatory VEGF plasma levels and normalisation of circulating VEGF levels in an in vivo study resolved AVMs [15]. Furthermore, enhanced ALK1 signalling has been shown to inhibit endothelial VEGF expression [14].

Management Before initiating treatment a full patient work-up is required. Unfortunately, treatments directly targeting dysfunctional ALK1 and ENG are not yet available for HHT patients to date.

Disease management focuses on supportive measures such as iron supplementation, blood pressure control and lesion-specific therapy involving interventional radiology and/or surgery [14]. Genetic counselling is also recommended since there is risk of genetic inheritance and pregnancy increases haemorrhaging risk in female HHT patients [14].

Bevacizumab, a monoclonal antibody directed against VEGF-A, is an approved drug for anti-angiogenic treatment in several types of cancers, age-related macular degeneration and diabetic retinopathy. There is a strong rationale for treating HHT patients with bevacizumab since these patients demonstrate increased systemic pro-angiogenic VEGF-A plasma levels [14, 15]. A single centre phase II study was able to show that intravenous (IV) administration of bevacizumab in 25 HHT patients with severe hepatic AVMs and high cardiac output resulted in a significant reduced duration and number of epistaxis episodes as well as a decrease in cardiac output [18]. Another 2017 single centre retrospective study demonstrated that IV administration of bevacizumab in 34 patients significantly decreased the number of red blood cell transfusions providing evidence that this treatment improves severe anaemia related to recurrent epistaxis and GI bleeding [19].

In addition, thalidomide, an immunomodulatory drug, promoted vessel maturation in *eng*-heterozygous mice by stimulating mural cell recruitment to improve vessel wall defects [20]. A subsequent small clinical study demonstrated that one daily dose of orally administered thalidomide significantly reduced the severity and frequency of epistaxis in HHT patients [20]. Furthermore, histological analysis of nasal mucosal biopsies revealed that thalidomide-treated patients displayed more SMC layers around nasal mucosal blood vessels demonstrating that this drug might be a novel strategy for treating vascular malformations [20].

13.4.2 Hereditary Pulmonary Arterial Hypertension

Definition and Epidemiology HPAH is a rare autosomal dominant genetic disease with incomplete penetrance that causes an abnormal elevation of pulmonary arterial pressure of greater than 25 mmHg at rest [21]. HPAH is a subcategory of class I pulmonary arterial hypertension (PAH). Class I PAH affects approximately 15 in 1,000,000 individuals, and in 50% of all cases, class I PAH in affected patients is caused by either idiopathic PAH (IPAH), HPAH or drug-induced PAH [21].

Clinical Symptoms Pulmonary arterial obstruction and hypercontractility result in PAH and increased right ventricular (RV) load resulting in impaired exercise tolerance [21, 22]. Pulmonary arterial remodelling causes impaired blood oxygenation and breathlessness [21]. Left untreated HPAH rapidly progresses to right ventricular failure (RVF) and death [21, 22].

Diagnosis Clinical diagnosis requires a full patient work-up. This includes taking a detailed patient history, performing a thorough clinical examination, performing a full blood work-up as well as performing extensive noninvasive (6-min walking distance, electrocardiogram, echocardiography, body plethysmography, chest radiograph, high-resolution computed tomography) and invasive (right heart catheter and vasoreactivity) diagnostic procedures to rule out other forms of PAH [21].

Pathogenesis Genetic defects in distinct genes encoding TGF-β superfamily signalling components are believed to be the underlying mechanism of disease onset and progression. Roughly 75% of all HPAH patients display a *BMPR2* gene mutation followed by more recent discoveries of defects in genes encoding ALK1, ENG, SMAD1, SMAD5, SMAD8 and BMPR1B (encodes bone morphogenetic protein receptor type 1B) [23, 24]. While a lot of recent research has focused on HPAH pathogenesis in *BMPR2* mutation carriers, very little is known about HPAH pathogenesis in patients carrying *ACVRL1* and *ENG* gene mutations [23]. Since *ACVRL1* and *ENG* mutations are also directly linked to HHT pathogenesis, these patients may exhibit both PAH and vascular malformations [23].

Since there is no evidence for direct loss of heterozygosity at the *BMPR2* locus in HPAH patients, decreased BMPR2 expression levels most likely occur as a consequence of increased mRNA degradation caused by nonsense-mediated mRNA decay (NMD) [24]. Since not all *BMPR2* mutation carriers develop PAH, disease onset is probably caused by a secondary pulmonary vascular insult [23, 24]. This most likely exacerbates already imbalanced pulmonary vascular TGF-β/BMP signalling resulting in reduced BMPR2 and increased ALK5 signalling resulting in dysfunctional pulmonary arterial

EC (PAEC) and SMC (PASMC) behaviour [22, 24, 25]. Overall, more chromosomal abnormalities are found in PAECs compared to PASMCs potentially highlighting the importance of dysfunctional pulmonary vascular endothelium in disease development and progression [24]. It is believed that initial PAEC apoptosis causes uncontrolled proliferation and expansion of dysfunctional surviving PAECs also resulting in increased PASMC proliferation and perivascular inflammation causing obstructive luminal arterial lesions [22]. Furthermore, pulmonary endothelial dysfunction results in impaired endothelial-dependent vasodilation and subsequently in pulmonary arterial hypercontractility [22]. Pulmonary arterial lesion formation as a consequence of imbalanced TGF-β/BMP signalling may also be regarded as a neoplastic process [24]. Microsatellite instability, a condition characterised by genetic hypermutability as a result of impaired DNA mismatch repair, is present in pulmonary arterial lesions from PAH patients [24]. In IPAH patients, microsatellite instability is commonly found within PAECs [26]. As a consequence of genetic hypermutability, frameshift mutations may also occur in certain tumour suppressor genes such as *BAX* (encodes bcl-2-like protein 4) preventing controlled PAEC apoptosis and facilitating uncontrolled clonal expansion [26].

Management To date, treatments directly targeting defective BMPR2 signalling are not yet available for HPAH patients. Therefore, disease management focuses on symptom relief including supervised rehabilitation, psychosocial support, oxygen supplementation, diuretic treatment in patients with signs of RVF and fluid retention, specific PAH drug treatment and ultimately lung transplantation [21]. Genetic counselling is also recommended since there is risk of genetic inheritance and pregnancy greatly increases cardiovascular risk in female HPAH patients [21, 23].

Pulmonary vasoreactivity is tested during right heart catheterisation by intra-arterial administration of calcium channel blockers (CCBs) [21]. Responsive patients are characterised by a significant drop in pulmonary arterial pressure following CCB administration [21]. Continued high-dose oral CCB treatment (nifedipine, amlodipin or diltiazem) may be considered in these patients [21]. HPAH patients unresponsive to intra-arterial CCB administration may be considered for further mono or combined antihypertensive treatment with phosphodiesterase inhibitors (sildenafil, tadalafil), endothelin receptor antagonists (ambrisentan, bosentan, macitentan), the guanylate cyclase stimulator riociguat, prostacycline analogues (epoprostenol, iloprost) and the prostacycline receptor agonist selexipag [21].

13.4.3 Loeys-Dietz Syndrome

Definition and Epidemiology LDS is a rare Marfan-like autosomal dominant genetic connective tissue disease causing widespread impaired organogenesis including arterial aneurysms and tortuosity, orbital hypertelorism (increased distance between the eyes), bifid (divided) uvula or cleft palate, cervical spine instability, club foot, joint hypermobility and early-onset osteoarthritis [27].

Clinical Symptoms Mutations in distinct genes dictate the clinical phenotype, and, therefore, symptoms vary between LDS patients [27]. In general, cardiovascular manifestations are more severe than in Marfan syndrome (MFS) patients with aortic and cerebral arterial dissections/ruptures occurring at a younger age [27]. Haemorrhaging poses a potentially life-threatening situation and often results in lifelong disability.

Diagnosis The combination of above-mentioned symptoms suggests the presence of LDS. Further assessment requires a full patient work-up and genetic testing to establish a full-scale picture of disease [28]. Diagnostic imaging should be performed in order to assess the number and extent of arterial aneurysms and tortuosity [28].

Pathogenesis Underlying disease mechanisms are caused by heterozygous nonsense mutations in the *TGFBR1* (causes LDS type I), *TGFBR2* (causes LDS type II), *MADH3* (causes LDS type III), *TGFB2* (LDS type IV) and *TGFB3* (LDS type V) genes [27]. Mutations in these genes result in loss of function (LOF) and subsequently in deregulation of vascular TGF-β signalling [27]. Although LOF mutations theoretically suggest dampened vascular TGF-β signalling, these defects cause a paradoxical over-activation of TGF-β signalling as evidenced by increased levels of TGF-β signalling components in aortic walls of LDS patients [27].

Genetically altered mice carrying *TGFBR1* and *TGFBR2* mutations known to cause LDS in

humans demonstrated thickened aortic adventitia containing increased numbers of CD45+ leukocytes displaying increased pSmad2 presence [29]. These findings were accompanied by increased aortic root wall *Tgfb1* expression [29]. Importantly, losartan, an angiotensin 2 type I receptor blocker (ARB), known to exhibit anti-TGF-β signalling properties but not propranolol, an unspecific β-blocker (BB), ameliorated aortic aneurysms in these LDS mice [29]. A further in vivo study found that SMC-specific *tgfbr2* deletion in mice resulted in thoracic aortic aneurysm (TAA) formation displaying increased adventitial fibrosis, increased TGF-β ligand expression and increased phospho-p38/phospho-ERK1/2 presence in the aortic wall [29]. While non-canonical TGF-β signalling was increased in this mouse model, canonical pSmad2 signalling was not affected. Based on these in vivo findings, LDS-associated genetic mutations most likely cause increased vascular ALK5 signalling promoting medial SMC and adventitial fibroblast/inflammatory cell dysfunction, resulting in progressive arterial wall deterioration facilitating aneurysm and tortuosity formation [29]. Arterial aneurysms and arterial tortuosity predispose LDS patients to potentially life-threatening bleeding events.

Overall, the TGF-β signalling paradox in LDS patients is still a matter of ongoing research. Several studies suggest that altered cell surface recycling of mutant receptors, imbalanced SMAD-dependent versus SMAD-independent signalling, a shift to increased *TGFB1* expression and autonomous cell signalling may explain this phenomenon [27].

Management Patient management can be categorised into conservative and surgical treatment strategies [28]. Physical exercise restriction should be implemented to protect the cardiovascular system from recurrent increases in heart rate and blood pressure [28]. Since administration of losartan to LDS mice ameliorated aneurysm formation, ARBs alone or in combination with BBs may be considered to lower systemic blood pressure, slow the heart rate and slow arterial aneurysm growth [29]. Surgical aortic root replacement may also be indicated to prevent TAA dissection [28, 29]. Lifelong follow-ups are necessary since even optimally managed LDS poses a continued risk of arterial dissection, development of cardiac arrhythmias and left ventricular dilation [28, 29]. Further patient management involves genetic counselling since genetic inheritance poses a risk for potential offspring and pregnancy greatly increases cardiovascular risk in female LDS patients [28, 29]. Additionally, further cardiovascular risk factors such as hypertension, dyslipidaemia and diabetes should be avoided [29].

Conclusion and Clinical Perspectives

- Normal vascular morphogenesis and homeostasis require functional TGF-β superfamily signalling.
- Advanced clinical mutation screening and basic scientific research have contributed substantially to a better understanding of how defective TGF-β signalling causes impaired vascular morphogenesis and HVDs and how this may lead to the development of novel therapeutic strategies.
- Identification of ALK1 and ENG as pro-angiogenic factors in vascular morphogenesis has led to the development of dalantercept, an ALK1-F_c fusion protein functioning as a ligand trap, PF-03446962, an ALK1 neutralising antibody, and carotuximab, an ENG antibody [30, 31]. These drugs have been or are currently being tested as mono- and combined therapy with receptor tyrosine kinase inhibitors (RTKI) in several phase I, II and III trials in patients with advanced solid tumours, head and neck squamous cell carcinoma, endometrial cancer, ovarian/Fallopian tube/primary peritoneal cancer, advanced renal cell carcinoma, advanced malignant mesothelioma, relapsed or refractory urothelial cancer and advanced angiosarcoma [30, 31].
- The identification of *ENG* and *ACVRL1* mutations in HHT patients causing increased systemic VEGF-A led to the novel therapeutic strategy of treating HHT patients with the monoclonal VEGF-A antibody bevacizumab. Bevacizumab now presents a promising therapeutic option for reducing recurrent bleeding events and anaemia. To date, there are several ongoing clinical trials for HHT patients investigating local and systemic delivery of bevacizumab, ranibizumab (monoclonal antibody fragment directed against VEGF-A), octreotide (somatostatin analogue) and pazopanib (RTKI) [14].
- Reduced pulmonary vascular BMPR2 expression levels are observed throughout many different

forms of PAH. Therefore, targeting this receptor may present a viable therapeutic strategy in the near future. Encouragingly, a recent study demonstrated that BMP-9-treated PAECs displayed increased BMPR2 expression levels and decreased apoptosis in response to tumour necrosis factor-α treatment. Furthermore, administration of recombinant BMP-9 to mice carrying the human R899X *BMPR2* mutation known to cause NMD and PAH restored pulmonary endothelial barrier integrity and reversed PAH [32]. Therefore, drugs aimed at restoring pulmonary vascular BMPR2/SMAD1 signalling may present a novel treatment strategy for HPAH patients.

- Enhanced vascular ALK5 signalling is the most likely cause for arterial aneurysm and tortuosity formation in LDS patients. The ARB losartan has been shown to dampen TGF-β signalling and effectively lowers systemic blood pressure. Therefore, ARBs are now routinely administered to LDS patients since this drug class dampens aortic root growth rate and also protects the cardiovascular system by lowering the systemic arterial pressure.

Gaps in Knowledge

- As not every HVD mutation carrier presents with an overt clinical phenotype, research now additionally has to focus on what kind of secondary vascular insults trigger disease onset and progression.
- Since vascular TGF-β superfamily signalling interacts with many other signalling pathways, a better understanding is needed on how this crosstalk is dysregulated in cancer and HVDs in order to find new therapeutic targets.
- Novel therapeutic strategies such as gene therapy are needed to specifically target vascular TGF-β superfamily signalling in order to avoid off-target effects since the TGF-β superfamily also critically regulates whole body homeostasis.
- More prospective, multicentre, double-blinded and randomised clinical trials are needed to test and confirm novel anti-angiogenic and HVD drug treatment strategies.

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MicroRNA and LncRNA in the Vascular System

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_14

Key Concepts

- MicroRNA and lncRNA constitute a major layer of transcriptional regulation within vascular cells.
- MicroRNAs play pervasive roles within vascular physiology and disease.
- LncRNAs are emerging as new players in vascular pathophysiology.

14.1 Introduction: Non-coding RNA

In the past, the conventional view of gene regulation was centred around the simple concept that DNA is transcribed into messenger RNA, which is then translated into proteins that support the structural, functional and regulatory requirements of cells. However, extensive investigation over the last decade has revealed considerable new insight into the complexity and various layers of gene regulation in eukaryotes. Sequencing of the human genome has revealed that upwards of 90% may be actively transcribed but, surprisingly, only ~2% codes for proteins [1]. Thus, the functional complexity of organisms would seem to depend not only on the absolute number of coding genes but also on the ability to leverage the extensive transcriptional repertoire of non-coding RNAs (ncRNAs). This concept is supported by the observation that the size of the non-coding transcriptome increases with increasing organismal complexity, while protein-coding content remains largely constant [2, 3].

Non-coding RNA (ncRNA) species vary widely in size, abundance, structure and function but can be broadly classified according to their length. Small non-coding RNAs are generally less than 200 nucleotides and include microRNAs (miRNAs, miRs), transfer RNAs, Piwi-interacting RNAs (piRNAs) and small nuclear/nucleolar RNAs. By definition, long non-coding RNAs (lncRNAs) are non-protein-coding transcripts greater than 200 nucleotides [4]. This is an arbitrary but practical threshold that distinguishes lncRNAs from most known classes of small regulatory and infrastructure-related RNA species. Among the various classes of small ncRNA, miRNAs have garnered considerable interest as key regulators of gene expression that have been predicted to control upwards of 60% of all protein-coding genes [5] and have been investigated as potential biomarkers, messengers and/or mediators of vascular biology and disease [6]. MicroRNAs are expressed in most types of mammalian cells at varying levels, with the number of molecules per cell estimated to be similar on average as messenger RNA (i.e. $1–3 \times 10^5$ versus $3–10 \times 10^5$ molecules per cell, respectively) [4]. MicroRNA sequences are also highly conserved across mammalian species, and more than 2500 unique mature miRNAs have been reported (miRBase release 22, Mar 2018) [7], though the function(s) of most remain to be elucidated.

Long non-coding RNAs have also been demonstrated to play important roles in vascular biology and disease via several different mechanisms involving the negative and positive regulation of protein gene expression, alternative RNA splicing, regulation of miRNA and the modulation of protein localization and activity [8]. LncRNAs are among the newest and therefore least well-characterized RNA molecules. Although estimates for the number of different human lncRNA transcripts range upwards of 53,000 [4], only a very small fraction of these have been functionally validated. Insight into the regulation and function of lncRNAs remains challenging, which can be attributed to several different factors including their relatively low expression levels (i.e. 1–2 orders of magnitude lower than miRNA), poor sequence conservation across species and high tissue/cell specificity [4]. Table 14.1 highlights some of the similarities and differences between microRNA and lncRNA.

14.2 MicroRNA

14.2.1 MicroRNA Biogenesis and Regulation

MicroRNAs are small (~18–22 nucleotide) non-coding RNA molecules that negatively regulate gene expression by binding to target messenger RNA (mRNA) in a sequence-dependent manner and subsequently inducing mRNA degradation or blocking translation of protein synthesis. miRNAs are generated through a regulated pathway shown in Fig. 14.1, which involves two major processing events that lead to the mature microRNA. First, long primary miRNAs (pri-miRNAs) are transcribed from the genome and processed in the nucleus via the DROSHA enzyme into one or several smaller

Table 14.1 Comparison between microRNA and lncRNA

Property	MicroRNA	LncRNA
Length	~18–22 nt	>200 nt
Number of different human transcripts	>2500 (mature species)	>50,000
Expression levels	Similar to messenger RNA	1–2 orders of magnitude lower than miRNA
Evolutionary conservation	High	Low
Function(s)	Negative regulation of gene expression	Negative and positive regulation of gene expression
		Alternative RNA splicing
		miRNA regulation
		Modulation of protein localization and activity

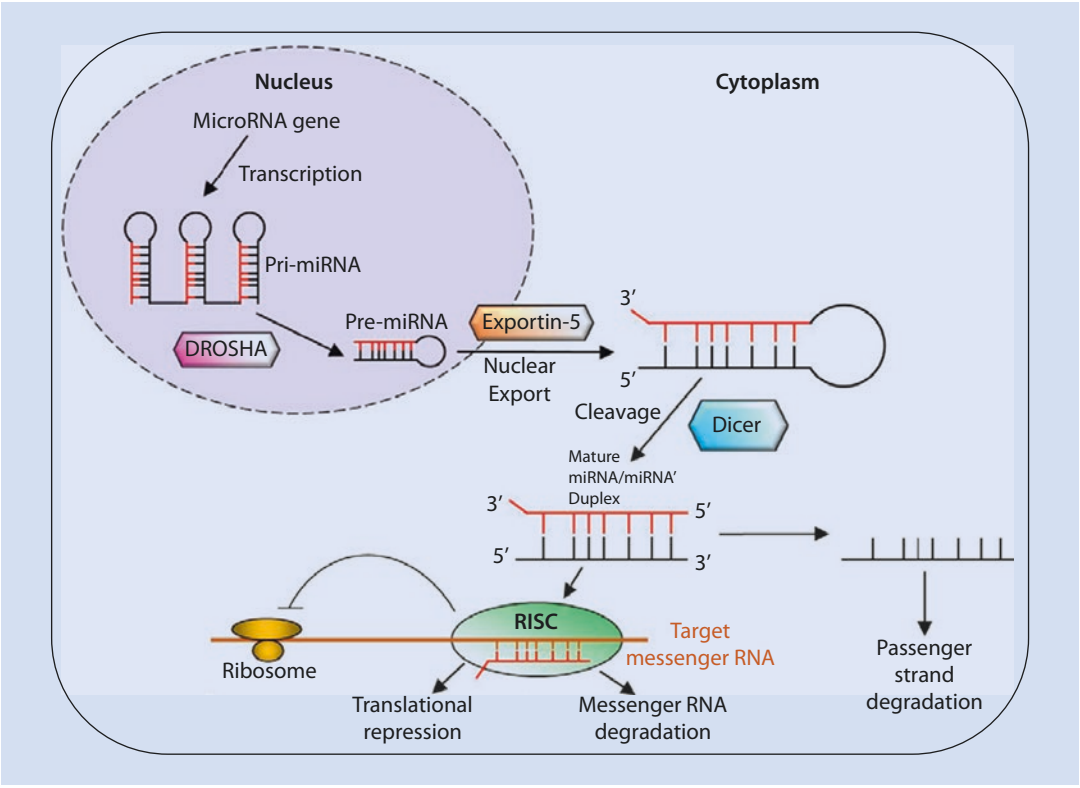

Fig. 14.1 MicroRNA biogenesis and processing pathway. Expression and maturation of miRNA begin with production of a longer primary miRNA transcript (pri-miRNA) followed by several successive processing steps involving the actions of DROSHA, exportin-5 and Dicer proteins to generate the final mature miRNA species. Mature miRNA binds the RISC ribonucleoprotein complex, which facilitates either the translational repression or degradation of target messenger RNA. (Adapted from [9])

precursor miRNAs (pre-miRNAs), which are then transported into the cytoplasm via exportin-5. The double-stranded 60–90 nt pre-miRNA forms the classical hairpin stem and loop structure shown in Fig. 14.1. In the cytoplasm, pre-miRs are cleaved by the enzyme DICER to generate mature duplex

miRNAs that subsequently unwind. One strand of the mature miRNA duplex, called the guide strand, is incorporated into a multisubunit protein complex known as the RNA-induced silencing complex (RISC) that serves to block mRNA translation or enhance mRNA degradation. The miRNA guide strand recognizes specific mRNA targets typically through partial base pairing within the 3′ untranslated regions (3′ UTRs) of mRNAs, while the other miRNA strand not incorporated into RISC (referred to as the passenger strand) is typically degraded [9].

The conventional nomenclature for mature miRNAs is comprised of the "miR" prefix followed by a hyphen and unique identifying number that is assigned sequentially in the order in which miRNAs were discovered, albeit with some notable exceptions such as the first miRNAs let-7 and lin-4 that have retained their original names for historical purposes. MicroRNAs from different species are denoted by the species abbreviation (e.g. human= *hsa*, mouse=*mmu*) followed by the miRNA name. The lowercase "mir" refers to the pre-miRNA, while uppercase "MIR" refers to the gene that encodes the microRNA. Mature miRNAs with closely related sequences are annotated with a lowercase letter suffix (e.g. miR-146a and miR-146b) and can be further distinguished with either a "-5p" or "-3p" suffix based on whether the mature sequence was produced from the 5′ or 3′ arm of the hairpin precursor, respectively [7, 10].

14.2.2 MicroRNA in Vascular Development and Physiology

The vascular system requires complex regulatory mechanisms to coordinate vessel development and normal physiological functions, and miRNA has been shown to be essential for normal endothelial cell (EC) activity. In vitro experiments designed to interfere with global miRNA biogenesis via targeted loss of key processing enzymes (e.g. DICER) have demonstrated substantial changes to EC phenotype including a reduction in EC growth, angiogenic properties and disruption in nitric oxide (NO) signalling pathways. Specific miRNA has also been shown to play important roles in the vasculature. For example, miR-155 is a negative regulator of endothelial nitric oxide synthase (eNOS), an enzyme that synthesizes NO. Inflammatory stimuli such as tumour necrosis factor alpha (TNFa) increases miR-155 expression, and knockdown of miR-155 has been shown to prevent the TNFa-induced downregulation of eNOS and NO and impairment of endothelium-dependent vascular relaxation [11].

Angiogenesis is controlled by molecular cues within endothelial cells. MiR-126 is abundantly expressed in ECs and has been shown to mediate developmental angiogenesis in vivo in zebrafish models; targeted deletion of this miRNA resulted in a loss of vascular integrity including leaky vessels and haemorrhaging. MiR-126 has also been shown to enhance the pro-angiogenic actions of vascular endothelial growth factor A (VEGF-A), via repression of Sprouty-related EVH1 domain 1 (SPRED-1), an intracellular inhibitor of angiogenesis. The expression of another miRNA, miR-125b, has been shown to be transiently increased following VEGF stimulation or ischaemia and inhibit in vitro tube formation (i.e. an angiogenic assay) via downregulation of VE-cadherin [12]. Additionally, miR-34b-5p and miR-205 have been shown to regulate angiogenesis in the context of cancer. Expression levels of miR-34b-5p were markedly decreased in a large number of thyroid carcinoma cell lines and patient tissue samples and were associated with pathological T-stages of thyroid carcinomas. miR-34b-5p was also shown to negatively regulate the expression of VEGF-A [13]. Similarly, the experimental overexpression of miR-205 in a mouse tumour xenograft model was shown to inhibit tumour growth, invasion and vascularization through a potential mechanism involving the regulation of VEGF-A and several other angiogenic and epithelial-to-mesenchymal (EMT) protein factors [14].

In addition to their impact on endothelial cells, miRNAs are also known to regulate the smooth muscle cell (SMC) phenotype, specifically by affecting the proliferative and migratory capacity of SMCs. MiR-21 was one of the first miRNA demonstrated to regulate vascular SMC proliferation and survival in vivo, through a mechanism involving decreased expression of phosphatase and tensin homolog (PTEN) and increased B-cell lymphoma 2 (Bcl-2) expression, which together help to confer a pro-proliferative and anti-apoptotic phenotype. MiR-21 has also been shown to regulate the SMC contractile phenotype via inhibition of programmed cell death protein 4 expression, which leads to an increase in SMC-restricted contractile proteins. In addition,

miR-143 and miR-145 have been shown to regulate SMC phenotypic switching in the context of vascular injury, leading to alterations in the relative contractile and proliferative states. These effects are thought to be mediated through multiple effectors including several transcription factors such as KLF4, KLF5 and ELK-1 [15].

MicroRNA-26a (miR-26a) has also been implicated in SMC phenotypic switching. Cells deficient in miR-26a (via experimental knockdown) showed a marked decrease in cell cycle progression and were less likely to migrate towards a growth factor/serum gradient. MiR-26a alters downstream components of the TGF-β signalling pathway in order to exert this function, with decreased levels of miR-26a leading to significant increases in key TGF-β signal transducers such as SMAD-1 and SMAD-4 [16].

14.2.3 MicroRNA in Vascular Pathology

Alterations in miRNA expression have been implicated in functional abnormalities associated with a wide spectrum of pathophysiological conditions including cardio- and pulmonary vascular diseases. In a number of cases, gain- and/or loss-of-function experiments in vascular cells or relevant animal models have demonstrated causative roles for miRNA in disease pathobiology.

14.2.4 MicroRNA in Pulmonary Hypertension

Pulmonary hypertension (PH) is a multifactorial and progressive disease characterized by pulmonary microvascular rarefaction and right heart failure. (See ▶ Chap. 41 for further details on the pathophysiology of PH.) An increasing number of miRNAs have been implicated in the pathobiology of this disease, supported by evidence from observational studies showing dysregulated expression of miRNAs in explanted tissues and cells from PH patients and experimental studies that have demonstrated the effects of miRNA overexpression and/or inhibition on specific molecular signalling pathways, as well as their effects on pulmonary vascular remodelling and haemodynamics in rodent models of PH. Collectively, these studies have helped to establish causative roles for miRNAs in the development and progression of PH and also revealed various hierarchical levels of regulation [17]. A comprehensive overview of the diverse roles played by miRNAs in pulmonary hypertension has been elegantly reviewed elsewhere [17]. In this chapter, we highlight a few miRNAs of interest.

Loss-of-function mutation of the bone morphogenetic protein receptor 2 (BMPR2) gene is associated with poor endothelial cell survival and proliferation in the pulmonary vasculature and is one of the genes known to contribute to heritable forms of PH. Several miRNAs have been implicated in dysregulated BMPR2 expression and/or related signalling including miR-302, miR-322, miR-17–92 cluster, miR-21, miR-145, miR-96, miR-140-5p and miR-130/301. The majority of these miRNAs have also been demonstrated to be causally involved in experimental PH induced in common mouse or rat models of PH [17].

A large number of miRNAs have also been implicated in PH triggered by exposure to chronic hypoxia (i.e. low oxygen exposure), which leads to perturbations in several pathways related to vascular proliferation and stiffness, inflammation and energy metabolism (i.e. from oxidative phosphorylation to glycolysis). These biological effects are mediated in large part through signalling of the hypoxia-inducible factor (HIF) pathway and other PH-relevant pathways that may alter miRNA expression and/or be the subject of reciprocal regulation by the miRNAs. Among the many miRNAs that have been linked to hypoxia, several including miR-210, miR-451, miR-130/301, miR-27b, miR-424/503, miR-124, miR-223, miR-204 and miR-143 have been shown to exert protective effects and modulate the severity of the PH phenotype in rodent models [17].

While most of the preceding miRNAs have been linked to PH individually, the miR-130/301 family is worth highlighting because it has been implicated as a master upstream regulator of two other miRNA-controlled pathways with important roles in cellular proliferation in the lung vasculature, providing insight into an integrated miRNA strategy for controlling PH progression. In particular, the miR-130/301 family was shown to regulate the apelin-miR-424/503-FGF2 signalling axis in endothelial cells while modulating STAT3-miR-204 signalling in smooth muscle cells. The miR-424/503 cluster and miR-204 had previously and independently been shown to be

causatively involved in PH, and the biological importance of miR-130/301 to the PH phenotype was likewise confirmed by evaluating the effects of both overexpression and inhibition in rodent models [18].

As a general note, while genetic and pharmacological strategies aimed at increasing or decreasing miRNA levels have demonstrated potential therapeutic effects in preclinical animal models of PH, the actions of miRNAs typically do not completely reverse the disease phenotype. While technical limitations (e.g. suboptimal pharmacologic dosing) may contribute to this effect and generally cannot be discounted in any experimental setting, these results also serve to highlight the notion that miRNAs function within broader regulatory networks with cooperative (i.e. multiple miRNAs targeting different components of a biological pathway) and/or redundant (i.e. multiple miRNAs targeting the same effector protein within a pathway) mechanisms designed to help fine-tune, rather than switch on or off, gene expression and related biologic activities. This is a quality of miRNA-mediated regulation that is not unique to PH but has been observed broadly in cardiovascular biology [6].

While miRNAs have been studied predominantly for their cellular roles in regulating gene expression, there is increasing interest in understanding their potential extracellular roles in circulation. Circulating cell-free miRNAs are protected from ribonuclease digestion in blood via complexation with RNA-binding proteins or inclusion within nano- or micro-vesicles and may facilitate intercellular communication between vascular and immune cells [17]. A number of studies have also reported the potential utility of circulating miRNAs to help diagnose, predict or classify disease. MiR-150 was the first miRNA reported as a potential circulating biomarker of pulmonary arterial hypertension (PAH) [19]. Following microarray profiling in a small cohort of PAH patients and healthy control subjects, 58 miRNAs were found to be differential altered in plasma. Among these altered miRNAs, miR-150 exhibited the greatest downregulation in patient plasma, and reduced levels were also associated with poor survival in PAH [19]. Notwithstanding the promise of these findings, further investigation is needed to determine whether miR-150 could be used as a robust clinical biomarker or whether it may also play a role in the pathogenesis of PAH. In an independent report, miR-26a was shown to be concordantly reduced in the plasma of PAH patients and rats with monocrotaline (MCT)-induced pulmonary hypertension as compared to their respective healthy controls [20]. In patients, plasma levels of miR-26a were shown to correlate directly with a clinical marker of functional impairment and disease severity related to exercise capacity (i.e. 6-min walk distance) and exhibit potential diagnostic utility reflected by a high area under the corresponding receiver-operator characteristic curve (AUC = 0.85). Furthermore, in MCT-exposed rats, the reduced plasma levels of miR-26a mimicked underlying changes in tissue expression, including a reduction in both the lung and right ventricle of the heart [20]. While these observations implicate miR-26a in PAH, further investigation is needed to determine whether it contributes causatively to disease biology.

14.2.5 MicroRNA in Atherosclerosis

Atherosclerosis is another progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. (See ▶ Chap. 20 for further details on the pathophysiology of atherosclerosis.) Lipid uptake and inflammatory responses in macrophages have been shown to be regulated by miR-155 and miR-125a, and inhibition of these microRNAs reduces neointimal accumulation of foam cells. A miRNA that has extensively been evaluated in atherosclerosis is miR-33, which controls the expression of cholesterol efflux machinery and inflammatory gene expression. Antagonism or deletion of miR-33 has been shown to reduce atherosclerosis lesion progression, promote cholesterol removal from macrophages and induce regression of existing lesions, making miR-33 an important pro-atherogenic miRNA [21].

The pathogenic feature of atherosclerosis is an inflammatory process by which blood vessels respond to injury. Portions of vessels from atherosclerosis susceptible regions such as the aortic arch have shown marked reductions in miR-10a expression. Furthermore, the knockdown of miR-10a in cultured endothelial cells followed by transcriptome microarray analysis revealed upregulation of prominent pro-inflammatory pathways such as the NFkB pathway, as well as an increase in

pro-inflammatory cytokines and cell adhesion molecules such as MCP-1, IL-6, IL-8, VCAM-1 and E-selectin. Collectively, these data suggest that miR-10a plays a role in regulating the pro-inflammatory endothelial phenotype associated with athero-susceptible areas of the vasculature [22].

14.2.6 MicroRNA in Vascular Remodelling

Vascular remodelling is the alteration in the structure and arrangement of blood vessels through biological processes including cell growth, cell death, cell migration and production or degradation of the extracellular matrix (see ▶ Chap. 18 for further details on vascular remodelling). As noted above, several microRNAs can alter smooth muscle cells to promote a migratory and proliferative phenotype. Although this switch is required for vascular repair, dysregulation of this process is a hallmark of vascular remodelling. MiR-221 and miR-222 have been shown to stimulate vascular smooth muscle cell switching from a "contractile" phenotype to the "synthetic" phenotype that is associated with induction of proliferation and motility. Interestingly, the opposite effect is observed in endothelial cells where proliferation has been shown to be inhibited by miR-221 and miR-222 [23]. The cause of this cell-type-specific differential effect is unclear.

MiR-21, as mentioned above, plays a role in regulating proliferation and apoptosis in several cell types. MiR-21 has been shown to be upregulated following mechanical injury of large vessels in a humanized rat model, and blocking miR-21 activity in this model prevented restenosis [24]. MiR-21 expression is also elevated in multiple models of vein graft disease, and miR-21 knockout mice exhibit a marked reduction in neointima following in vivo vein grafting [25].

Genetic strategies using miR-143/145 knockout mice have shed additional insight into the biological roles of miR-143/145 in the vasculature. Microscopy studies of tissue specimens from these knockout mice have revealed structural differences in contractile smooth muscle cells and reduced smooth muscle cell layers in the aorta and other arteries. The reduction in vessel thickness was attributed to a decrease in actin-based fibres, suggesting that these microRNAs affect cytoskeletal assembly. In addition to their role in SMC function, miR-143/145 also play important roles in the responsiveness of SMC to vascular injury. Several studies have shown that miR-143/145 are downregulated in experimentally induced diseased vessels following balloon injury or ligation of carotid arteries. Accordingly, the restoration of miR-143/145 levels with a miR-143/145 adenovirus delivery vector was shown to reduce balloon injury-induced neointimal formation via mechanisms involving proliferation and migration of vascular SMCs, demonstrating that miR-143 and miR-145 can modulate vascular remodelling in vivo [23].

MicroRNAs help to fine-tune changes in vascular processes to maintain homeostasis and can alter endothelial and smooth muscle cell phenotypes by controlling cellular migration and proliferation. Additionally, intracellular microRNAs can be released into circulation and might serve as markers of disease activity. The important roles of miRNAs in vascular remodelling and disease suggest that therapeutic strategies designed to either increase miRNA levels or inhibit activity might be a promising approach for further clinical investigation.

14.3 LncRNA

14.3.1 LncRNA Biogenesis and Function

LncRNA is transcribed from regions throughout the genome and share several characteristics with messenger RNA in that they are transcribed by RNA polymerase II and typically, but not always, alternatively spliced and polyadenylated. LncRNA utilizes several different mechanisms to regulate gene expression, which may involve interactions with DNA, RNA or proteins. The various types of molecular mechanisms used by lncRNA have previously been categorized into four basic functional archetypes including (i) signal archetype, in which the lncRNA functions as a molecular signal or indicator of transcriptional activity; (ii) decoy archetype, wherein lncRNA can bind to and titrate away other regulatory RNAs or proteins; (iii) guide archetype, in which lncRNA directs the localization of specific ribonucleoproteins to their specific targets; and (iv) scaffold archetype, wherein lncRNA serves as a supporting structural platform for other

relevant components to help stabilize nuclear structures or signalling complexes [9].

Of note, there is at present no uniform system for naming lncRNAs, which are typically assigned a unique abbreviation or acronym of a descriptive name that provides some insight into their function (or genomic context if the function is unknown). However, several additional guidelines have been proposed by the HUGO Gene Nomenclature Committee to provide clear and informative names for all lncRNA genes in the human genome, which has been described in detail elsewhere [26].

14.3.2 LncRNA in Vascular Physiology and Pathology

LncRNAs have gained widespread interest due to their role in developmental biology and diseases such as cancer. While comparatively little has been reported on the roles of lncRNA within the vasculature, an increasing number of studies have shed insight on the potential roles of lncRNAs in endothelial and smooth muscle cell biology and pathology. Here, we highlight a few examples of lncRNAs implicated in vascular physiology and disease including *Lnc-Ang362*, *MALAT1*, *Tie-1AS* and *LEENE*.

The identification of *Lnc-Ang362* provided insight into how lncRNAs may function as host transcripts for miRNAs that are co-regulated in response to vasoactive stimuli. *Lnc-Ang362* and two proximally located miRNAs, miR-221 and miR-222, were found to be concordantly upregulated in rat vascular smooth muscle cells (VSMC) after stimulation with angiotensin II, a peptide hormone and vasoconstrictor agent implicated in atherosclerosis and hypertension. Administration of targeted small interfering RNA to knockdown *Lnc-Ang362* expression was shown to reduce the expression of both miRNAs, indicative of their co-regulation. Furthermore, the reduction in *Lnc-Ang362* levels was associated with a decrease in VSMC proliferation, which is a biologic function that miR-221 and miR-222 had previously been shown to regulate [8].

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is an intergenic lncRNA that was found within the nucleus of cells. *MALAT1* has been shown to play an important role in endothelial physiology and disease and is significantly upregulated under hypoxic conditions. In vitro silencing of *MALAT1* has been shown to promote migration of endothelial cells while inhibiting their proliferation. In addition, the genetic ablation or pharmacological inhibition of *MALAT1* has been shown to reduce vascular growth in vivo [8].

Tie-1AS is an antisense transcript of tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain-1 (*Tie-1*), an endothelial cell-enriched orphan receptor shown to be essential for normal vascular development and function and implicated in atherosclerotic progression. Interestingly, while lncRNA is typically poorly conserved between species, *Tie-1AS* was found to be present within the zebrafish, mouse and human genomes, suggesting a common functional link in vascular biology. In embryonic zebrafish, the *Tie-AS1* transcript was shown to be spatially and temporally expressed with *Tie-1 messenger RNA*. In vitro and in vivo studies have shown that *Tie-1AS lncRNA* binds to and regulates the transcript levels of *Tie-1*, thereby regulating endothelial cell junction integrity. Thus, the interplay between the *Tie-1* coding and Tie-1AS non-coding transcripts may represent an important mechanism of control in vascular development [8].

More recently, the lncRNA LEENE has been identified as an enhancer of endothelial nitric oxide synthase (eNOS) expression, further demonstrating the importance of lncRNA to vascular function and particularly endothelial homeostasis [27]. LEENE was shown to improve recruitment of RNA polymerase II to the promoter of eNOS to increase RNA transcription. In addition, experiments designed to increase or decrease LEENE function demonstrated that both eNOS expression and endothelial function were differentially altered in response [27].

Conclusion and Clinical Perspectives

The development of next-generation RNA sequencing technology over the last decade has greatly expanded our understanding of the transcriptome and the functional impact of both short and long non-coding RNA species in vascular biology. MicroRNAs and lncRNAs have been shown to be important and pervasive regulators of protein-coding gene expression, thereby exerting control over diverse biological functions in endothelial and smooth muscle cells, as well as

many other cell types that were not covered in this chapter. The dysregulated expression of miRNAs and lncRNAs has been implicated in a number of vascular diseases. An increasing number of preclinical studies suggest that targeted interventions designed to restore normal levels or activity of these RNA species may have therapeutic benefits that warrant further clinical investigation.

Gaps in Knowledge

Although thousands of miRNAs and lncRNAs have been identified through RNA sequencing efforts to date, the potential functions of most of these non-coding RNAs have yet to be clearly defined. Whether all of these transcripts exert discrete biological functions or alternatively represent non-functional transcriptional noise remains an important question. Additional investigations of how RNA structure dictates function could reveal insights to help distinguish between these two possibilities. Similarly, integrated analyses designed to better understand how miRNAs, lncRNAs and other biomolecules interact within larger co-regulatory networks may yield further functional clues by association. Finally, additional translational research is needed to determine whether the preclinical therapeutic effects of miRNA and lncRNA reported in animal models can be translated into meaningful health outcomes for humans with vascular disease.

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Microparticles and Exosomes in Cell-Cell Communication

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_15

Key Concepts

- There are two main categories of extracellular vesicles (EV): exosomes/small EV and microparticles/large EV, which are different in size and molecular composition.
- Exosomes and microparticles are released into the extracellular space under normal and stressed conditions and can be easily identified by size and the presence of proteins from the parent cell. They may be used as biomarkers of the health status of the cell of origin.
- Although exosomes and microparticles are formed by different mechanisms, they both modify the physiological state of target cells by carrying significant amount of protein, RNA, microRNA, peptides, transcription factors, and lipid mediators.
- Mechanisms whereby extracellular vesicles interact with target cells include receptor-ligand interaction, internalization by endocytosis and/or phagocytosis, or fusion to the membrane of the target cell, where the cargo is delivered.
- The number and characteristics of circulating exosomes and microparticles are altered in different cardiovascular diseases.

15.1 Introduction

Extracellular vesicles (EV) comprise a group of circulating small vesicles that contain a phospholipid bilayer around a soluble core. They are released from parent cells into the extracellular space including body fluids such as plasma, breast milk, urine, and saliva (Fig. 15.1) and may reflect the activation status and phenotype of the parent cell from which they derive. EV are able to interact with cells and influence their function in an autocrine, paracrine, or endocrine manner [1]. Extracellular vesicles transfer their content (cargo) including proteins, lipid mediators, RNA, microRNA (miR), or enzymes to local or distant cells and have been implicated in the pathogenesis and progression of several pathologies, including cardiovascular disease, immune disorders, inflammation, and tumour growth [1].

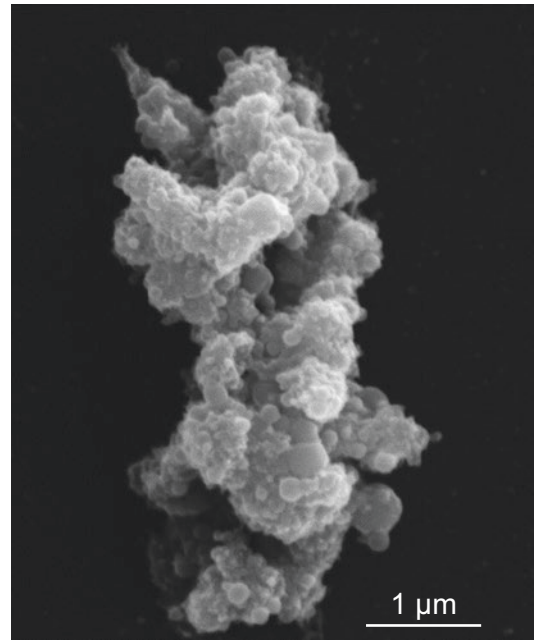

Fig. 15.1 Extracellular vesicles. Cluster of EV isolated from human plasma by ultracentrifugation and visualized by transmission electron microscopy. Scale bar = 1 μm. (Personal file)

Extracellular vesicles comprise any membrane-enclosed vesicle with the best-characterized populations being exosomes, microparticles (MP), and apoptotic bodies. Each of these populations is typified by a distinct size and mechanism of production (Fig. 15.2). Exosomes are between 40 and 100 nm in size and are identified by enrichment in ubiquitinated protein and the presence of lysosomal-associated membrane protein 1 (Lamp1, also known as CD107a), Lamp3 (CD63), CD9, CD81, or tumour susceptibility gene 101 (TSG101). Microparticles (also termed microvesicles or ectosomes) are 100–1000 nm in size and are identified by the presence of phosphatidylserine (PS) on their surface as well as membrane markers of the parent cell. Apoptotic bodies (1–5 μm) are formed during apoptosis and are larger than exosomes and MP. Apoptotic bodies contain cellular proteins, lipids, nuclear fragments, and cellular organelles. They are also identified by the presence of phosphatidylserine on their surface; however they can be differentiated from MP by size and increased membrane permeability [1, 2].

Many studies have focused on EV, especially exosomes and MP in the pathogenesis and progression of several diseases. EV also show great potential to be used as novel diagnostics and

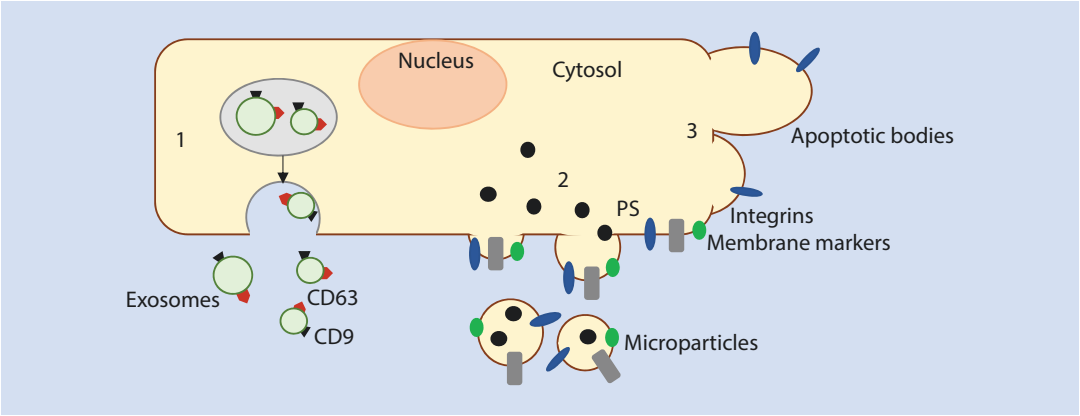

Fig. 15.2 Formation of extracellular vesicles (EV). (1) Exosomes are formed by endosomal trafficking, gated by multivesicular endosomes (MVE), followed by fusion to the plasma membrane, and release their contents to the extracellular environment. Exosomes express CD63, CD9, CD81, and/or tumour susceptibility gene 101 (TSG101). (2) Microparticles are formed from the outward "blebbing" of the plasma membrane and posterior shedding to the extracellular space. Microparticles are identified by the external presence of phosphatidylserine (PS) and present the membrane marker of the parental cell. (3) Apoptotic bodies are formed during the cell apoptosis process and are larger than exosomes and microparticles. Apoptotic bodies also express phosphatidylserine

therapeutics. In particular they may be biomarkers of disease and, through their cargo-carrying potential, may act as a strategy for drug delivery.

15.2 Exosomes

15.2.1 Exosome Formation

Exosomes are originating from the late phase of endosomal trafficking, gated by multivesicular endosomes (MVE), followed by fusion to the plasma membrane and release of their contents to the extracellular environment as exosomes (Fig. 15.2). Exosomes from vascular smooth muscle cells, endothelial cells, fibroblasts, platelets, monocytes/macrophages, lymphocytes, mast cells, haematopoietic cells, and tumour-associated cells have been identified in the circulation. Cells from adipose tissue are also an important source of exosome production. They are found in all body fluids, including plasma, breast milk, saliva, urine, and cerebral fluid. They contain lipid raft microdomains, fragments of the membrane, and cytosol of their parent cells; are significant carrier of RNA, microRNA (miR), proteins, and lipid mediators; and are enriched in ubiquitinated proteins [3].

The exosome formation requires a complex intracellular machinery and is first formed as intraluminal vesicles by inward budding of the limiting membrane of late endosomes or multivesicular bodies that fuse with the plasma membrane and release their content as exosomes to the extracellular environment. Among several mechanisms described for exosome formation, the best characterized involves the participation of the endosomal sorting complex required for transport (ESCRT), which is a complex or proteins able to drive the sorting of ubiquitin-conjugated membrane proteins into vesicles that bud into the lumen of multivesicular endosomes and subsequently fuse with lysosomes. Proteomic analyses in purified exosomes from various cell types have also identified ESCRT components, such as TSG101 and ALIX, which are also a regulator of the endo-lysosomal system, and ubiquitylated proteins in their cargo. The formation of exosomes also can be independent on ESCRTs and dependent on lipid-metabolizing enzymes such as sphingomyelinase and phospholipase D2 (PLD2) that hydrolyse sphingomyelin to ceramide and phosphatidylcholine to phosphatidic acid, respectively [4]. The enzyme heparanase also plays an important role in the exosome formation by cleaving and activating the heparan sulphate of the syndecans. Syndecans are a small family of transmembrane proteoglycans that carry three to five heparan sulphate and chondroitin sulphate chains. Because syndecans are co-receptors of several growth factors and adhesion molecules and also participate in cytoskeleton organization, it might play an important function in the formation of the exosome cargo and incorporation of phosphorylated

receptors, growth factors, and other proteins that depend on heparan sulphate for activity [5]. Another mechanism involved in exosome formation requires tetraspanin-mediated organization of proteins such as amyloidogenic protein and premelanosome protein [6]. Apart from their ability to mediate physiologic effects, some miRNA and other non-coding RNAs present some specific motif that controls their location in the exosomes. These mechanisms require one type of posttranscriptional modification called sumoylation of the protein heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), which increases its capability to bind to motifs present in the non-coding RNAs and controls their loading into exosomes [7]. Once the exosomes are formed in the intracellular space, they are released to the extracellular environment, and part of these mechanisms is dependent on Rab GTPases, specifically Rab27a and Rab27b, which are proteins already described to have been involved in the secretion of secretory granules and lysosome-related organelles [4, 6]. These multiple mechanisms involved in exosome formation amplify considerably the heterogeneity of secreted vesicles with different cargo contents and consequently their functional effects on different target cells. Besides, exosomes carry fragments of membrane and cytosol from their parent cell.

Several proteins have been identified in exosomes, such as tetraspanin family members Lamp1, CD9, CD63, CD89, CD81, and CD82, flotillin, annexin, cofilin-1, heat shock proteins (HSP70 and HSP90), TSG101, and enzymes [enolase-1, aldolase-A, phosphoglycerate kinase (PGK-1), and lactase dehydrogenase A (LDH-A)] [3].

According to a current exosome database, exosomes from various organisms and cell types have been characterized as containing 4563 proteins, 194 lipids, 1639 mRNAs, and 764 miRNA. Exosomes promote cell activation by transferring protein and genetic material between similar and different cell populations. Therefore, exosomes are considered an important source of gene transfer between cells in a paracrine and endocrine fashion. In fact, exosomes carry significant amount of mRNA and non-coding RNA. Non-coding RNA is a class of RNA that does not translate proteins and is classified according to length of the sequence: long (more than 200 bases) and small (less than 200 bases) non-coding RNA. MicroRNA (miRNA) is defined as non-coding RNA, with approximately 22 bases. MicroRNA binds complementary to multiple sites in the 3′-UTR of the target mRNA, leading to cleavage of the mRNA and inhibition of protein synthesis. Most part of the microRNA present in plasma and saliva is carried in exosomes, although stoichiometric measurement suggests that most exosomes contain few copies of a given miRNA [8].

15.2.2 Exosomes in Cell Activation and Cardiovascular Diseases

Cells from the vascular and immune systems are important players in the atherogenic process, mediating the chronic inflammatory response and development of the atherosclerotic plaque [9]. Endothelial cells are the vascular cells in direct contact with the blood, which makes them main targets for soluble factors present in the circulation including exosomes. Physiologically, exosomes are generated in normal conditions playing important functions in maintenance of homeostasis. However the amount of exosomes increases significantly when vascular cells are stimulated, for example, by inflammatory mediators or hypoxia conditions. Under stimulation of the pro-inflammatory cytokine, such as tumour necrosis factor-alpha (TNF-α), endothelial cells produce exosomes carrying genes and proteins related to oxidative stress (superoxide dismutase 2, SOD2), inflammatory and immune responses [monocyte chemoattractant protein 1 (MCP-1), interleukin-8 (IL-8), vascular cell adhesion molecule 1 (VCAM-1)], and the NF-κB pathway. Additionally, under hypoxia conditions, endothelial cells release exosomes carrying genes involved in stress response [N-myc downstream-regulated 1 (NDRG-1), cold-inducible RNA binding protein (CIRBP), and apoptosis (BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3)] [10]. Exosomes derived from red blood cells carry TNF-α, inducing inflammatory profile, whereas exosomes from platelet may increase the apoptosis of endothelial cells through mechanisms dependent on generation of reactive oxygen species (ROS). Exosomes produced by macrophages induce the expression of the intercellular adhesion molecule-1 (ICAM-1) and activation of the transcription factor NF-κB in endothelial cells. Exosomes derived from lymphocytes, macrophages, and dendritic cells acti-

vate apoptotic mechanisms by expressing Fas-L (CD95L) that binds to the Fas receptor in the target cell inducing apoptosis. Moreover, exosomes derived from lymphocytes or adipocytes induce macrophage differentiation to foam cells, which are cells present in all stages of atherosclerosis development [11]. Additionally, exosomes transfer atherogenic mediators between adipocytes and vascular tissues. Exosomes from stimulated macrophages and platelets express miR-150, which has pro-inflammatory properties in immune cells and promotes endothelial cell migration and apoptosis [12]. Of note, patients with severe atherosclerosis and type 2 diabetes present high plasma levels of miR-150, which was reversed by anti-platelet therapy. Similarly, atherogenic macrophage-derived exosomes carry miR-146a, which inhibits macrophage migration [13]. Platelet-derived exosomes express miR-320, which has anticoagulant and anti-inflammatory properties, through effects that reduce expression of the adhesion molecule ICAM-1. Anticoagulant mediators are also present in exosomes from mast cells. Experimental studies have also shown that endothelial progenitor cell-derived exosomes enriched in miR-486-5p protect against experimental kidney injury with reductions in endothelial cell apoptosis [14].

Long non-coding RNA (lncRNA) represents a heterogeneous subtype of nonprotein-coding RNA longer than 200 bases. lncRNAs have been demonstrated to play important roles in epigenetic modification and regulation of transcription, translation, RNA metabolism, as well as stem cell maintenance and differentiation, cell autophagy and apoptosis, and embryonic development. In addition, lncRNAs have been implicated in major diseases including different types of cancer and neurological and cardiovascular diseases. Importantly some lncRNAs that have been identified to be involved in cardiac microvascular dysfunction are myocardial infarction-associated transcript (MIAT) and PUNISHER, which are associated with myocardial infarction and angiogenesis [15]. lncRNA with tissue- and cell-type specific expression is present in plasma exosomes and also might be used as a biomarker for disease progression and diagnostics [16]. The function of lncRNA in exosomes is of high interest, since the genetic material delivered to other cells will induce changes in the cell activation phenotype programming the target cell to a pro-activated profile [17].

Cardiovascular diseases, such as atherosclerosis and hypertension, are characterized by excessive production of ROS and oxidative stress. In atherosclerotic conditions macrophages have been reported to produce exosomes enriched with thioredoxin (TRX-1) and peroxiredoxin (PRDX-1) [18]. Thioredoxin and peroxiredoxin are markers for oxidative stress, and high concentrations are observed in plasma from patients with unstable angina and aneurysms and are correlated with atherosclerosis severity. Exosomes express angiotensin II type 1 receptors (AT1R) and have been shown to contribute to AT1R expression in resistant arteries, where they amplify Ang II vascular effects [19].

Urinary exosomes may reflect kidney physiology. Approximately 3% of total protein in the urine from normal subjects is present in exosomes; 1 study reported 295 different proteins in urinary exosomes by proteomic analysis [20, 21]. Urinary exosomes from patients with diabetic kidney disease exhibit decreased gelatinase activity and increased levels of ceruloplasmin, which are biomarkers for kidney dysfunction. Additionally, urinary exosomes from patients with acute kidney injury have high concentrations of fetuin-A, an osteogenic factor synthesized mainly by the liver that is strongly associated with vascular calcification and diabetes. Emerging evidence indicates that sodium transporters are found in urinary exosomes, with altered expression levels in various hypertensive disorders. For example, individuals with pseudohypoaldosteronism type II display increased NaCl cotransporter (NCC) levels in EV compared with healthy controls. The levels of vesicular sodium transporters may be dependent on activation status of the renin-angiotensin-aldosterone system (RAAS). Increased activation of the RAAS increases exosomal epithelial sodium channel (ENaC) peptides [22]. Interestingly, exosomes have also been reported to inhibit ENaC activity through mechanisms involving the glyceraldehyde-3-phosphate dehydrogenase [23].

Urinary exosomes also carry miRNAs and may modulate tubular transporters in the kidney [24]. Urinary exosomes from patients with microalbuminuria show reduced levels of miR-155, miR-424, and miR146a, whereas miR-130a and miR-145 are increased. These processes may be important in effects mediated by transforming growth factor-β (TGF-β) which plays a role in renal fibrosis.

15.3 Microparticles

15.3.1 Microparticle Formation

Microparticles (MP) are EV formed from the outward "blebbing" of the plasma membrane and shedding into the extracellular space (Fig. 15.2). MP from many cell types have been identified in the circulation including cells from vessels (endothelial cells, smooth muscle cells, fibroblasts), circulating cells (platelets, erythrocytes, leukocytes), immune cells (neutrophils, lymphocytes, monocytes/macrophages), and progenitor cells. They are formed in physiological conditions and during cell activation and cell stress. Important molecular processes in parent cells that give rise to MP include increased intracellular calcium levels, oxidative stress, Rho kinase activation, caveolae, and cytoskeletal organization [2]. Rearrangement of membrane phospholipids and phosphatidylserine exposure are critically involved in membrane blebbing and MP formation and are characteristic features of MP.

The in vivo importance of phosphatidylserine exposure in MP formation is evidenced in patients with Scott syndrome, a genetic condition characterized by impaired ability to expose phosphatidylserine in the plasma membrane and deficiency in coagulation. These individuals have reduced numbers of circulating platelet MP. Reductions in circulating MP are also found in patients deficient in the ATP-binding cassette transporter ABCA1, a protein involved in cellular cholesterol metabolism and lipid homeostasis including shuttling of phosphatidylserine. Cholesterol-enriched microdomains in the cell membrane (e.g. caveolae, lipid raft microdomains) contribute to MP formation, since disruption of caveolae/lipid rafts results in decreased MP formation [2].

15.3.2 Microparticles in Intercellular Communication

Microparticles may be considered as important mediators of cell activation since they transmit information in a paracrine and endocrine fashion. Similar to exosomes, MP carry RNA, microRNA, proteins, lipid mediators, and growth factors. There is considerable overlap in content between exosomes and MP, but MP are distinguished by their size, membrane proteins, and surface phosphatidylserine. The MP content changes according to the status of the parent cell, for example, MP content differs if cells are activated, stressed, apoptotic, or necrotic. Their content also changes depending on the stimulus to which the parent cell is exposed, e.g. glucose, vasoactive peptides, growth factors, and cytokines [25].

Microparticle-mediated intercellular communication occurs not only between similar cell types, e.g. endothelial MP influencing endothelial cells, but also between different cell types, e.g. platelet MP influencing endothelial cells, etc. Such interactions facilitate crosstalk between different cell populations.

15.3.3 Extracellular Vesicles in Cell Activation and Cardiovascular Diseases

The interaction between MP and exosomes with target cells is complex and may involve multiple mechanisms (see Fig. 15.3), including (i) ligand-receptor binding, (ii) direct fusion with cell membranes, or (iii) uptake by recipient cells.

(i) Ligand-receptor binding. The membrane of EV expresses bioactive lipids, including arachidonic acid, which are metabolized by stereospecific lipid peroxidation to generate various signalling molecules. By expressing phosphatidylserine, MP can interact with scavenger receptors expressed mainly in cells from the phagocytic system. Scavenger receptors CD36 interact with phosphatidylserine motifs in MP, apoptotic cells, and oxidized phospholipids and have been implicated in MP signalling and clearance [26]. Similarly, some EV carry angiogenic growth factors such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) that may bind to EGFR or VEGFR, respectively, in the target cell.

(ii) Direct fusion with cell membranes. A second way that EV interact with the cell membrane is by fusion with the recipient cell lipid bilayer, which is common in cells from the same lineage, since they carry a similar lipid composition [27].

(iii) Uptake by recipient cells. Endocytosis followed by internalization is another mechanism whereby EV can interact with target cells. These processes result in release of the EV content to recipient cells, where they influence cell signalling and function (Fig. 15.3).

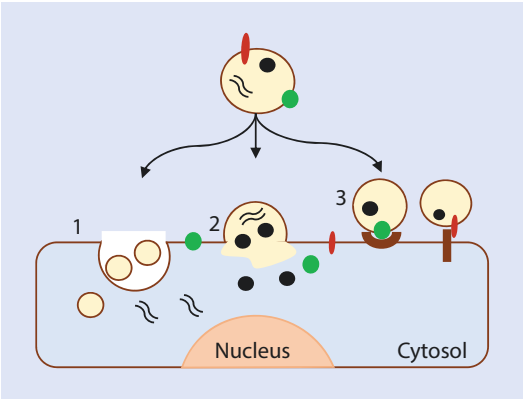

Fig. 15.3 Interaction of extracellular vesicles to the target cell. Extracellular vesicles can interact with target cells and transfer mRNA, miRNA, proteins, and signalling molecules through (1) endocytosis, (2) membrane fusion, or (3) receptor interaction, inducing target cell activation

Oxidative stress is a significant contributor to cell activation and injury in cardiovascular disease. Experimental data have shown that cultured human umbilical vein endothelial cells (HUVECs) stimulated with the pro-inflammatory cytokine TNF-α produce MP that are enriched in proteins related to oxidative stress. Endothelial MP reduce cell proliferation and nitric oxide (NO) production and influence vasorelaxation through redox-dependent mechanisms. An increase in MP number is observed in cell senescence by mechanisms involving Rho-associated kinase (ROCK) activity. Endothelial cells exposed to high-glucose conditions also cause an increase in MP production. Reactive oxygen species play an important role in both MP production and in MP-induced cell activation. In fact, endothelial MP express functionally active nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which promote ROS production in target cells [28, 29].

Microparticles also carry genetic material important for cell activation. For example, mRNA for endothelial nitric oxide synthase (eNOS), phosphoinositide 3-kinase (PI3k), and Akt has been observed in MP. Transfer of such material could alter key cellular processes including nitric oxide (NO) production, glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. Endothelial MP also carry mediators that increase endothelial cell differentiation from bone marrow cells. Moreover, MP induce a pro-inflammatory phenotype in endothelial cells by increasing expression of interleukin-6 (IL-6), MCP-1, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) through mechanisms dependent on MAPK and NF-κB activation. MP-mediated transfer of metalloproteinases -2, -3, -9, and -13 (MMP associated with plaque atherosclerotic instability and rupture) has been implicated in vascular remodelling in atherosclerosis [30].

Vascular smooth muscle cell-derived MP may also affect endothelial cells by releasing MP which reduce nitric oxide production and impair vasorelaxation by mechanisms that still need to be elucidated. Another example of MP carrying protein between different cell populations is observed in the lungs. Alveolar macrophages constitutively produce MP carrying suppressor of cytokine signalling (SOCS) proteins that interact with alveolar epithelial cells and reduce the inflammatory response by mechanisms involving signal transducer and activator of transcription (STAT) [31]. This protection is reduced in individuals after cigarette smoking. Of note, SOCS proteins are present in the extracellular environment only if carried by MP or exosomes.

15.3.4 Microparticles as Biomarkers in Cardiovascular Diseases

Microparticles can be detected in low levels in plasma from healthy individuals. In various cardiovascular diseases, diabetes, obesity, kidney disease, and cancer, circulating levels of MP are increased. As such, an active area of research is to identify MP populations that are differentially expressed in healthy and in disease states that can be used as biomarkers. In this regard, there is an increase in MP derived from leukocytes correlated with levels of C-reactive protein (CRP) in patients with metabolic syndrome and atherosclerosis. Likewise, the numbers of MP are correlated with cardiovascular disease score risk observed in the Framingham study [32], a long-term, ongoing cardiovascular cohort study on residents of the city of Framingham, Massachusetts. In patients with end-stage kidney disease, an elevated level of circulating endothelial MP is more predictive of future cardiovascular risk than the Framingham risk score or age [33]. High-fat diet, which impairs endothelial function and increases oxidative stress, also induces an acute increase in MP in young healthy men. Hypertensive patients have

elevated plasma MP derived from endothelial cells, platelets, and monocytes.

Certain therapeutic interventions have been shown to influence circulating MP levels. In patients with hypertension and type 2 diabetes, treatment with simvastatin and losartan decreased levels of MP derived from endothelial cells, platelets, and monocytes. The reduction observed relates not only to the total number of circulating MP but also to the expression profile [34]. Statins reduced MP levels as well as MP expression of fibrinogen receptors in type 2 diabetes patients, which is related to a decrease in thrombus formation and cardiovascular events [35]. Fish oil supplementation also decreases circulating endothelial-derived MP levels [36]. More broadly, treatment of hypertension is associated with a reduction in circulating endothelial MP, although not to the levels of healthy individuals. Similarly, glycaemic control is associated with a reduction in circulating endothelial- and platelet-derived MP which are correlated with glucose tolerance in diabetes [37, 38].

Interestingly, disease may also impact the bioactivity of MP. In stroke, MP are derived from monocytes and platelets and influenced vascular calcification, plaque instability, rupture, and stroke development [39]. Similarly, MP from patients with metabolic syndrome impair NO production and endothelium-dependent vasorelaxation in isolated vessels, while those from healthy individuals have minimal effect [40].

15.4 Isolation of Extracellular Vesicles

Previously in this chapter, we mentioned that microparticles and exosomes are differentiated by size, and these physical characteristics are important for isolation of extracellular vesicles to allow a more specific molecular investigation. The isolation of MP (size 100–1000 nm) from plasma samples requires a two-step centrifugation: (1) plasma samples are centrifuged at 1500–2500 × g for 10 min to remove cell debris, followed by ultracentrifugation at 17,000–20,000 × g/20 min. Pellet containing MP can be solubilized in saline for further studies [41]. Specific details regarding ultracentrifugation protocols should be found in specialized literature.

The isolation of exosomes requires more steps to avoid contamination with proteins, cell organelles, or microparticles. To remove this contamination, after microparticle isolation as previously described, the supernatant should be filtered at 0.1 μm, in order to remove any source of contamination larger than 100 nm, followed by ultracentrifugation at 100,000–120,000 × g for 90 min at 4 °C. These are the most used techniques; however, possible drawbacks of using differential centrifugation for isolating exosomes are the co-sedimentation of protein aggregates and copurifying non-specifically bound proteins. To avoid such contamination, other protocols have been developed, including density-gradient centrifugation (DGC), sucrose cushion centrifugation, and gel permeation chromatography (GPC). All these protocols present some differences in purity, interfering also with the final concentration of exosomes. Additionally, antigen affinity capture is also considered to increase the purity of extracellular vesicles. The affinity is based on specific markers' content in exosomes (tetraspanin membrane proteins CD9, CD81, and CD63) and microparticles (anexin V) [4].

15.5 Extracellular Vesicles as Therapeutic Delivery Systems

Extracellular vesicles display a number of characteristics that make them attractive drug delivery systems. They are capable of transporting proteins and genetic material throughout the body while providing protection to their cargo. Additionally, by the nature of their cargo, EV can be exploited for therapeutic approach. These effects can be observed as immunotherapy, where EV can contribute to the activation of lymphocytes, therefore, improving the treatment for infectious diseases and cancer. The usage of EV as a drug delivery presents some advantages, and it is of great interest to improve the diseases' treatment. EV are compatible, and depending on the parental cell, they can be immunologically inert, such as mesenchymal stem cells or immature dendritic cells. One very important characteristic is that EV are able to cross the blood-brain barrier. In fact, specialized investigations reported the delivery of exosomes contained silencing RNA to the brain [42, 43].

Exosomes can be engineered in artificial systems to express foreign proteins, miRNA, and siRNA as well as to carry drugs. They can be

loaded with adenovirus, improving the delivery to target cells. As such there is enormous potential to develop EV as therapeutic modalities. However, despite the advantages, there are important challenges including the lack of specificity, unknown pharmacokinetics, and insufficient cytosolic delivery efficiency [44]. Therefore, additional studies aimed at establishing EV as drug delivery systems are still needed.

Conclusion and Clinical Perspectives

EV, including exosomes and MP, are important players in cell-cell communication involved in physiologic and pathological processes. All cells have the potential to generate EV, which are normally found in the circulation in low levels. Cardiovascular disease, diabetes, metabolic syndrome, obesity, kidney disease, and cancer are associated with increased plasma levels of EV. How EV interact with target cells is still elusive and likely involves multiple processes including ligand-receptor interaction, cell membrane fusion, endocytosis, and transfer and release of MP cargo into recipient cells. In addition to their role as mediators of crosstalk between cells, EV are important biomarkers of disease and have the potential to act as therapeutic delivery systems for drugs, proteins, miRNA, and other molecules.

Gaps in Knowledge

- Methods to isolate pure fractions of EV need to be improved in order to avoid contamination.
- EV can interact with the target cell by endocytosis, receptor interaction, or membrane fusion, and it is unknown if these different processes of interaction result in difference in the activation profile of the target cell.
- EV comprise a diverse group of vesicles, and their complexity increases in pathological states. The specific functions of different subclasses of EV produced by the same cell are still a challenge for future investigations as are the identities of molecules central to their bioactivity.
- MP as diagnostics and therapeutics in disease still need to be validated clinically.

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Pathophysiology of Vascular Diseases

Contents

Genomics of Hypertension

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_16

Key Concepts

- Blood pressure and hypertension are polygenic traits.
- Genome-wide association studies have identified over 900 SNPs associated with blood pressure that explains nearly 20% of its heritability.
- Rare monogenic forms of high and low blood pressure exist, and they are predominantly due to single-gene mutations affecting renal and adrenal pathways.

Genomics has contributed greatly to our understanding of the molecular basis of disease and, to a lesser but growing extent, to the development of effective therapies. Until the publication of the draft sequence of the human genome in 2001, cardiovascular genetics focused mainly on a small number of rare Mendelian syndromes [1]. However, the vast majority of cardiovascular diseases of major public health importance, like coronary artery disease (CAD) and hypertension (HTN), have a multifactorial inheritance, representing the complex interplay between polygenic risk alleles and environmental triggers. The dissection of the genetic architecture of cardiovascular diseases is therefore challenging. From an epidemiological and clinical perspective, blood pressure (BP) at the higher end of the normal population distribution is associated with an increased risk of cardiovascular mortality and morbidity. The main determinants of BP, cardiac output and total peripheral resistance are controlled by a complex network of interacting pathways involving renal, neural, endocrine, vascular and other mechanisms. Multiple genes within each of these systems contribute to the specialized functions regulating BP, and hence it is likely that many genes will participate in the development of HTN. Thus, by definition, BP is a complex trait which refers to any phenotype that does not exhibit classic Mendelian inheritance attributed to a single gene and result from the interactions between multiple genes and environmental factors (see Table 16.1 for differences between simple and complex traits and Fig. 16.1 for the complex causation of HTN). The distribution of BP in the population is a normal unimodal distribution which

Table 16.1 Simple versus complex traits

Simple or monogenic trait	Complex trait
Usually due to a single gene defect	Polygenic trait with involvement of multiple genes and environmental factors
Due to deleterious mutations	Not due to rare deleterious mutations but due to natural variations frequent in the population
Clear pattern of inheritance – autosomal dominant, autosomal recessive or sex-linked	Aggregates in families, but do not show a clear Mendelian pattern of inheritance
Complete penetrance	Variable penetrance
No phenocopies	Phenocopies present
High allelic heterogeneity	Phenotypic and genetic heterogeneity
Usually occur in early life	Usually late-onset diseases
Gene discovery is through parametric linkage analysis	There are a multitude of approaches to discover the causal gene variant. Association studies are the most commonly used. No ideal solution
Examples	Examples
Long QT syndrome	Coronary artery disease
Familial hypercholesterolaemia	Hypertension
Liddle syndrome	Diabetes

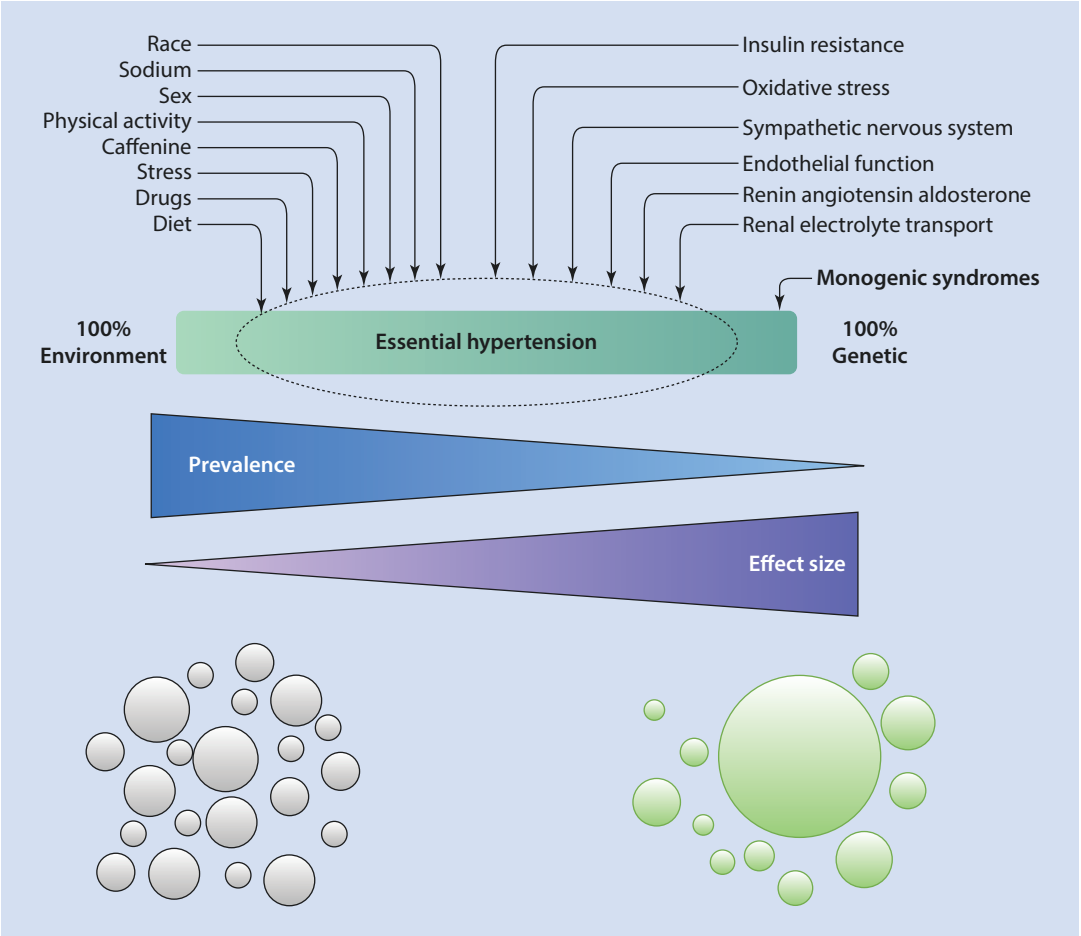

Fig. 16.1 The complex multifactorial interplay of genetic and environmental factors in the causation of essential hypertension. Monogenic forms of hypertension are completely genetically determined and are a rare cause of hypertension in the general population

supports the complex multifactorial basis of BP regulation [2]. However, there are rare monogenic forms of HTN or hypotension ("simple trait") as illustrated in Fig. 16.1 at the right end of the spectrum of BP. These monogenic forms of HTN or hypotension are very rare in the population and have little to no impact on public health in contrast to essential HTN, which has a prevalence of over 25% among the adult population worldwide. The monogenic forms of BP dysregulation have provided valuable insights into BP regulation and expanded our understanding of both the mechanisms and the treatment of HTN. Mutations in around 25 genes are now linked to perturbed gene function and consequent BP dysregulation [3]. The challenge is to extend these successful examples to mapping of genes associated with essential HTN.

16.1 Complex Causation of Hypertension

Though BP and HTN reflect a single phenotype at a clinical level, they may actually be a heterogeneous group of potentially overlapping disorders on a genetic or aetiologic level [3]. For example, high BP may be due to modifiable environmental factors such as high salt consumption, structural factors such as renal artery stenosis and anthropometric factors such as obesity or male sex, and thus the BP or HTN label does not indicate a pure phenotype. This becomes extremely important in genetic dissection as overlapping underlying mechanisms has a major impact on interpreting results of genetic studies and may lead to negative studies [4, 5]. The complex causation of HTN is illustrated in

Fig. 16.1. One strategy for dealing with underlying genetic heterogeneity and potentially a complex network of aetiologies contributing to variation in a phenotype is to directly study quantitative risk factors that may index different underlying aspects of disease aetiology in the hope that these intermediate phenotypes may be genetically less complex with potentially stronger genetic signals. For example, to identify genetic markers for salt-sensitive HTN, it would be reasonable to focus on salt sensitivity as a phenotype to be studied rather than BP. However, this entails accurate measurement of the trait, and the associated costs for screening and measurement need to be considered.

16.2 Evidence for a Genetic Basis of Essential Hypertension

While monogenic syndromes of HTN provide evidence that disruption of gene function can have a major impact on BP, to venture into formal genetic dissection of BP or essential HTN requires evidence for a genetic contribution to these traits. Strong indication that BP and essential HTN may have a genetic component came from family studies demonstrating correlation of BP among siblings and between parents and children with part of this correlation attributable to genetic factors [2, 3]. The Montreal Adoption Study [6] demonstrated correlation coefficients of 0.38 and 0.16 between biological and adoptive sibs, respectively, while the Victorian Family Heart Study estimated correlation coefficients of 0.44 for non-twin siblings, 0.78 for monozygous twins, 0.50 for dizygous twins and 0.12 for spouse-spouse pairs. All these data indicate presence of a genetic component if the environmental influence is assumed to be similar between comparison groups. Two additional measures that are commonly used to assess the genetic component of a trait are heritability (h^2), which is the fraction of variation in disease susceptibility due to genetic factors, and sibling recurrent risk (λs) which is the degree of elevated risk of disease for a sibling of an affected individual compared with a member of the general population [7]. The heritability of clinic systolic BP is around 15–40% and 15–30% for clinic diastolic BP, whereas for ambulatory night-time systolic and diastolic BP, the heritabilities are 32–70% and 32–50%. It is pertinent to point out that though the heritability estimates are considerable, this does not equate to magnitude of genetic effect. This is because the denominator in the estimate of heritability comprises measurement error and variances attributable to genes, shared environment, non-shared environment and unmeasured determinants. Heritability is also a property of the population studied, and low heritability estimates would suggest that genetic mapping would be difficult for that phenotype. The sibling recurrent risk of HTN is around 1.2–1.5, and taking this along with heritability and correlation estimates, HTN and BP can be considered a trait with relatively modest genetic effect [1, 7].

16.3 Common Variants or Rare Variants

A good understanding of the genetic architecture of a trait is required for successful gene mapping. The genetic architecture of a trait refers to the number of disease variants that exist, their allele frequencies, the risks that they confer and the interactions between multiple genetic and environmental factors. Mutations that account for Mendelian forms of HTN are highly penetrant and are usually under very strong selection, which keeps them at low frequencies with high levels of allelic heterogeneity. In contrast, susceptibility variants involved in essential HTN are likely to have low or medium penetrance and are probably not subject to such strong selection resulting in lower allelic heterogeneity. There is an ongoing debate whether common or rare variants contribute to essential HTN. Single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) >1% account for more than 90% of the genetic differences between individuals and thus are likely to contribute to the population variation in BP rather than rare variants. This is the basis of the common disease/common variant (CDCV) hypothesis which states that genetic variants underlying complex traits occur with a relatively high frequency, have undergone little or no selection in earlier populations and are likely to date back to more than 100,000 years ago [2]. Indeed from an evolutionary perspective, essential HTN is a disease of civilization (i.e. high salt intake, decreased physical activity, increased intake of processed food, reduced fruit and vegetable intake) and may be an undesirable pleiotropic effect of a preserved genotype that could have optimized fitness in the ancient environment. It is well recognized that

HTN occurs earlier and with more severity in people of African ancestry compared to those of European ancestry [8]. Differing predispositions to HTN in different populations may simply reflect different evolutionary selection pressures ("bottlenecks") and the fact that populations do not share the same ancestral histories. Also, an allele with no effect on reproductive fitness is expected to achieve high equilibrium frequency, and this is likely to be the case for genes influencing HTN. The other competing model for HTN is the common disease rare variant hypothesis, with an inverse relationship between the magnitude of genetic effect and allele frequency. This model argues that diseases are common because of highly prevalent environmental influences, not because of common disease alleles in the population. Support for this hypothesis or model has come from studies of rare variants of three genes *SLC12A3*, *SLC12A1* and *KCNJ1* (major mutations of which cause Gitelman syndrome, Bartter syndrome type 1 and Bartter syndrome type 2, respectively) in the general population producing clinically significant BP reduction and protection from development of HTN [9]. The most likely scenario would be that the allelic spectrum of the disease variants is the same as the general spectrum of all disease variants. Under this neutral model, although most susceptibility variants are rare with MAF <1%, SNPs with MAF >1% would account for more than 90% of the genetic differences between individuals. It is plausible that these common variants might contribute significantly to those common diseases in which susceptibility alleles might not be under intense negative selection. For the genome as a whole, it has been predicted that of the expected 10–15 million SNPs with MAF >1%, approximately half have MAF >10%. Given that the number of disease variants conferring mild to moderate risks might be large, there are likely to be hundreds of common and rare variants contributing to the familial clustering of HTN.

16.4 Monogenic Forms of Hypertension and Hypotension

Table 16.2 summarizes the rare monogenic forms of HTN that have contributed to our understanding of blood pressure regulation and targeted treatment [1].

16.5 Polygenic Hypertension

Major advances in identifying common variants associated with BP and HTN arose from genome-wide association studies (GWAS). GWAS are large-scale association mapping making no assumptions of the genomic location or function of the causal variant and provide a comprehensive approach to testing the hypothesis that common alleles contribute to heritable phenotype variation. GWAS rely on the linkage disequilibrium (LD) or correlation patterns of SNPs with functional variants and, therefore, identified SNPs are usually proxies of untyped functional variants. A typical GWAS experiment consist of genotyping 500,000 to 1 million SNPs across the genome, as depending on the population this number of SNPs is adequate to interrogate 80% of common SNPs with MAF >5%. To adjust for multiple testing and to decrease type I error (false-positive rates), the statistical burden of proof relies on stringent *P*-values usually $P < 5 \times 10^{-8}$. Figure 16.2 provides a summary the top loci identified from GWAS for BP and HTN, which have mapped common variants in over 901 (including results from Warren et al. Nature Genetics 2018, *in print*) [10] loci which collectively explain about 20% of the heritability for systolic and diastolic BP [11–15]. One successful GWAS for HTN used an extreme case-control design representing the top 2% and bottom 9% of the BP distribution in Sweden. Combined with follow-up validation analyses in 19,845 cases and 16,541 controls, a locus near the Uromodulin (*UMOD*) gene was identified [12]. *UMOD* is exclusively expressed in the kidney, suggesting that the discovered variant may have an effect on sodium homeostasis. Trudu et al. [16] showed that furosemide treatment significantly enhanced natriuresis and reduced BP levels both in the transgenic mice and in the hypertensive individuals homozygous for the *UMOD*-increasing allele, making this a potentially interesting locus for both HTN and renal disease. This is now the basis of a clinical trial (► www.clinicaltrials.gov NCT03354897) to reposition a loop diuretic in the HTN care pathway.

An important limitation of GWAS is that genome-wide significant SNPs often merely tag but do not provide direct information on the causal variants. To translate those signals to biological function, follow-up studies are necessary.

Table 16.2 Monogenic forms of hypertension

Locus	Position (GRCh38/hg38)	Gene/nearest genes	Inheritance	Genomic and phenotypic annotation	Hypertension treatment strategies
1p36.13	16043752–16057308	*CLCNKB*	Autosomal recessive	Bartter syndrome, type 3 OMIM #607364	Sodium and potassium supplements
				Low blood pressure. Impaired chloride reabsorption in the thick ascending loop of Henle leads to impaired sodium reabsorption. Hypokalaemic metabolic alkalosis. Increased plasma renin and aldosterone	Aldosterone inhibitors and angiotensin-converting enzyme (ACE) inhibitors
					Indomethacin
1p36.13	17018730–17054170	*SDHB*		Paragangliomas 4 OMIM #115310	Alpha adrenergic blockers for pheochromocytoma
				Multiple catecholamine-secreting head and neck paragangliomas and pheochromocytomas. Adult onset	
1q23.3	161314376–161364745	*SDHC*	Autosomal dominant	Paragangliomas 3 OMIM #605373	Alpha adrenergic blockers for pheochromocytoma
				Tumours or extra-adrenal paraganglia-associated pheochromocytoma	
2q36.2	224470150–224585397	*CUL3*	Autosomal dominant	Pseudohypoaldosteronism, type IIE OMIM *603136	Thiazide diuretics
				Hypertension, hyperkalaemia, hyperchloraemic metabolic acidosis	
3p25.3	10141635–10153670	*VHL*	Autosomal dominant	von Hippel-Lindau syndrome OMIM #193300	Alpha adrenergic blockers for pheochromocytoma
				Associated with retinal, cerebellar and spinal haemangioblastoma, renal cell carcinoma (RCC), pheochromocytoma and pancreatic tumours	
4q31.2	148078764–148442520	*NR3C2*	Autosomal dominant	Hypertension exacerbation in pregnancy OMIM #605115	Spironolactone contraindicated
				Missense mutation (S810L) in the mineralocorticoid receptor. Low-renin, low-aldosterone, hypokalaemia. Progesterone and other steroids lacking 21-hydroxyl groups, normally MR antagonists, becoming potent agonists	
			Autosomal dominant	Pseudohypoaldosteronism type I OMIM #177735	Sodium chloride treatment
				Failure to thrive. Renal unresponsiveness to mineralocorticoids. Hyponatraemia, hyperkalaemia, metabolic acidosis. Increased renin and aldosterone	

5p15.3	218241–256699	*SDHA*		Paragangliomas 5 OMIM # 614165 Tumours or extra-adrenal paraganglia-associated pheochromocytoma	Alpha adrenergic blockers for pheochromocytoma
5q31.2	137617500–137736090	*KLHL3*	Autosomal dominant/recessive	Pseudohypoaldosteronism, type IID OMIM # 614495 Hyperkalaemia. Hyperchloraemic metabolic acidosis	Thiazide diuretics
7p22.3-7p22.1	10001–7239940		Autosomal dominant	Familial hyperaldosteronism type 2 OMIM #605635 Hyperaldosteronism due to adrenocortical hyperplasia not suppressed by dexamethasone	Aldosterone antagonists
7q36.1	150991056–151014599	*ABP1*, *KCNH2*, *NOS3*, *ACCN3*		NOS3 – pregnancy-induced hypertension OMIM +163729 Nitric oxide plays an important role in the maintenance of cardiovascular and renal homeostasis	
8q24.3	142872357–142917843	*CYP11B1*, *CYP11B2*	Autosomal dominant	Familial hyperaldosteronism type 1 Glucocorticoid-remediable aldosteronism (GRA) OMIM #103900 Chimeric gene. Plasma and urinary aldosterone responsive to ACTH; dexamethasone suppressible within 48 h. Increased aldosterone and low renin	Hypertension suppressed by dexamethasone
			Autosomal recessive	Corticosterone methyloxidase II deficiency OMIM #61060 CYP11B2 Enzymatic defect results in decreased aldosterone and salt-wasting, high plasma renin	Sodium chloride supplementation Fludrocortisone
			Autosomal recessive	Steroid 11β-hydroxylase deficiency OMIM #202010 CYP11B1 Neonatal onset. Virilization, short stature, suppressed aldosterone and renin	Glucocorticoids to reduce the ACTH-driven adrenal hyperplasia and production of the various hormone precursors. Potassium-sparing diuretics

(continued)

Table 16.2 (continued)

Locus	Position (GRCh38/hg38)	Gene/nearest genes	Inheritance	Genomic and phenotypic annotation	Hypertension treatment strategies
10q11.2	43077069–43130349	*RET*	Autosomal dominant	Multiple endocrine neoplasia, type IIA OMIM #171400	Alpha adrenergic blockers for pheochromocytoma
				Associated with multiple endocrine neoplasms, including medullary thyroid carcinoma, pheochromocytoma and parathyroid adenomas	
10q24.3	102830531–102837533	*CYP17A1*	Autosomal recessive	17-alpha-hydroxylase deficiency OMIM #202110	Glucocorticoids to reduce the ACTH-driven adrenal hyperplasia and production of the various hormone precursors. Potassium-sparing diuretics
				Hypertension, hypokalaemic alkalosis. Increased ACTH and FSH. Absent sexual maturation	
11q12.2	61430125–61446767	*SDHAF2*	Autosomal dominant	Paragangliomas 2 OMIM #601650	Alpha adrenergic blockers for pheochromocytoma
				Tumours or extra-adrenal paraganglia-associated pheochromocytoma	
11q23.1	112086847–112095794	*SDHD*	Autosomal dominant	Paragangliomas 1 OMIM #16800	Alpha adrenergic blockers for pheochromocytoma
				Tumours or extra-adrenal paraganglia-associated pheochromocytoma	
11q24.3	128838020–128867373	*KCNJ1*	Autosomal recessive	Bartter syndrome, antenatal, type 2 OMIM #241200	Sodium and potassium supplements
				Reduced potassium recycling leads to impaired sodium reabsorption. Elevated plasma renin and aldosterone. Hypokalaemia, hypochloraemia, hyperprostaglandinuria	Aldosterone inhibitors and angiotensin-converting enzyme (ACE) inhibitors
					Indomethacin
12p12.3–12p11.1	19847067–33147066	*PDE3A*	Autosomal dominant	Hypertension with brachydactyly Bilginturan syndrome OMIM %112410	
				Brachydactyly, short phalanges, short metacarpals	
12p12.3	752923–911452	*WNK1*	Autosomal dominant	Pseudohypoaldosteronism type IIC Gordon's syndrome OMIM #614492	Alkalizing agents, potassium-binding resins, prostaglandin inhibitors and diuretics
				Gain-of-function mutations in WNK1. Hyperchloraemic metabolic acidosis. Low plasma renin, normal or elevated K^+	

15q21.1	48206301–48304078	*SLC12A1*	Autosomal recessive	Bartter syndrome, antenatal, type 1 OMIM #601678 Homozygous or compound heterozygous mutation in the sodium-potassium-chloride cotransporter-2 gene	Sodium and potassium supplements Aldosterone inhibitors and angiotensin-converting enzyme (ACE) inhibitors Indomethacin
16p12.2	23302270–23216879	*SCNN1B*, *SCNN1G*	Autosomal dominant	Liddle syndrome OMIM # 177200 Constitutive activation of epithelial sodium transporter, ENaC. Low plasma renin and aldosterone. Hypokalaemia	Amiloride or triamterene
16q13	56865207–56915850	*SLC12A3*	Autosomal recessive	Gitelman syndrome OMIM #263800 Low BP. Loss-of-function mutation leads to lower sodium reabsorption. Increased plasma renin. Renal potassium and magnesium wasting	Potassium and magnesium supplements. NaCl intake
16q22.1	67431133–67437551	*HSD11B2*	Autosomal recessive	Apparent mineralocorticoid excess OMIM # 218030 Increased plasma ACTH. Increased urinary cortisol/cortisone ratio. Low plasma renin and aldosterone	Spironolactone
17q21.2	42780631–42797066	*WNK4*	Autosomal dominant	Pseudohypoaldosteronism type IIB Gordon's syndrome OMIM #614491 Loss-of-function mutations in WNK4. Low plasma renin, normal or elevated K+	Alkalizing agents, potassium-binding resins, prostaglandin inhibitors and diuretics

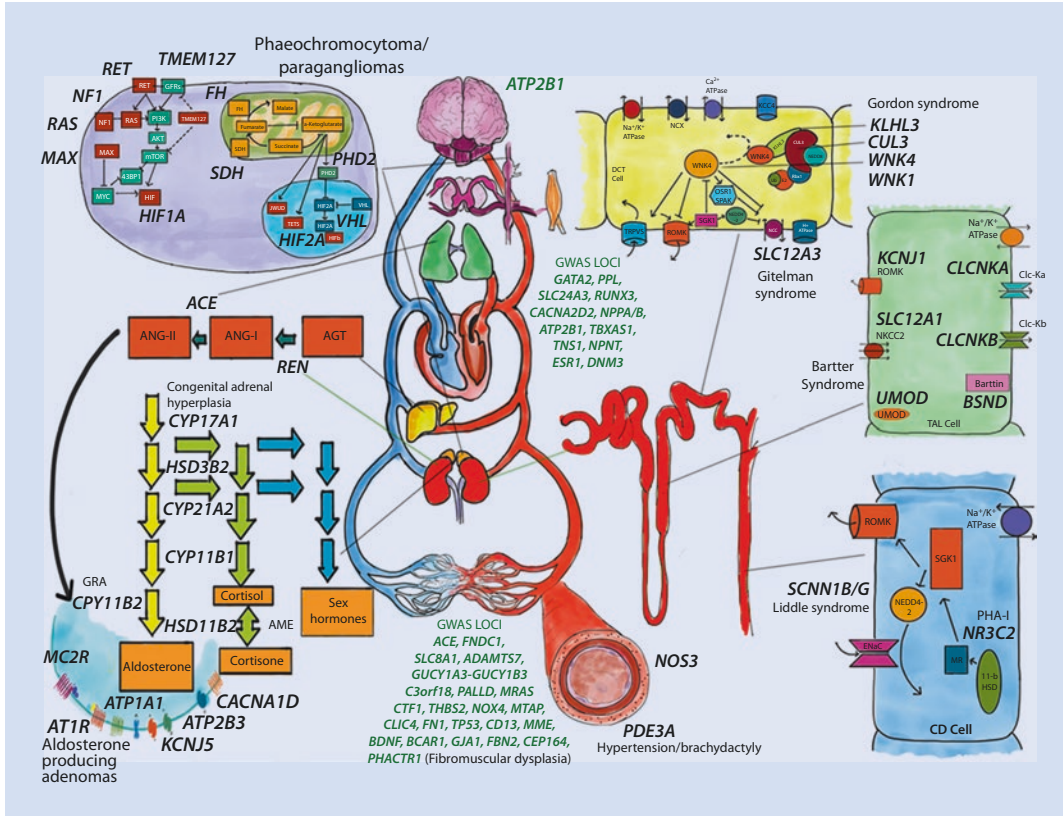

Fig. 16.2 Monogenic and polygenic genomic loci for BP and HTN from genome-wide association studies and linkage studies

Another issue arising from GWAS is the small fraction of population variance of BP (<1%) and BP heritability (~2%) that are explained by the collective effect of all the GWAS loci identified so far. The missing heritability [17] conundrum is not unique to BP genetics but is observed in most of the common phenotypes.

Despite the increasing pace of discovery of variants associated with BP and HTN, the limited predictive utility of these variants either singly or as part of a composite risk score is striking [18]. One way of maximizing information about the genetic signals is to create a composite genetic risk score coding for the presence or absence of risk alleles and their numbers for all the BP GWAS SNPs [18]. In the International Consortium for Blood Pressure Genome-Wide Association Study [15], between the top and the bottom quintiles of the risk score, a 4.6 mmHg systolic and 3.0 mmHg diastolic BP difference was detected, and the prevalence of HTN was 29% compared to 16% in the top and bottom deciles. The score was also associated with early and advanced target organ damage including left ventricular hypertrophy, stroke and CAD but not chronic kidney disease or markers of renal function [15]. The lack of association between BP risk score and kidney function would indicate high BP and renal disease do not necessarily have the same molecular origin, and this opens a new avenue for research to validate or refute the observation. It is clear that using panels of genetic markers to predict risk has very poor discrimination and that the utility of GWAS approaches is primarily in identification of novel pathways.

Conclusions and Clinical Perspectives

Advances in high-throughput genomics have vastly increased our understanding of the genetic architecture of BP and HTN. The burgeoning list of genomic variants associated with BP and HTN provides a realistic basis for refining the molecular taxonomy of HTN and directing

the discovery of new drugs for precision medicine. The clinical applications of genomic discoveries are evident in the treatment strategies for monogenic forms of HTN. However, for polygenic HTN, clinical translation of GWAS needs functional dissection of the signals and drug discovery or repurposing.

Gaps in Knowledge

- GWAS offer population-based estimates of risk, and thus application as predictive tests at an individual level is not appropriate and needs further research.
- GWAS signals reflect association and not causality. Establishing the causal variant and gene is a critical first step in clinical translation.
- The biggest challenge in genomics of complex traits is unravelling the effect of pleiotropy, gene-gene interactions and gene-environment interaction in the development of the final phenotype.

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Endothelial Dysfunction

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_17

Key Concepts

- The endothelium plays a key role in vascular function by mediating vascular tone, transport of molecules across the vascular wall, cell adhesion, angiogenesis, coagulation and fibrinolysis.
- In vitro and ex vivo studies interrogate endothelial function in more detail, where myography and organ bath setups play a major role.
- Due to the multitude of its tasks, no single test exists that comprehensively assesses endothelial function.
- In clinical research, non-invasive assessment of endothelium-dependent vasodilation provides a useful surrogate of endothelial function, especially when complemented by biomarker studies.

17.1 Introduction to the Endothelium

The endothelium is a unicellular layer composed of simple squamous cells that line the internal wall of the vasculature. Once considered to act simply as a semipermeable membrane between the interstitium and the vascular wall, we are now aware the endothelium is a tissue capable of a wide range of biological functions including secretory, synthesising, metabolic and immunological [1]. The endothelium plays a central role in vascular biology through various functions:

- Active transport and degradation of small molecules
- Blood coagulation and fibrinolysis
- Adhesion and migration of inflammatory cells
- Angiogenesis

Endothelial dysfunction is a widely used term that, in this context, will be used to describe a significant reduction in the production and release of vasoactive factors resulting in a loss of endothelium-dependent vasorelaxation. One central mechanism of endothelial function is the delicate balance between reactive oxygen species (ROS) and the vasodilator, NO. Superoxide anion ($\bullet O_2^-$) and NO react more quickly than $\bullet O_2^-$ and the main intracellular antioxidant, superoxide dismutase; therefore, during physiological conditions there will be some loss of NO to this reaction. In cardiovascular disease where there is an increase in superoxide, a shift in the balance results in larger amounts of NO being lost due to an increase in this reaction to form peroxynitrite ($ONOO^-$) which is a powerful oxidant molecule.

In the endothelium, endothelial nitric oxide (eNOS) produces NO in the reaction:

$$2\,\text{L}-\text{arginine} + 3\,\text{NADPH} + 3\,\text{H}^+ + 4\,\text{O}_2 \rightleftharpoons$$
$$2\,\text{L}-\text{citrulline} + 2\,\text{NO} + 4\,\text{H}_2\text{O} + 3\,\text{NADP} +$$

eNOS must be dimerised to produce NO and can bind five cofactors, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4) and calmodulin. eNOS transfers electrons from NADPH, via FAD and FMN to the heme group. The electron is then used to reduce an oxygen molecule, which oxidises L-arginine to L-citrulline and NO. Heme is critical for dimerisation of eNOS, which allows the flow of electrons to move from one monomer to the other. BH4 is critical in supplying an additional electron for this reaction.

In order to function normally, eNOS requires an excess of substrate, L-arginine, and the cofactor BH4. When either of these is absent, eNOS stops producing NO and can produce ROS; this is eNOS uncoupling. eNOS uncoupling can become a self-perpetuating mechanism whereby ROS are produced, this oxidises the BH4 cofactor which means it is not available to bind eNOS leading to further eNOS uncoupling [2] (Fig. 17.1).

17.2 Assessment of Endothelial Function In Vitro and Ex Vivo in Basic and Translational Research

17.2.1 In Vitro Study of Endothelial Function

In vitro culture of endothelial cells is a useful technique to study molecular signalling as well as providing a defined system in which to study endothelial cell function such as migration, proliferation, immune cell adhesion and tube formation.

Two of the most common and straightforward in vitro experiments to assess endothelial cell function are a scratch assay and tube formation

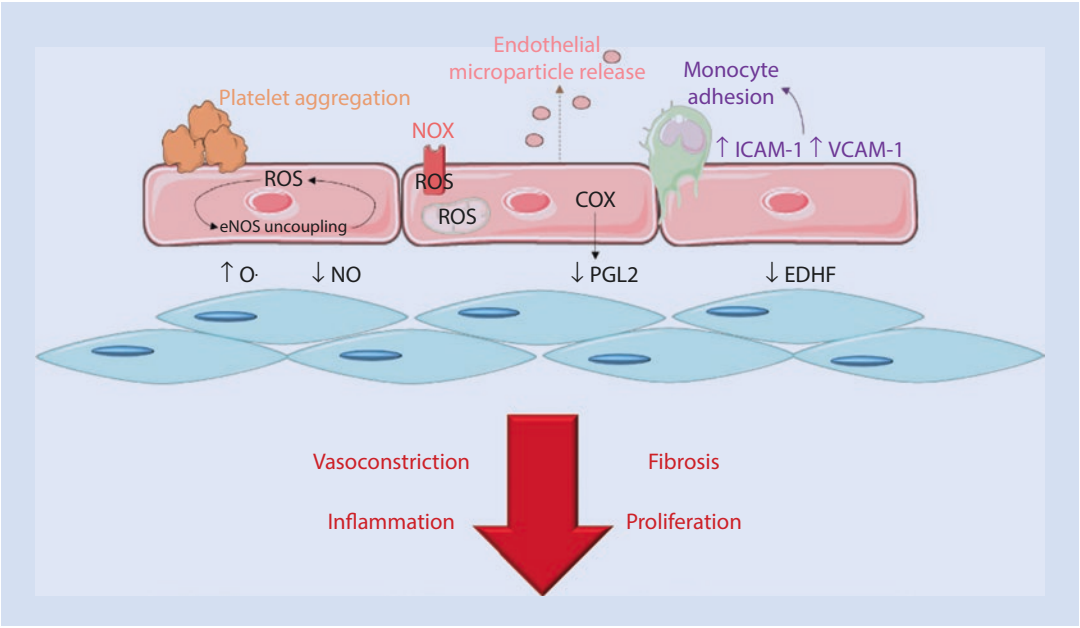

Fig. 17.1 Mechanisms of endothelial dysfunction. Endothelial cell (pink) dysfunction affects the vascular smooth muscle cells (blue) resulting in pathologies central to cardiovascular disease such as excessive vasoconstriction, inflammation, fibrosis and uncontrolled proliferation. An imbalance in the presence of prostaglandin and thromboxane results in platelet aggregation. Platelet aggregation occurs because of an increase in the expression of surface glycoproteins and is further potentiated by circulating fibrinogen. The combined activation of platelets by factors such as thromboxane and adherence to each other and the vascular wall can result in blockage of the vessel causing ischaemia and potentiation of atherosclerotic lesions. Reactive oxygen species (ROS) are increased within the endothelial cell through several mechanisms, for example, NADPH oxidases (NOX) enzymes or mitochondrial dysfunction. This can result in oxidative damage by the superoxide molecules themselves and reduce the bioavailability of the vasodilator nitric oxide by reacting with it to form peroxynitrite or contributing to a feed-forward loop of endothelial nitric oxide synthase (eNOS) uncoupling. Endothelial cells can shed microparticles formed by blebbing of the plasma membrane under periods of stress. Microparticles are under investigation as a biomarker for endothelial damage but may also play an active role in mediating endothelial cell repair. A lack of nitric oxide and the presence of pro-inflammatory cytokines such as TNFα and IL-6 result in endothelial cell activation defined as the expression of adhesion molecules (VCAM-1, ICAM-1) on their cell surface. These adhesion molecules attract the infiltration of immune cells. Endothelial cell activation contributes to the inhibition of eNOS and the induction of reactive oxygen species. Therefore, the main mechanisms of endothelial dysfunction are closely linked and often induce similar pathways. In summation, endothelial dysfunction can contribute to vasoconstriction, inflammation, fibrosis and proliferation – key hallmarks of CVD. (This figure was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0. Generic License at ► http://smart.servier.com/)

assay. A *scratch assay* involves making a scratch through a confluent monolayer of endothelial cells and monitoring their migration to heal this wound over time. A scratch assay must be verified by using an alternative migration assay such as a Boyden chamber and accompanied by a proliferation assay to ensure that closure of the gap is a result of migration and not an increase in cell number. A *tube formation assay* is a read-out of the endothelial cell's ability to contribute to angiogenesis. The induction of angiogenesis is important in the recovery from a period of tissue ischaemia such as during a myocardial infarction.

Human umbilical vein endothelial cells (HUVECs) are the most widely used in vitro endothelial cell model. These primary cells are readily harvested from the umbilical vein and have been widely used by the scientific community since their characterisation in 1973. HUVECs will only last in culture for around ten passages before reaching senescence, and cell characteristics can vary dependent upon the harvest and culture protocol. HUVECs also do not reflect the known biological differences between endothelial cells from large vessels and the microvasculature. Therefore, immortalised endothelial cell lines

have been developed to overcome these issues. Immortalised cell lines are available for endothelial cells from large arteries (e.g. EA.hy926) and from small vessel endothelium (e.g. HMEC-1). HUVECs and endothelial cell lines are excellent tools for studying endothelial cell biology as they are readily available, mostly reproducible and easy to culture [3]. However, the most translational model for in vitro endothelial studies is to use primary culture from human or animal tissues. Primary culture is more technically challenging and expensive than using a cell line.

Communication between the endothelium and the vascular smooth muscle cells is vital for the maintenance of healthy vasculature. Endothelial cells can be cocultured with vascular smooth muscle cells using specialised plates that more closely reflect conditions in situ. The expression of different vasoactive factors such as NO or the expression of various proteins such as eNOS can be measured in cultured cells to give an indication of endothelial function; however, the gold standard method is ex vivo functionality of isolated vessels using myography.

17.2.2 Ex Vivo Study of Endothelial Function

Vessels can be dissected from animal models or patient samples and mounted on a myograph. Myography can be used to assess vessels from larger arteries such as the aorta to small resistance vessels dependent upon the pathology being investigated.

In *wire myography*, a vessel is mounted upon two wires that are fed through the vessel and fixed by the experimenter in a bath filled with physiological salt solution at 37 °C. The vessels are then normalised to give a baseline tension so that comparisons can be drawn between the various baths during one experiment and between groups in separate experiments. Vasoactive substances can then be added to the bath in increasing concentrations where tension is measured [4]. Large arteries and veins greater than 1 mm can be assessed in a similar way using an *organ bath*. In this technique, the vessel is mounted but onto fixed pins [5].

A common protocol is to first assess vasoconstriction to noradrenaline, followed by vasorelaxation to acetylcholine and sodium nitroprusside. The relaxation of the vessel to carbachol (synthetic form of acetylcholine) is dependent on functional eNOS within the endothelial cells, whereas sodium nitroprusside (SNP) is a nitric oxide donor and causes endothelial independent relaxation.

The *pressure myograph* differs from the wire myograph in that the vessels are mounted on two cannulas which retain the vessel under isobaric conditions where changes in vessel diameter rather than tension are measured. Whilst pressure myography is not as highly sensitive to changes in vascular tension as wire myography, it does offer a number of benefits: it provides a measurement of vascular structure (e.g. wall thickness and cross-sectional area) as well as function, and there is less endothelial damage and no limitation of intraluminal perfusion; thus it is more "physiological" as such flow elicits shear stress and some basal release of NO. Pressure myography can also be coupled to confocal microscopy allowing for visualisation of cellular composition, functional aspects such as calcium flux and endpoints such as apoptosis [6].

17.3 Assessment of Endothelial Function in Humans

The early appearance of endothelial dysfunction in cardiovascular disease and the concept of the endothelium as an overall gauge of vascular health have prompted significant interest in its clinical assessment. The heterogeneous functions of the vascular endothelium render this assessment particularly challenging, as no single test can provide a comprehensive physiological overview of the entire vascular tree. As such, the majority of techniques focus primarily on regulation of vascular tone as a surrogate measure of the NO-mediated vasodilator response and the effect of specific agonists or shear stress. Many of these techniques were originally developed in coronary vessels but have since migrated to the forearm or digital circulation allowing more practical, non-invasive and repeatable studies, which are thought to be representative of coronary microvascular function.

17.3.1 Invasive Methods to Assess Endothelial Function in Humans

The principles from the ex vivo myography studies above can also be applied to clinical studies in

humans. Several techniques are available where the effect of an endothelium-dependent vasodilating agent such as acetylcholine can be assessed. There are, however, notable technical challenges:

- Systemic administration of vasodilating or vasoconstricting agents will inevitably have systemic effects such as a reduction or an increase in blood pressure. This will not only affect blood flow and make measurements unreliable but will also put the volunteer at risk.
- Local administration into an artery is possible. This takes advantage of the short half-life of vasoactive substances combined with a dilution effect once the agent leaves a local vascular bed and enters the central veins. Such local administration is only possible by placing arterial lines.
- Vasodilation cannot be measured precisely as tension such as in organ bath or myography experiments. Instead, assessment of blood flow or vascular diameter has been used to estimate the vasodilatory or vasoconstrictive effects.

In *coronary angiography*, an arterial catheter will be forwarded to the coronary vessels with the main aim of injecting a contrast agent to visualise the coronary arteries under X-ray. Once the catheter is in place, it can also deliver vasoactive substances such as acetylcholine or sodium nitroprusside to the coronary artery, and changes in blood flow or arterial diameter in the brachial artery can be measured [8]. This technique is regarded as the gold standard of invasive endothelial function tests in humans as it is carried out in a vascular bed that really matters for cardiovascular disease and because diameter and flow can be measured quite precisely.

The principle of applying vasoactive substances locally (or in lower doses systemically) and measurement of changes in blood flow has also been applied to organ-specific vascular beds including the renal [9] and the retinal circulation [10].

17.3.2 Non-invasive Methods to Assess Endothelial Function in Humans

Although predictive of cardiovascular events, assessment of the coronary circulation is an invasive and expensive technique limited to those subjects requiring or willing to undergo coronary angiography. These features mean that invasive studies of the coronary arteries are challenging if serial measurements are required. For this reason, many techniques have now migrated to the peripheral circulation to allow more repeatable and non-invasive assessment of endothelial function in the clinical setting.

17.3.2.1 Brachial Artery Flow-Mediated Dilatation

Flow-mediated vasodilatation (FMD) of the forearm arteries is the most widely used non-invasive method of assessing endothelial function. This technique relies on brachial artery imaging with high-resolution ultrasound during a period of reactive hyperaemia. With the patient lying in supine position, forearm or hand ischaemia is induced through interruption of arterial flow using a tourniquet or blood pressure cuff inflated to supra-systolic pressure for 5 min. Cuff release results in dilation of the distal microvasculature, blood flow-associated shear stress and therefore reactive hyperaemia. The magnitude of change in vessel diameter from baseline to the peak observed during hyperaemic phase is indicative of overall endothelial function. Simultaneous acquisition of pulsed-wave velocity signals is recommended to quantify the shear stress generated by the procedure (◘ Fig. 17.2).

Importantly, FMD of the brachial artery has been shown to correlate with coronary artery endothelial dysfunction. Impaired FMD is predictive of cardiovascular events in patients with coronary artery disease and hypertension [7]. More recent studies have suggested that FMD may be predictive of cardiovascular events in healthy subjects and when used in conjunction with Framingham score could improve cardiovascular risk prediction [11]. As such, this technique is currently accepted as the gold standard for non-invasive assessment of peripheral vasoreactivity [12].

17.3.2.2 Digital Peripheral Arterial Tonometry

Digital peripheral arterial tonometry (PAT) is based on measuring pulse amplitude in the fingertip at baseline and following induction of reactive hyperaemia. This technique involves capturing beat-to beat plethysmographic recordings of the digital pulse amplitude with bilateral placement of pneumatic finger probes. As with FMD, a blood pressure cuff is placed on the upper arm, and after a period of baseline recording, the cuff is inflated

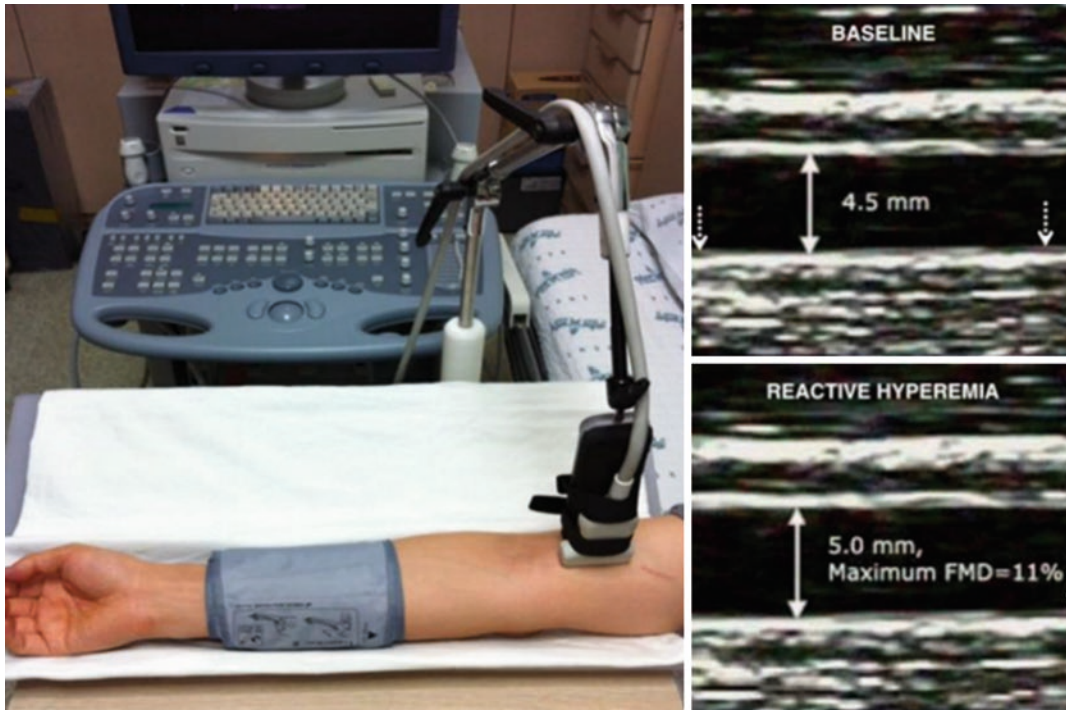

Fig. 17.2 Standard FMD examination with cuff inflated around distal forearm and linear ultrasound probe held over brachial artery in longitudinal view. (Images on right show appearance of vessel before and during reactive hyperaemia)

above systolic pressure. After a 5 min occlusion, the cuff is deflated to induce reactive hyperaemia on the occluded arm at which point signals are absent from the affected digit. An increase in arterial blood volume in the affected fingertip generates an increase in the measured signal. This system allows the contralateral finger not experiencing hyperaemia to be used as an internal control to adjust for systemic effects. The magnitude of flow-mediated hyperaemia corrected for readings in the uncuffed arm is termed the "reactive hyperaemia index" (RHI) (Fig. 17.3).

Assessment of digital microvascular function using PAT has been shown in small studies to be reproducible, and the automated analysis software means that interobserver variability is limited. Although PAT is more standardised and operator independent, it should be borne in mind that digital microvascular vasodilatory capacity is heavily reliant on sympathetic tone; thus findings from a controlled research environment may not be generalisable at population level. Studies have shown that lower RHI is associated with traditional cardiovascular risk factors such as obesity, diabetes, cholesterol and smoking [13] and correlates with coronary endothelial dysfunction. PAT has not however been shown to correlate consistently with FMD as the two techniques measure different aspects of vascular biology: FMD examines macrovascular arterial vasodilatory capacity, whilst PAT assesses microvascular function in a terminal vascular bed.

17.3.2.3 Venous Occlusion Plethysmography

In contrast to FMD which measures changes in arterial diameter, venous occlusion plethysmography measures changes in forearm blood flow usually combined with administration of vasoactive agents via the brachial artery [14]. The technique is based on the principle that whilst brief interruption of venous drainage is applied, arterial inflow remains unchanged; thus blood can enter the forearm but cannot leave it. The result is a linear increase in forearm volume which is proportional to arterial inflow. It is standard practice to exclude the hand from circulation during these measurements, as it contains a high number of arteriovenous shunts. In practice, this is done by rapid inflation of a wrist cuff well above normal systolic

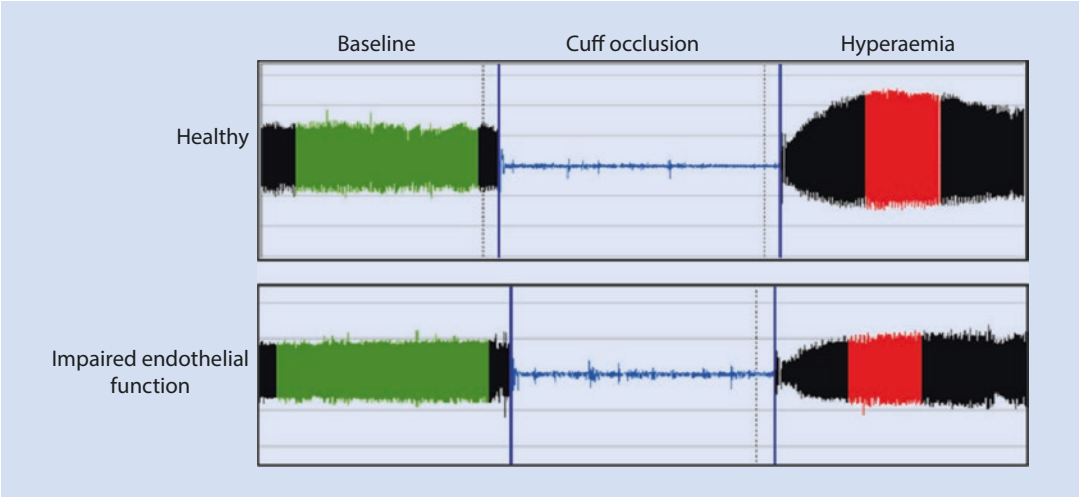

Fig. 17.3 Sample three-phase PAT recording comparing healthy control with impaired endothelial function. Phase 1 records baseline pulse amplitude bilaterally; phase 2 marks the period of unilateral cuff inflation where flow is occluded in test finger but continues unchanged in the control finger; and phase 3 is recorded following cuff release. In a healthy individual, pulse amplitude should rise rapidly following cuff release with minimal change in the control finger. In impaired endothelial function, pulse amplitude does not respond as briskly

blood pressure 60 s prior to taking any measurements. Since the hand is ischaemic, this limits the measurement period; however, intervals up to 13 min have been used without ill effects.

One of the most important uses of this technique is the ability to study local effects of vasoactive mediators in the forearm vascular bed administered intraarterially. In addition, the opposite arm can be used as an internal control.

Venous occlusion plethysmography has been shown to be predictive of cardiovascular events in patients with coronary artery disease and hypertension [7]. However, different initial arterial pressures, forearm size and blood flow between individual patients mean that although well suited to measuring differences in blood flow in a single patient, comparisons between patient groups or even serial studies in the same patient may have limited value.

17.3.2.4 Laser Doppler Flowmetry

The accessibility of the skin makes it another appropriate site for peripheral assessment of endothelial function. The microvascular function of the skin can be measured using laser Doppler flowmetry to determine response to a stimulus such as pharmacological agents, arterial occlusion or thermal alterations [15]. The process is based on reflection of a beam of laser light which undergoes change in wavelength termed "Doppler shift" upon hitting moving blood cells. The magnitude and frequency of such wavelength changes correspond to the number and velocity of blood cells. Red cell flux is the most commonly used signal and is the product of velocity and concentration of moving blood cells within the measured volume, expressed as arbitrary perfusion units (PU). The basic procedure involves placement of a probe on the skin surface which is connected to a delivery fibre from the source laser as well as a collection fibre for signal detection and processing [16].

17.3.2.5 Summary of Non-invasive Techniques

Although our ability to measure endothelial function in day-to-day clinical research has been greatly advanced by the minimally invasive methodological approaches described above, their use as tools in clinical practice has not yet been established, nor have they yet been endorsed by national or international guidelines for primary or secondary cardiovascular prevention.

17.4 Circulating Biomarkers of Endothelial Function

Biochemical measures are generally inexpensive and non-invasive and offer greater reproducibility, as well as insight into the mechanisms

Table 17.1 Summary of example biochemical measures of endothelial function

Biological process	Example biomarker(s)
NO availability	ADMA
	ROS
Cell adhesion	s-VCAM
	s-ICAM
	E-selectin
	vWF
Inflammation	CRP
	IL-6
	TNF-α
Coagulation	PAI-1

ADMA asymmetric dimethylarginine, *ROS* reactive oxygen species, *s-VCAM* soluble vascular cell adhesion molecule, *s-ICAM* soluble intercellular adhesion molecule, *vWF* von Willebrand factor, *CRP* c-reactive protein, *IL-6* interleukin-6, *TNF-α* tumour necrosis factor α, *PAI-1* plasminogen activator inhibitor-1

underlying endothelial pathology, as such recent years has seen heightened interest in their use as surrogate markers of endothelial dysfunction and therefore cardiovascular risk. A detailed review of the literature on circulating biomarkers with potential for assessment of endothelial dysfunction is out with the scope of this chapter; however a few of interest are discussed below and summarised in Table 17.1.

17.4.1 Markers of Inflammation

C-reactive protein (CRP) has emerged as one of the most important inflammatory markers of atherosclerosis and has been shown to be an independent predictor of cardiovascular morbidity and mortality [17]. Moreover, evidence suggests that CRP is also a mediator of atherosclerotic lesion formation with direct effects on cytokine production, expression of adhesion molecules and modulation of NO and endothelin-1 (ET-1) availability as well as facilitating endothelial cell apoptosis. Relationships between cardiovascular outcomes and other inflammatory markers such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) have also been shown [17], but CRP appears by far the most powerful at present.

17.4.2 Markers of Nitric Oxide Production and Availability

Endothelial cell NO production is a major determinant of endothelium-dependent vasodilation. As NO is a free radical with a short half-life, attention has focussed on markers of NO synthesis or degradation as alternative measures. Plasma levels of NO degradation products are not thought to accurately reflect endothelial NO production as these mediators are known to be influenced by external factors. Measurement of asymmetric dimethylarginine (ADMA) offers an alternative surrogate marker of NO production. As a potent competitive inhibitor of NO production from NOS plasma, ADMA levels correlate with endothelial NOS activity and with endothelial dysfunction [18]. $\bullet O_2-$ impairs vasorelaxation by reducing NO availability, and measurement of reactive ROS has also been considered a non-specific biomarker of endothelial dysfunction [19].

17.4.3 Cellular Adhesion Molecules

Cellular adhesion molecules regulate migration of leukocytes to the vessel wall and therefore play a regulatory role in the inflammatory process. Soluble forms of these adhesion molecules such as vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) and E-selectin have been shown to correlate with endothelial dysfunction, and their levels have been shown to increase in association with traditional vascular risk factors such as diabetes, hyperlipidaemia, smoking and hypertension. In addition, some clinical studies have associated ICAM with risk of future cardiovascular events and E-selectin with structural and functional measures of atherosclerotic disease [20].

17.4.4 Coagulation Factors

The procoagulant consequences of endothelial activation can be assessed by investigating the balance between tissue plasminogen activator and plasminogen activation inhibitor-1 (PAI-1) its endogenous

inhibitor. Increased PAI-1 has been shown to correlate with endothelial dysfunction. Similarly, levels of von Willebrand factor (vWF), a key mediator of platelet aggregation released in response to endothelial cell damage, have been shown to be increased in hypertension and vascular disease [20].

17.5 Cellular Biomarkers of Endothelial Function

To date, most endothelial biomarkers used in clinical research have been circulating or protein-based markers. Emerging evidence suggests that cellular markers such as microparticles or endothelial progenitor cells may prove to be useful tools for assessment of endothelial dysfunction.

17.5.1 Microparticles

Microparticles are formed by all cell types and typically measure up to 1.0 μm in diameter. These are nuclear cell membrane fragments containing cell surface proteins and cytoplasmic material which are shed from cells under stress. Flow cytometry of plasma samples is typically used to identify microparticles based on size and expression of cell surface antigens. Accumulating evidence links microparticles originating from endothelial cells, platelets and leukocytes with multiple disease states such as hypertension, diabetes and kidney disease, and some studies have shown that these markers correlate with functional endothelial assessments [21]; however their predictive potential has yet to be fully evaluated.

17.5.2 Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are detectable in plasma and marrow. These small immune precursor cells can be difficult to distinguish from circulating endothelial cells and are thought to reflect the endothelial repair process, rather than endothelial cell damage, i.e. a reduction in EPCs may indicate endothelial dysfunction. EPCs can be identified by cell surface markers (CD133, CD34 and VEGFR2 is accepted as the most reliable combination) using flow cytometry or by colony-forming assays in vitro. Again, the absolute number of EPCs has been shown to be reduced in conditions associated with endothelial dysfunction such as diabetes, ageing, hypertension and kidney disease and to correlate with clinical measures of endothelial function such as FMD. In addition, reduced plasma EPCs have been related to cardiovascular morbidity and mortality in patients with coronary artery disease [19].

Conclusion and Clinical Perspectives

- The endothelium plays a crucial role in regulating vascular tone not only by controlling release of vasoactive mediators but also through modulation of platelet activation and aggregation, leukocyte adhesion and thrombosis. In this way, the endothelium balances the counter-regulatory pathways controlling vasoconstriction, inflammation, oxidative stress, fibrosis and thrombosis – the key mechanisms underlying cardiovascular disease.
- Endothelial dysfunction has been implicated in a variety of cardiovascular diseases, including hypertension, atherosclerosis, coronary artery disease, Type 2 diabetes, obesity and metabolic syndrome, chronic kidney disease and pre-eclampsia.
- Endothelial dysfunction not only contributes to atherogenesis but has been shown to precede the development of morphological vascular changes and appears to be an independent predictor of cardiovascular events in patients with pre-existing vascular disease, as well as at population level. In this context, assessment of endothelial function is a key focus of cardiovascular research.

Gaps in Knowledge

- More research is required to understand the details of endothelial function and particularly the interplay between reactive oxygen species and nitric oxide availability.
- Standardised approaches to study endothelial function in basic and clinical research settings are required in order to facilitate comparison of data across research groups.
- The role of endothelial function as a risk stratification tool in "real-world" clinical practice is yet unclear.

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Vascular Remodeling

Carmine Savoia

R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_18

Key Concepts

- Vascular remodeling is defined as the structural modifications of large and small arteries causing alteration of lumen size and change in diameter as well.
- Activation of hormonal systems, including renin-angiotensin system, and vascular inflammation are key elements in the pathophysiology of vascular remodeling.
- Functional and structural alterations of resistance arteries are thought to be the earliest vascular alterations that may occur in hypertension, and these changes may contribute through wave reflection to stiffness of large arteries and have prognostic significance. In particular, increased media-to-lumen ratio in small resistance arteries strongly correlates with cardiovascular prognosis.
- A cross talk between the small and large artery may exaggerate arterial damage, following a vicious circle.

18.1 Introduction

Vascular remodeling is defined as the structural modifications of large and small arteries causing alteration of lumen size and change in diameter as well. This may begin as a functional and/or structural process induced by physical forces (change in endovascular pressure and shear) as well as by hormonal mediators. Remodeling of resistance arteries (that includes small arteries and arterioles – vascular diameter ranged between 100 and 300 μm) is characterized by a narrowing of the lumen, which may increase vascular resistance even at full dilation (in the absence of vascular tone) and increased wall thickness. Therefore an increased media-to-lumen ratio of small resistance arteries may occur and play an important role in the enhancement of vascular resistance. This may be an adaptive response to the increased hemodynamic load as well as a result of enhanced response to vasoconstrictor stimuli and impaired endothelial function associated with a proinflammatory and prothrombotic state. Increased resistance arteries, in turn, can play an important role in the development and maintenance of hypertension and contribute to the pathogenesis of cardiovascular complications.

The vascular phenotype of hypertension is age-dependent. In younger individuals with elevated blood pressure, vascular remodeling occurs mainly in small arteries and arterioles. As blood pressure remains elevated for a prolonged time, or in subjects older than 50 years of age, vascular changes occur predominantly in large conduit arteries, such as the aorta, which becomes stiffer as arteriosclerosis develops, resulting in increased pulsatility and therefore pulse pressure. In particular, chronically elevated blood pressure induces vascular stretch that initiates complex signal transduction cascades leading to vascular remodeling in large conduit arteries. Both the remodeling of small and large arteries contributes to the development and complications of hypertension.

The molecular events involved in the development of the remodeling processes in systemic hypertension are still not fully understood. Elevated blood pressure has an impact on the vasculature as a consequence of both the mechanical effects of blood pressure and shear stress. In addition, the action of hormonal systems influences remodeling such as the renin-angiotensin-aldosterone system, endothelins, catecholamines, agents generated in perivascular fat, inflammatory mediators, such as different cytokines and chemokines, as well as immune mediators, such as lymphocytes and macrophages and their products. In this chapter, we briefly review the fundamentals and the pathophysiology of arterial remodeling that may occur in hypertension.

18.2 Remodeling of Small and Large Conduit Arteries in Hypertension

18.2.1 Remodeling of Small Arteries

The pathophysiology of systemic hypertension is complex and multifactorial; nevertheless the hallmark of essential hypertension is an increase in peripheral vascular resistance. The diameter and the compliance of resistance vessels predominantly determine the peripheral resistance and therefore are key elements in the control of blood pressure as resistance arteries increase peripheral vascular resistance to blood flow, which occurs generally as a result of energy dissipation in small arteries and arterioles, particularly in younger individuals. Structural changes in the microcirculation may

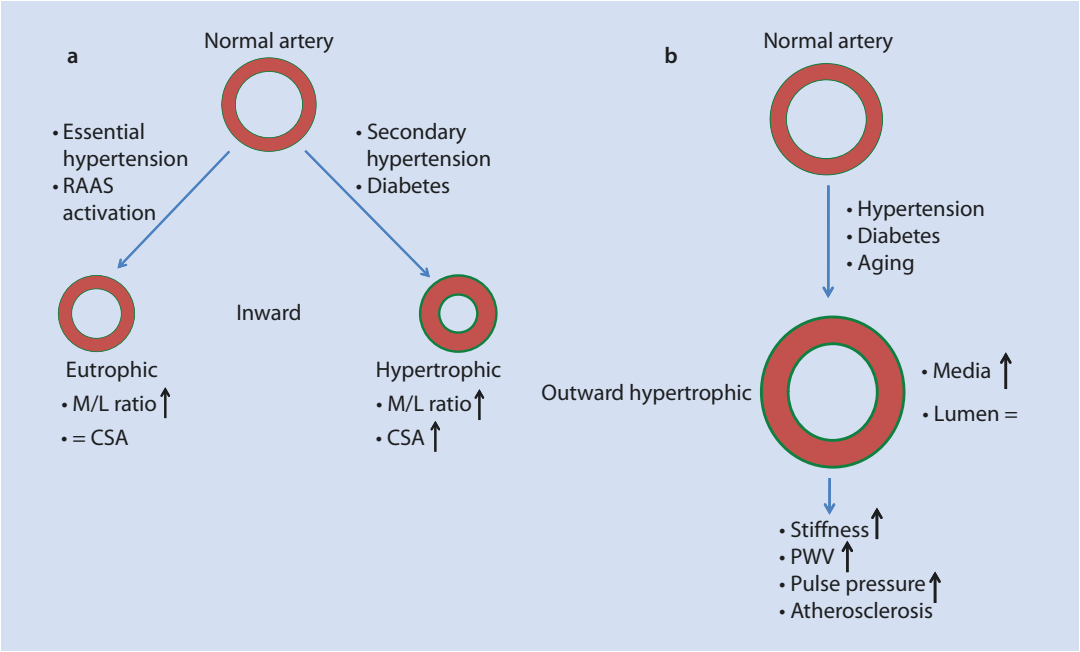


Fig. 18.1 Vascular remodeling in hypertension: **a** in resistance arteries; **b** in large conduit arteries. RAAS: renin-angiotensin-aldosterone system; M/L ratio: media-to-lumen ratio; CSA: cross-sectional area; PWV: pulse wave velocity

directly affect blood pressure values. In hypertension, resistance arteries undergo vascular remodeling (reduced vascular lumen with increased media thickness) that may be functional, mechanical, and structural. In small arteries smooth muscle cells and the extracellular matrix components are restructured (or remodeled) around a smaller lumen with a thickened arterial wall which also may play an important role in increasing vascular resistance (Fig. 18.1a). Based on Poiseuille's law, resistance is inversely proportional to the radius to the fourth power; therefore, slight alterations in the lumen of resistance arteries result in significant effects on vascular resistance. Thus, a thickened arterial wall together with a reduced lumen (with increased media-to-lumen ratio) would play an important role in the increase of vascular resistance. This is considered an adaptive response to the increased hemodynamic load, yet the mechanisms of vascular remodeling are not fully understood. It has been suggested that on an exposure to acute elevations in arterial pressure, smaller arteries and arterioles undergo constriction through the myogenic reflex (the intrinsic reflex that occurs independent of neural innervation or endothelial function in response to an increase in intraluminal pressure). Among the mechanisms involved in the control of myogenic tone are changes in intracellular calcium, protein kinases, diacylglycerol, modulation of transient receptor potential-like channels, and ion transport. The activation of the myogenic reflex could lead to decrease of lumen size and increase of wall thickness in order to normalize the increased wall stress, according to Laplace law. In hypertension, the reflex constriction in resistance arteries becomes persistent and is eventually associated with endothelial dysfunction and mild inflammation in the media of the arteries. Hence, with chronic vasoconstriction, vessels become embedded in a remodeled extracellular matrix, and this can prevent vasodilation. Thus the alterations become permanent, and structural changes occur (i.e., vascular remodeling). These processes result in a greater media thickness in the resistance arteries with a reduced lumen and increased media-to-lumen ratio in patients with essential hypertension.

18.2.1.1 Types of Vascular Remodeling

Different types of vascular remodeling have been described. The process of change in lumen without change in amount (wall cross-sectional area) or characteristics of materials is called eutrophic

remodeling. The process may involve increase (hypertrophic remodeling) or decrease (hypotrophic remodeling) in wall cross-sectional area. Increase or decrease in lumen diameter is classified as external and internal remodeling, respectively. It has been shown that in hypertension at least two types of remodeling may occur, depending on whether the media cross-sectional area is enlarged which is expression of true hypertrophy, respectively, inward eutrophic and inward hypertrophic remodeling. In essential or primary hypertension, eutrophic remodeling is usually found in humans and in experimental models such as spontaneously hypertensive rats (SHRs, where the renin-angiotensin system is even mildly activated); in this type of vascular remodeling rearrangement of the same amount of wall material around a smaller vessel lumen is typically found, without cell growth (Fig. 18.1a). The mechanisms leading to inward eutrophic remodeling are poorly understood but could result from inward growth combined with peripheral apoptosis or from vasoconstriction embedded in an expanded extracellular matrix. On the other hand, in secondary hypertension such as in renovascular hypertension, primary aldosteronism, and salt-dependent form of hypertension or in pheochromocytoma as well as in hypertension associated with diabetes mellitus and in acromegaly, hypertrophic remodeling has been described (Fig. 18.1a). This type of vascular remodeling is characterized by a more evident contribution of cell growth including vascular smooth muscle cell hypertrophy (volume increase) or hyperplasia (cell number increase).

It has been postulated that an increase in the media-to-lumen ratio of small resistance arteries and the structural narrowing of the lumen amplifies vasoconstriction in response of any hypertensive stimulus. This can occur as a consequence of increased concentration of specific agents at the level of receptors, greater receptor density, or post-receptor signaling alterations associated with enhanced angiotensin II signaling leading to increased reactive oxygen species (ROS) generation and vessel growth and enhanced constriction (i.e., hypothesis of the "vascular amplifier"). Thus the effect of some vasoconstrictor agents could be more pronounced in the presence of an increased media-to-lumen ratio both in animal models of hypertension and in humans. Nevertheless, this hypothesis has been recently challenged.

It is not clear whether an increase in blood pressure values precedes or follows the onset of these microvascular alterations. However it has been shown that small artery remodeling may be the first manifestation of target organ damage in hypertension, since an increase in the media-to-lumen ratio in small resistance arteries might be present very early, but its severity parallels the increase in blood pressure values.

18.2.1.2 Remodeling of Large Conduit Arteries

Hypertension is an important factor in accelerated aging of the vasculature (i.e., early vascular aging), resulting in premature cardiovascular disease. Arterial hypertension as well as aging and other cardiovascular risk factors (including diabetes) may induce remodeling and increase arterial stiffness in large conduit arteries. Remodeling allows arteries to withstand the increased pressure load, but as a result the vessels become more rigid than in their native state, and the reduced compliance decreases their ability to dampen the cyclical changes in blood pressure, resulting in increased pulse pressure (i.e., increased pulsatility of pulse wave). Large arteries such as the aorta cannot autoregulate with constriction in case of raised arterial pressure and hence undergo hypertrophy in order to normalize the increased wall stress (outward hypertrophic remodeling) while maintaining the lumen size. The degree of these adaptive responses depends on associated cardiovascular risk factors, progression of atherosclerosis, and inflammatory accumulation of lipids in plaques in the intima, triggered in part by endothelial dysfunction (which will be discussed elsewhere in the book). In this context, extracellular matrix (ECM) remodeling is thought to be an important step in the pathogenesis of several vascular diseases associated with hypertension. In hypertension, the vascular smooth muscle cells of large elastic arteries are exposed to stretch from the elevated arterial pressure. In the steady state, the cyclic stretching of artery walls sustains a quiescent, contractile VSMC phenotype and slow turnover of ECM proteins. However, when physical or chemical conditions change, arteries react to the new environment by remodeling of vascular wall, whereby the structure and function of the vessel wall are modified to accommodate the new settings. Remodeling involves the activation of a wide range of intracellular signaling pathways

leading to the modulation of vascular cell migration, proliferation, and death as well as synthesis and degradation of the ECM. In the context of hypertension, for example, the arterial wall is chronically subjected to an exaggerated tensile strain. To resist this force, small arteries and arterioles tend to respond by VSMC hyperplasia, whereas large arteries display VSMC hypertrophy and ECM reorganization.

18.2.1.3 Interaction Between Small and Conduit Arteries

A cross talk (i.e., functional interaction) between the small and large arteries may exaggerate arterial damage. Microvascular structure is not only the site of vascular resistance but possibly the origin of most of the wave reflections generating the increased central systolic blood pressure particularly in the elderly, although the proper location of a reflection site may be elusive. As a consequence, increased wall-to-lumen ratio of small arteries is one of the major factors for an increase in mean blood pressure which, in turn, may increase large artery stiffness through the loading of stiff components of the arterial wall at high blood pressure levels resulting in increased pulse wave pulsatility. It has been postulated that in younger people with a compliant large artery wall, pulse pressure increases progressively from central to peripheral arteries. This is due to the merging of the incident waves (pulse waves traveling toward the periphery of the arterial tree) and the reflected waves (pulse wave traveling back from the periphery). This occurs centrally during the diastolic time, after the dicrotic notch in the aortic pulse waveform (◘ Fig. 18.2a). Reflected waves originate at different sites possibly where vessels branch out and particularly at the level of resistance arteries (a site of increased vascular impedance). As the conduit arteries become stiffer (for aging, hypertension, or other risk factors), these reflected waves return at faster velocity and add pressure to the incident pulse wave merging ahead of the dicrotic notch in the aortic pulse waveform, resulting in increased systolic pressure. The effect is more pronounced proximally in the aorta where more reflected waves arrive than in distal arteries (◘ Fig. 18.2b). Stiffer vessels result in acceleration of both forward and reflected waves, which arrive earlier in the cardiac cycle and amplify the aortic central systolic pressure. The increase in pressure due to the arrival of the reflected wave determines the augmentation index (Aix) which contributes to increased aortic and peripheral systolic blood pressure and pulse pressure and results in absence of amplification toward the periphery. It also contributes to further increased stiffness of central elastic arteries. On the other hand, the increased pulse pressure in stiffer conduit arteries, in turn, may be transmitted to small arteries and may contribute to vascular injury of small arteries in different organs (heart, brain, retina, kidney) and, in general, favors the development of target organ damage.

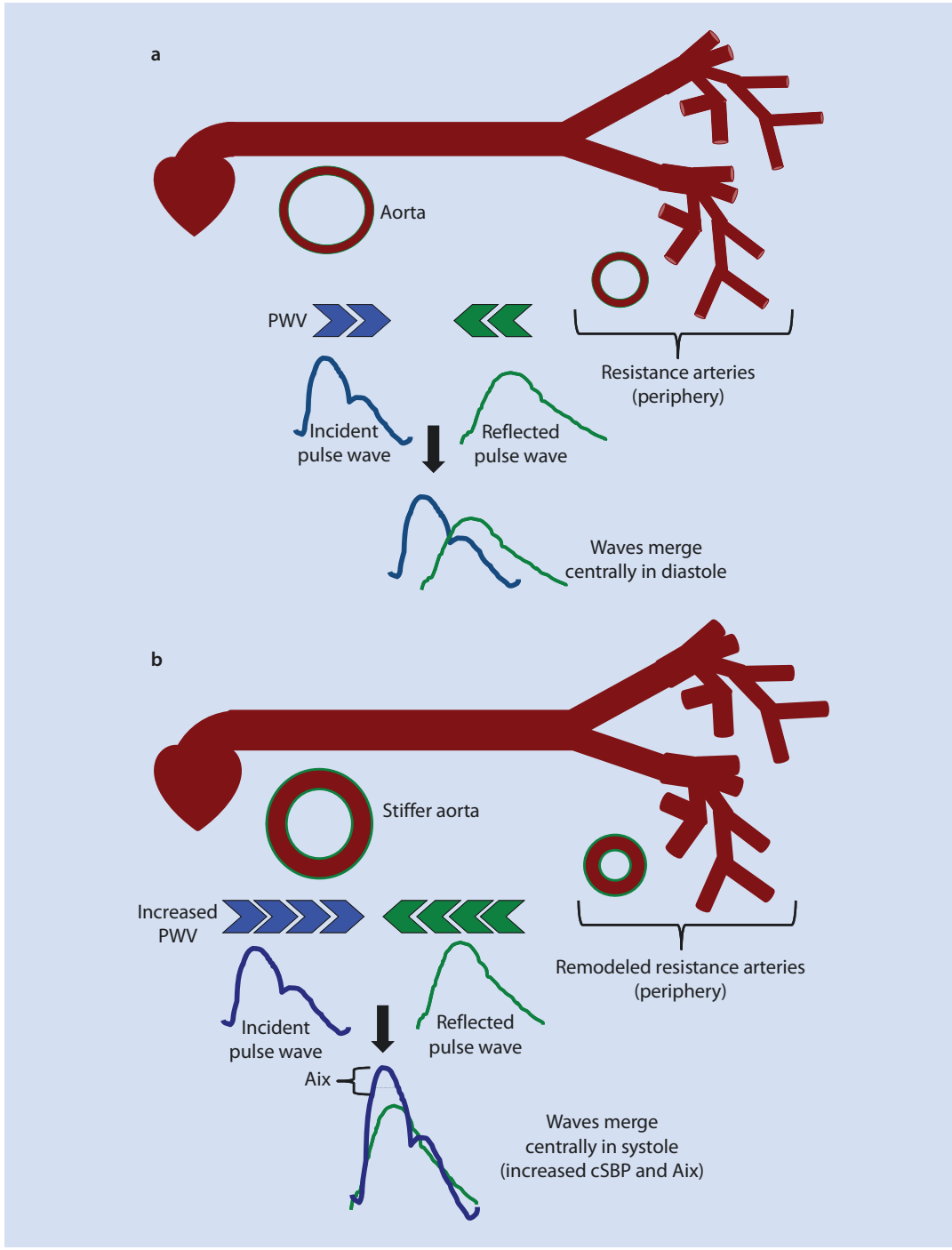
For clinical purpose vascular stiffness in the aorta can be evaluated by carotid-femoral pulse wave velocity (PWV) as suggested by the European Guidelines of Hypertension. Indeed, as previously explained, a stiffer artery conducts faster the pulse wave to the periphery. Carotid-femoral PWV is probably the most accurate measure currently available of aortic stiffness which is significantly and independently associated with both target organ damage and increased risk for cardiovascular morbidity and mortality.

18.3 Mechanism of Vascular Remodeling

The molecular mechanisms underlying the remodeling of resistance arteries and, in particular, the mechanisms leading to eutrophic remodeling as well as the age and disease-related arterial stiffness are presently not fully understood. Because a smaller lumen and a thicker wall decrease circumferential tension and media stress (according to Laplace's law), eutrophic remodeling may represent a protective mechanism for the vessel wall against the elevation of blood pressure values. Recent experimental data suggest that chronic vasoconstriction may contribute to eutrophic remodeling. Activation of neurohormonal systems, including renin-angiotensin-aldosterone system (RAAS, as will be extensively discussed in another chapter) and endothelin system, intracellular signaling mainly related to increased ROS production, the activation of inflammatory and growth pathways, as well as the modification of extracellular matrix components may all be involved in these processes. In hypertension, hyperplasia and hypertrophy of vascular smooth muscle cells (VSMCs) contribute, to varying degrees, to vascular remodeling with associated

apoptosis, cell elongation, reorganization, altered production of extracellular matrix proteins, and inflammation.

Several components of the RAAS play a key role in the pathophysiology of hypertension and CVD. Angiotensin II stimulates cell growth by inducing hyperplasia and hypertrophy of VSMCs from resistance arteries of patients with essential hypertension and small arteries from hypertensive rats. Angiotensin II and aldosterone, as well as endothelin-1 (ET-1), modulate basal superoxide production by activation of



reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and expression of its subunits via cSrc, PKC (protein kinase C), PLA2 (phospholipase A2), and PLD (phospholipase D) pathways (which will be further discussed in other chapters of the book).

There is increasing evidence that stimulation of ROS generation and mediators of inflammation may contribute to the remodeling process, activating cell signaling pathways, such as mitogen-activated protein kinases, which act on nuclear and cytoplasmic targets inducing cell growth. Both inflammatory processes and the pro-fibrotic properties of several hormones may be involved in the increased collagen deposition associated with a reduction in elastin content within the small vessels' wall. These alterations are frequently observed both in diabetic patients and in patients with primary aldosteronism. The adaptations in the extracellular matrix component may be involved in the development of changes in the mechanical properties of the microvessels and increased stiffness.

Low-grade inflammation localized in vascular and perivascular tissue, including fat, is recognized as an important contributor to the development of vascular remodeling in small and large conduit arteries as well as to the pathophysiology of hypertension. In particular, inflammation of large arteries exerts its effects in part by contributing to endothelial dysfunction and increasing vascular stiffness. Inflammation contributes to vascular remodeling promoting cell growth and proliferation of VSMCs. Inflammation is characterized by the increased expression in the vascular wall of adhesion molecules and ligands, leukocyte extravasation, increased oxidative stress, cytokine production, activation of immune cells, and pro-inflammatory signaling pathways.

Endothelial dysfunction in turn may also promote vascular inflammation by inducing the production of vasoconstrictor agents, adhesion molecules, and growth factors. Blood pressure itself or activation of the RAAS may induce an inflammatory process, which participates in vascular remodeling and may contribute to accelerated vascular damage in aging and cardiovascular disease (CVD).

Arterial stiffness is associated with vascular fibrosis, which involves accumulation of extracellular matrix proteins, such as collagen, elastin, fibrillin, fibronectin, and proteoglycans in the vascular wall. Deposition of collagen and other components of the extracellular matrix contribute to media thickening in hypertrophic remodeling of resistance arteries and to reorganization of the vessel wall components in eutrophic remodeling. Cell growth end extracellular matrix deposition may result from blood pressure elevation or from growth-promoting factors including angiotensin II, aldosterone, ET-1, and catecholamines. Increased extracellular matrix may also result from diminished activity of matrix metalloproteinases (MMPs), which play a central role in the homeostasis of the extracellular matrix in the vascular wall, resulting in accumulation of different types of collagen.

A role of innate immunity in mechanisms that contribute to the low-grade inflammatory response in hypertension has also been described. Recent evidence suggests that different subsets of T lymphocytes may be involved in the mechanisms leading to the inflammatory response described in cardiac and metabolic diseases when an imbalance exists between the proinflammatory Th1, Th2, and Th17 and the anti-inflammatory T regulatory (Treg) subsets. In particular, it has been shown that Treg-adoptive transfer lowered blood pressure and protected from vascular remodeling mice infused with either angiotensin II or aldosterone. One of the mechanisms whereby T lymphocytes participate in hypertension and

Fig. 18.2 Interaction between conduit and resistance arteries: **a** in normotensive condition in young people; **b** in hypertension, diabetes, and aging. **a** In young normotensive individuals, incident waves (pulse waves traveling toward the periphery of the arterial tree) merge with the reflected waves (pulse wave traveling back from the periphery) centrally during the diastolic time, after the dicrotic notch in the aortic pulse waveform. **b** As the conduit arteries become stiffer (for aging, hypertension, or other risk factors), the reflected waves return at faster velocity and add pressure to the incident pulse wave merging ahead of the dicrotic notch in the aortic pulse waveform, resulting in increased systolic pressure. The effect is more pronounced proximally in the aorta where more reflected waves arrive than in distal arteries. Thus, stiffer vessels result in acceleration of both forward and reflected waves, which arrive earlier in the cardiac cycle and amplifying the aortic central systolic pressure. PWV: pulse wave velocity; Aix: augmentation index; cSBP: central systolic blood pressure

peripheral inflammation is in response to increased oxidative stress. The central and pressor effects of angiotensin II are also critical for T-cell activation and development of vascular inflammation.

Interestingly, circulating endothelial progenitor cells (EPCs) appears to be an important determinant of endothelial function. Circulating EPCs are significantly reduced in hypertensive Type 2 diabetic patients and in salt-loaded hypertensive rats. Furthermore, decreased EPC numbers are associated with arterial stiffness and decreased endothelial function.

Conclusions and Clinical Perspectives

Complications of hypertension include changes in the structure and function of large and small arteries, as well as accelerating the progression of atherosclerosis. In particular, the vascular disease of hypertension, by promoting tissue underperfusion and progression of atherosclerosis, contributes to myocardial ischemia and cardiovascular events, heart failure, stroke, nephrosclerosis and chronic kidney disease, and peripheral vascular disease.

Microvascular structural alterations and changes in the mechanical properties of the large conduit arteries represent potent predictors of prognosis. Moreover, clinical observation has shown that some indexes of large artery stiffness are related with the media-to-lumen ratio of subcutaneous small resistance arteries in hypertensive patients. Small artery remodeling has prognostic significance because hypertensive patients with the highest media-to-lumen ratio particularly if associated with net growth (increased CSA) had increased incidence of cardiovascular events. Several studies have suggested that structural changes in the microcirculation (increased media-to-lumen ratio of subcutaneous small arteries) and alterations on mechanical properties of large arteries are two most important factors in predicting cardiovascular outcome. Moreover the media-to-lumen ratio was significantly related to both brachial systolic pressure and pulse pressure and to central systolic and pulse pressure. A positive correlation was observed between media-to-lumen ratio and carotid-femoral PWV as well as to aortic augmentation index; these correlations remained statistically significant after adjustment for age and mean blood pressure.

The hemodynamic markers of arterial stiffness pulse pressure and PWV have been associated with stroke, dementia, and lowered levels of cognitive function. Furthermore, it seems that a close relationship has been established between brain and kidney microvascular damage and indices of age and large artery stiffness (pulse pressure, PWV, and augmentation index).

Hypertension-related damage to the micro- and macrovascular system may be corrected by pharmacological agents. Among them, beta-blocking agents and diuretics have a minor effect on microvascular structure, while RAAS antagonists and calcium entry blockers have favorable actions, improving large artery mechanics and possibly reducing central wave reflections. Some intervention studies have demonstrated an improvement or even an almost complete normalization of the structure of subcutaneous small resistance arteries with angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, and angiotensin II receptor blockers (ARBS). Conversely, the beta-blocker atenolol and the diuretic hydrochlorothiazide were devoid of effects on resistance vessels and on brachial pulse pressure, despite a blood pressure reduction similar to that observed with ACE inhibitors.

Gaps in Knowledge

- Arterial stiffness also increases with cardiovascular risk factors, including hypertension, the metabolic syndrome, diabetes, obesity, and hypercholesterolemia. It is not, however, a parameter that can be easily measured in the clinic to stratify risk. In particular, the study of remodeling of resistance arteries is performed by invasive techniques such as a direct investigation of changes in small resistance arteries obtained from subcutaneous and omental fat tissue of essential hypertensive patients by using the wire or pressure micromyographic methods. Alternatively the study of arteries from the retina could be also performed by scanning laser Doppler flowmetry, although this technique is not extensively used, and further validation and standardization of the technique is required.

- The mechanisms of vascular remodeling particularly at molecular level are not fully understood; further studies are required to better understand the development of vascular remodeling in several pathological conditions in order to tailor specific therapies.
- Although it has been well established that increased media-to-lumen ratio in resistance arteries correlates with cardiovascular prognosis, it is not well defined whether its reduction by therapeutic intervention may correlate with improvement of cardiovascular prognosis.

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Arterial Stiffness

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_19

Key Concepts

- The large arteries buffer the cyclical changes in blood pressure during ventricular ejection to keep systolic pressure low and maintain the diastolic (perfusion) pressure.
- Arterial stiffness increases with age and blood pressure and is a key, independent predictor of cardiovascular disease events.
- Arterial stiffening promotes cardiovascular disease processes by a number of mechanisms, including causing increased load on the left ventricle, transmission of damaging pulsatile forces to target organs and a vicious cycle of elastin degeneration leading to even higher pulsatile forces.
- Arterial stiffness can now be assessed using simple-to-use, non-invasive devices, suitable for use in the routine clinical setting.

19.1 Introduction

The concept of 'hardening of the arteries' is not new. Indeed, the arterial pulse has attracted interest for thousands of years as a diagnostic tool, and it was recognized very early that features of the pulse such as its shape and 'feel' could provide information about the elasticity of the arteries. Although the nineteenth century hailed a so-called golden era of assessing and interpreting the shape of the arterial waveform, this was diminished by the introduction of the sphygmomanometer, which allowed more precise quantification of the systolic and diastolic blood pressure, which greatly improved risk prediction. However, with computerization and rapid advances in technology, we now have available a wide array of techniques and devices to assess arterial stiffness. This has driven intensive investigations of arterial stiffening and its underlying determinants over the last 30–40 years, such that arterial stiffness is now recognized as an important independent determinant of cardiovascular risk. Nevertheless, much is still to be understood regarding the processes underlying arterial stiffening and its place in routine clinical assessments.

19.2 Physiological Role of Large Arteries

In order to understand the pathophysiology of arterial stiffening, it is important to examine the physiological role of the large arteries in healthy individuals. Blood is ejected intermittently from the left ventricle into the aorta causing cyclic changes in the arterial blood pressure (measured clinically as the systolic and diastolic blood pressure). In healthy young individuals, the large arteries are highly elastic and (i) expand to accommodate the stroke volume during systole and then (ii) recoil due to stored energy, during diastole. This continuous cycle of expansion and recoil has often been likened to a windkessel – the German word meaning 'air chamber', used in fire carts in the 1700s to convert the pulsatile flow of water from a hand-operated water pump into a continuous flow of water from the nozzle of the fire hose. The arterial 'windkessel' keeps the systolic pressure low during the expansion phase yet maintains the diastolic pressure as a result of the recoil phase. Maintenance of the diastolic pressure is important to allow adequate perfusion of the coronary circulation, which occurs during diastole, and effective distribution of blood towards the tissues. Thus, the pulsatile stroke volume is transformed into a 'smoothed' and continuous flow of blood to the microcirculation and peripheral tissues, maximizing the chances of efficient tissue perfusion.

Consideration of the structural properties of the large arteries is key to understanding their ability to expand and recoil, in response to ventricular ejection. As discussed in previous chapters, the vascular wall contains an integrated assembly of vascular smooth muscle cells and extracellular matrix, and both of these components contribute to the mechanical properties of the large arteries. The main load-bearing elements of the arterial wall are the elastin and collagen fibres contained within the extracellular matrix. Elastin fibres are much more elastic or distensible, while collagen fibres are inherently stiffer. Due to the arrangement of these fibres in the vessel wall, the elastin fibres engage, i.e. take on the primary load-bearing function, at lower distending pressures, allowing the artery to expand in order to take up the stroke volume. In contrast, the stiffer collagen fibres become increasingly engaged in load-bearing with increasing distending pressures

[1]. This pattern of engagement helps to prevent overexpansion of the artery during systole and drives the recoil force during diastole in healthy large arteries. Therefore, the arterial wall becomes stiffer with increasing distension and in a non-linear manner. Moreover, the relationship between elastin and collagen explains why the vessel distending pressure (measured clinically as the mean arterial pressure) is perhaps *the* most important physiological determinant of vessel stiffness [2]. The arterial wall also contains a 'ground substance', which is rich in glycosaminoglycans or GAGs. The water- and ion-binding potential of GAGs may also affect arterial wall mechanics either directly or indirectly [3], although the actual significance of GAGs on large artery stiffness is unknown.

19.3 Mechanisms of Arterial Stiffening

The mechanisms underlying arterial stiffness are not well established and remain an active area of research. Nevertheless, arterial stiffening is generally associated with changes in the physical and mechanical properties of the arterial wall. Due to the pulsatile nature of the circulatory system, the large arteries are subjected to continuous, cyclical strain throughout life. Indeed, it is accepted that in almost all societies worldwide, there is progressive arterial stiffening with age. This appears to be a degenerative process termed *arteriosclerosis*, affecting the walls of large arteries. An important point is that arteriosclerosis should not be confused with *atherosclerosis*, which is characterized by inflammation and plaque formation on the intimal surface of the arteries, although it is highly likely that the two processes occur in tandem. Elastin has a half-life of approximately 40 years, making it one of the most stable proteins in the body. But despite this stability, fatigue of elastin fibres can occur, especially considering that, by the age of 60 years, for example, the proximal aorta will have experienced more than two billion expansions during ventricular contraction. This long-standing cyclic stress in elastin-containing arteries produces structural disorganization, fatigue and eventual fracturing of elastin fibres – a situation made worse if the vessel distending pressure is high. Given the strong effect of ageing on elastin fatigue and arterial stiffening, it is tempting to speculate that arterial stiffening is simply inevitable. However, stiffness appears to increase much less rapidly in truly rural or indigenous populations [4] suggesting that a major part of age-related arterial stiffening seen in 'westernized' societies is actually *pathological*. Other structural changes in the extracellular matrix are also thought to occur, including proliferation of stiffer collagen fibres and deposition of calcium. Accumulation of advanced glycation end products, which alter the physical properties of elastin and collagen fibres, and accumulate over time and with increased plasma glucose levels [5] are also recognized as important determinants of arterial stiffness. In addition, changes in smooth muscle tone are able to actively modulate the stiffness of the arterial wall, even in large arteries such as the aorta [6]. All of these processes have the effect of changing the stress-strain characteristics of the arterial wall, resulting in a stiffer wall for a given distending pressure and an overall loss of the important buffering of cyclical changes in blood pressure during the cardiac cycle.

19.4 Clinical Consequences of Arterial Stiffening

Cardiovascular risk factors such as hypertension, hypercholesterolaemia, smoking and diabetes are all associated with increased arterial stiffness, leading to the notion that arterial stiffness is an important biomarker of cardiovascular disease. However, it is becoming increasingly accepted that arterial stiffening itself drives various disease processes and is therefore an important risk factor in itself. Indeed, the most compelling evidence of the importance of arterial stiffness comes from a recent meta-analysis of outcome studies, conducted in 17,635 individuals [7]. Using the individual participant data from 17 different study cohorts, the meta-analysis demonstrated that aortic pulse wave velocity (PWV), a robust measure of arterial stiffness, predicts future fatal and non-fatal coronary and stroke events, with a hazard ratio of ~1.30 after adjustment for established cardiovascular risk factors including age, systolic blood pressure, smoking, diabetes and cholesterol. To put this into context, using the Emerging Risk Factors Collaboration study [8] comprising over 165,000 individuals for comparison, the predictive value of PWV was similar to the predictive

value of systolic BP for cardiovascular disease but appeared to be better than the predictive value of total cholesterol (HR of ~1.2). However, both smoking (HR~1.8) and diabetes (HR~2.0) had greater predictive value for cardiovascular disease. Interestingly, there was a stronger association with outcomes in younger individuals in the PWV meta-analysis, and it is tempting to speculate that the greatest clinical value of arterial stiffness measurements might be in *younger* individuals (i.e. those individuals at intermediate rather than high risk) in whom knowledge of arterial stiffness might add value to risk prediction algorithms.

The factors linking increased arterial stiffness with increased risk of cardiovascular disease fall into three broad categories, as follows:

1. *Widening of pulse pressure leading to isolated systolic hypertension and increased ventricular afterload*

   The progressive stiffening of the large arteries with ageing results in a widening of the pulse pressure. This is because the normal arterial expansion seen during systole in healthy young arteries becomes limited over time, leading to a rise in systolic pressure for a given stroke volume. In addition, the recoil forces needed for maintenance of normal diastolic pressure are diminished, leading to a *lowering* of the diastolic pressure. Ultimately, this process manifests itself as a condition called isolated systolic hypertension (ISH), which is now the most common form of hypertension in the UK and USA and carries a threefold increase in the risk of stroke and a doubling in the risk of heart disease [9]. Elevated systolic pressure also increases left ventricular afterload, promoting left ventricular hypertrophy, ventricular stiffening and increased myocardial oxygen demand. The reduction in diastolic pressure exacerbates this situation by reducing coronary blood flow, predisposing to ischaemia. Ultimately, these changes create an unfavourable mismatch between myocardial oxygen demand and supply, the consequences of which are likely to be diastolic dysfunction and heart failure. High systolic pressure also increases circumferential arterial wall stresses, causing further or accelerated fatigue fracture of elastin fibres. This results in further arterial stiffening, further loss of the arterial buffering capacity and further increases in systolic pressure, resulting in a vicious cycle.
2. *Increased speed of reflected pressure waves*

   The arterial pressure waveform is composed of a forward travelling wave, generated by left ventricular ejection, and a backward travelling reflected wave arising from sites of impedance mismatch – i.e. arterial taper and differences in vessel stiffness [10]. Arterial stiffening increases the speed of both the forward and backward travelling waves, resulting in a faster return of reflected waves and earlier summation with the incident (forward travelling) wave. This augments the aortic systolic pressure, leading to further increases in left ventricular afterload.
3. *Reduced dampening of pulsatile forces and increased transmission of pulsatility to low-resistance organs*

   The reduced buffering of pulsatile forces within a stiffened aorta is transmitted to other arteries such as the carotid, which undergo a process of remodelling to reduce wall stress, leading to intima-media thickening. Moreover, there is now robust evidence that stiffened arteries permit increased transmission of these pulsatile forces towards the microvasculature, which damages capillaries in high-flow, low-resistance organs such as the brain and kidneys [11].

19.5 Assessing Arterial Stiffness

Arterial stiffness can be assessed using a variety of techniques and approaches, as outlined in Table 19.1. These broadly fall into *direct* measures of stiffness, taken at one discrete location, or more *global* measures, which indicate regional or systemic arterial stiffness. Each measure has its own advantages and disadvantages. It is also important to realize that each measure yields subtly different information concerning vessel structure and function. Therefore, different measures of arterial stiffness are not necessarily interchangeable. Moreover, and as mentioned earlier, the elastic properties of the arterial wall are highly dependent on the vessel distending pressure, and this should always be taken into account when measuring arterial stiffness or interpreting arterial stiffness data.

Table 19.1 Methods of assessing arterial stiffness

Term	Definition	Formula	Common measurement methods
Direct measures of arterial stiffness			
Young's modulus	Elastic modulus per unit area; the pressure step per square cm required for theoretical 100% stretch from resting length	$(\Delta P \times D)/(\Delta D \times h)$ mmHg/cm	Tensile testing (ex vivo) Ultrasound, MRI (in vivo)
Arterial compliance	Absolute diameter (or area) change for a given pressure step	$\Delta D/\Delta P$ cm/mmHg^{-1} or cm^2/mmHg^{-1}	Tensile testing (ex vivo) Ultrasound, MRI (in vivo)
Arterial distensibility	Relative change in diameter (or area) for a given pressure change; inverse of elastic modulus	$\Delta D/(\Delta P \times D)$ mmHg^{-1}	Tensile testing (ex vivo) Ultrasound, MRI (in vivo)
Stiffness index	Ratio of logarithm (systolic/diastolic pressures) to relative change in diameter	$\mathrm{Ln}(P_s/P_d)/[(D_s - D_d)/D_d]$ Non-dimensional	Ultrasound, MRI
Characteristic impedance	Relationship between pressure change and flow velocity in the absence of wave reflections		Ultrasound, MRI
Global measures of arterial stiffness			
Pulse wave velocity	Speed of travel of the pulse along an arterial segment	$\Delta t/D$ m/sec or cm/sec	Pressure sensing catheter (invasive) MRI Ultrasound Tonometric pressure sensors Volumetric cuffs
Ambulatory arterial stiffness index	One minus the regression slope of the linear regression line between systolic and diastolic blood pressure recordings over a 24 hour period	1 – slope	Standard oscillometric cuff
Cardio-ankle vascular index	The natural logarithm of systolic-diastolic pressure ratio against the arterial wall extensibility	$a[(2\rho/\Delta P)\cdot\ln(P_s/P_d)\cdot \mathrm{PWV}^2] + b$	Oscillometric cuffs
Indices derived from waveform analysis			
Systemic arterial compliance	Relationship between change in flow for a change in systemic arterial pressure	$\mathrm{Ad}/[R \times (P_s - P_d)]$	Tonometry and Doppler velocimetry
Augmentation index	The difference between the second and first systolic pressure peaks expressed as a percentage of the pulse pressure	$((\mathrm{P2}-\mathrm{P1})/\mathrm{PP}) \times 100\%$	Ultrasound Tonometric pressure sensors Volumetric cuffs

19.5.1 Direct Measures of Arterial Stiffness

Stiffness can be defined as the resistance by an elastic body (i.e. the arterial wall) to deformation when force is applied, and all measures of arterial stiffness directly, or indirectly, deal with the relationship between forces applied to the arteries (stress), which result in mechanical strain.

Young's elastic modulus The ratio of stress/strain or, more precisely, the slope of the stress-strain

relationship from the unstressed state is given by Young's elastic modulus (E). In the context of arteries, E describes how much an artery will deform, from an unstressed state, in response to the pressure exerted by the blood on the arterial wall. Stiff arteries will resist deformation; therefore they are described as having a high E. However, the pressure-deformation (or stress-strain) relationships of blood vessels are strongly non-linear, so it is impossible to assign a single value of E to an artery. Moreover, it is impossible to determine stress-strain characteristics of arteries, from the unstressed state, in vivo. Instead, it has become common to use the incremental elastic modulus (E_{inc}). E_{inc} represents the slope of a vessel's stress-strain curve at a specific pressure, such as 100 mmHg. In this way, the mechanical stiffness of the vessel at physiological pressures can be analysed.

A number of other indices of local arterial stiffness can be used, which relate changes in pressure within the artery to changes in volume. In practice, changes in arterial volume are difficult to assess in vivo, and so changes in vessel diameter or cross-sectional area are used instead. An important point is that while these indices are influenced by the wall stiffness, they are also influenced by other factors such as wall thickness and arterial size (diameter).

Arterial compliance and distensibility Arterial compliance relates changes in volume to changes in pressure. Arterial distensibility is similar to compliance but normalizes for arterial size. Importantly, both compliance and distensibility are not only influenced by wall stiffness but also by arterial diameter and wall thickness, and these parameters need to be measured.

ß stiffness index This measure overcomes the dependence on distending pressure of other direct measures of stiffness, because it is relatively independent of transient blood pressure changes. However, it is also influenced by wall thickness.

Characteristic impedance (Zc) This is an important haemodynamic index, which relates directly to the stiffness of the arterial wall. Rather than being derived from stress (or pressure)-strain relationships, Zc is derived from pressure-flow relationships and describes the slope of the pressure-flow relationship in the absence of any reflected pressure waves. Therefore, the Zc of an artery determines the change in pressure for a given change in flow.

Direct measurements of arterial stiffness can be obtained using both ex vivo and in vivo approaches, although most measurements relating to arterial stiffness in humans have been performed using in vivo methods. Ex vivo approaches typically involve tensile testing of intact cylindrical aortic rings or strips of aortae, allowing direct characterization of aortic wall stiffness by calculation of E or E_{inc} (Fig. 19.1). However, this approach is limited by a reliance on using animal models of aortic stiffening, which might be considered a poor surrogate for human aortic stiffening due to marked differences in aortic wall structure between species. Further limitations relate to loss of viability of the vascular smooth muscle, meaning that tensile testing cannot necessarily predict the contribution of the smooth muscle to mechanical stiffness and difficulties in obtaining ex vivo human aortic tissue for investigation. In vivo approaches typically use ultrasound wall tracking techniques, although tissue Doppler imaging and magnetic resonance imaging (MRI) are increasingly being employed. These techniques allow measurement of wall thickness, although high spatial resolution is required, limiting ultrasound-derived measurements to superficial sites such as the carotid or femoral arteries. Given the dependence of wall stiffness on distending pressure, then measurements of blood pressure should ideally be obtained at the same time or very close in time as arterial diameter measurements. Blood pressure should also ideally be measured at the same site. This is because systolic and pulse pressures are amplified moving from central to peripheral arteries and the extent of this amplification is variable between individuals [12]. In contrast, mean and diastolic blood pressures do not change markedly. It is now possible to obtain local (i.e. carotid) pressure waveforms non-invasively, using probes or transducers. The waveforms can then be calibrated using the mean and diastolic blood pressure values obtained with a standard cuff at the brachial artery. The key advantage of direct arterial stiffness measurements is that very precise information concerning arterial wall properties can be obtained by carefully conducted mechanistic studies. Disadvantages are the expense and level of operator expertise required to obtain high-quality data, limiting such techniques to specialist research settings.

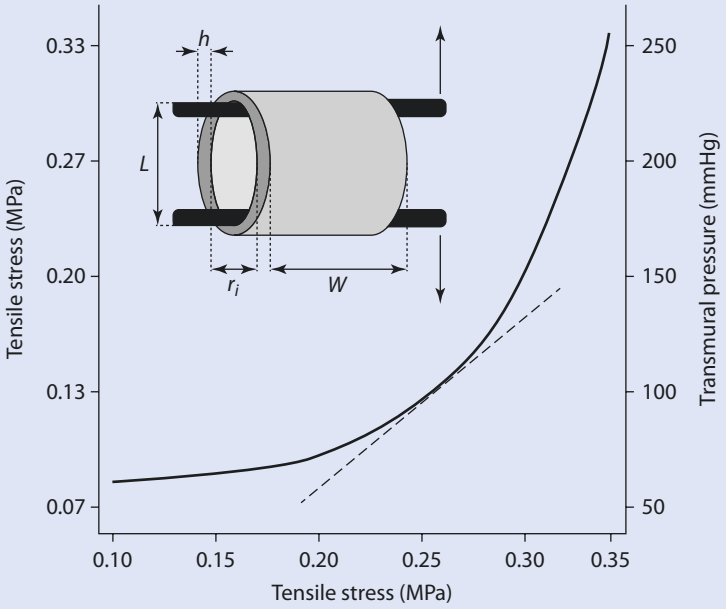

Fig. 19.1 Tensile testing of aortic samples. Aortae can be cut into rings and mounted in a tensile testing machine. The resulting stress-strain curve is used to calculate elastic modulus. *L* = lumen diameter (2ri); the cross-sectional area is twice the wall thickness (*h*) multiplied by the width (*w*) of the ring

19.5.2 Regional Measures of Arterial Stiffness

Regional or global measures of arterial stiffness are much more commonly reported than direct, local measures, due in large part to the widespread availability of simple-to-use, non-invasive devices to assess arterial stiffness, and a growing body of evidence that measures derived from these devices predict clinical outcomes.

Pulse wave velocity (PWV) Perhaps the most widely reported and clinically relevant measure is the PWV, which indicates the speed with which the pressure waveform propagates along a segment of the arterial tree; the stiffer the artery or segment of the arterial tree, the faster the wave travels. Pulse wave velocity is inversely related to arterial distensibility according to the 1922 Bramwell-Hill equation:

$$\mathrm{PWV} = \sqrt{\frac{1}{D\rho}}$$

where D = *distensibility;* $(\Delta V/V)/\Delta p$, *and* ρ = *density of blood.*

Therefore, the major determinants of the pulse wave velocity (PWV) are the elastic properties of the arterial walls, the geometry of the artery and blood viscosity, although blood density tends to vary within narrow limits and does not have a major impact on measurements of PWV.

In practice, measuring the PWV involves recording pressure waves from two different arterial sites with calculation of the wave transit time from the 'foot' of each waveform (foot-to-foot method). Measurement of the distance between the recording sites (path length) allows calculation of the PWV. Waveforms can be recorded simultaneously, or sequentially, with sequential recordings requiring a simultaneously measured ECG a reference. PWV can be measured invasively, typically in the aorta, using a catheter with dual pressure sensors, spaced a known distance apart, or a single-sensor catheter which is pulled back a known distance in order to calculate PWV (Fig. 19.2). MRI can also be used to calculate aortic PWV, either over the entire aortic path or within predefined segments, which provides information concerning regional variations in aortic stiffness, which might occur with ageing or disease. Aortic or segmental path lengths can be

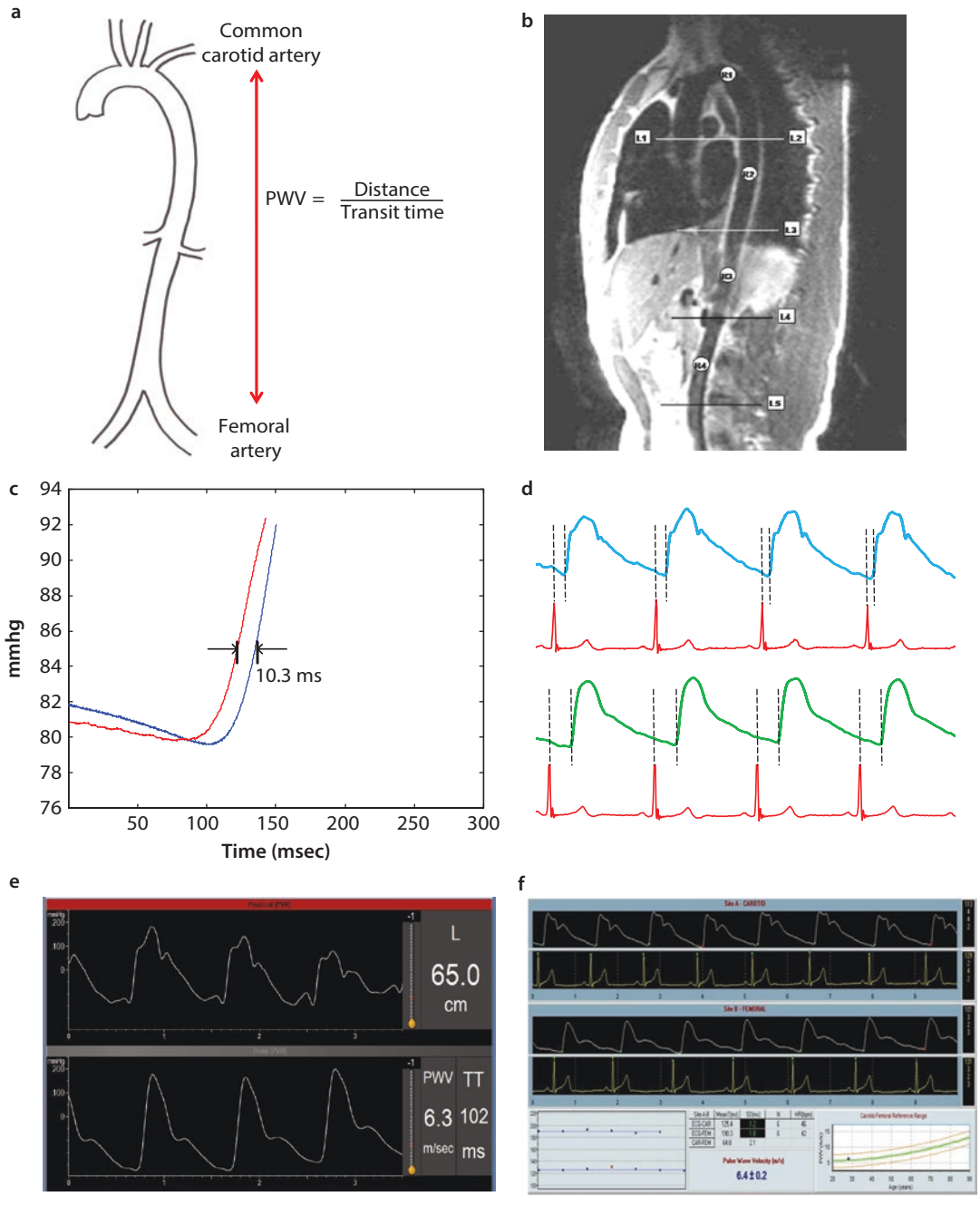

Fig. 19.2 Approaches to measuring aortic pulse wave velocity. **a** Aortic PWV can be calculated from waveforms recorded at carotid and femoral sites, if the distance and time delay in arrival of the pressure waveform between the sites is measured; **b** MRI can also be used to measure 'segmental' aortic PWV, providing information on regional variations in stiffness along the aorta; **c** PWV can be calculated from simultaneously measured pressure (or flow) waveforms by identifying the "foot" of each pressure waveform and measuring the time delay between them (foot-to-foot method); **d** Waveforms can also be measured sequentially, using a simultaneously recorded ECG as a reference. In this case, the difference between the ECG reference point and the foot of the pressure waveform is calculated i.e. distance/Δt; **e** Simultaneously measured waveforms using the Vicorder device; **f** Sequentially measured waveforms using the SphygmoCor device

measured with reasonable accuracy, except in cases where the aorta is highly tortuous [13].

Non-invasive measurements of PWV can also be made with various devices, employing pressure or volume sensors or cuffs. Ultrasound methods can also be used. Path length is measured using a tape measure or, ideally, callipers, which avoid overestimations of path length in obese persons but cannot overcome measurement inaccuracies in persons with a tortuous aorta. The PWV can be assessed non-invasively between any peripheral arterial sites where an arterial pulse can be recorded. However, the carotid-femoral PWV is most often reported, because it reflects aortic stiffness and is the measure with which most outcome data are associated. As such, carotid-femoral PWV is considered the gold standard measure of arterial stiffness. Brachial-ankle PWV is easy to measure and predicts cardiovascular events [14]. However, outcome data are mainly limited to Japanese populations, and further data are required in other populations. Finally, as with direct measures of arterial stiffness, it is imperative to measure blood pressure (preferably mean arterial pressure) either immediately before or after the PWV assessment and to factor this into any data interpretation, especially when comparing groups in whom blood pressure values might differ (e.g. men and women, hypertensives versus normotensives, metabolic syndrome versus healthy).

Ambulatory arterial stiffness index (AASI) This index, computed as 1 minus the slope of the linear regression line between ambulatory systolic and diastolic blood pressure readings taken over a 24 hour period, has been proposed as a surrogate measure of arterial stiffness. However, the AASI is also likely to be influenced by haemodynamic factors such as heart rate, stroke volume and vascular resistance, in addition to arterial stiffness, and so does not appear to be a strong surrogate of stiffness per se. Nevertheless, AASI independently predicts cardiovascular mortality [15] and stroke [16] in large, prospective investigations, proving it to be an interesting systemic haemodynamic measure which appears to provide added value for risk prediction. This may be of particular relevance given the increasing use of out-of-office blood pressure measurements in routine practice.

Cardio-ankle vascular index (CAVI) This is a relatively new surrogate measure of arterial stiffness proposed by Shirai in 2006 [17]. CAVI is thought to represent the stiffness of the arterial tree from the origin of the aorta to the ankle and is based on the observation that blood pressure and arterial diameter relate in an exponential manner. This pressure-diameter relationship, when combined with the Bramwell-Hill equation relating the PWV to changes in blood pressure and diameter, yields the measure of CAVI. In practice, the heart-to-ankle PWV is calculated from an ECG and volume plethysmography, using a cuff placed around the ankle. Brachial blood pressure is also assessed. The PWV is then converted to the CAVI, using the equation presented in ◘ Table 19.1. Non-invasive and simple to measure, CAVI is supposedly independent of blood pressure. It has been heavily studied in Japanese populations, and further data are required in other populations.

19.5.3 Indices Derived from Arterial Waveform Analysis

Various indices can be derived from analysis of arterial pressure or flow waveforms, such as systemic arterial compliance and augmentation index, although these measures do not relate directly to arterial wall stiffness.

Systemic arterial compliance This can be derived from measurements of aortic blood flow with a Doppler flow velocimeter placed at the suprasternal notch and measurements of pressure using tonometry at the carotid artery. The total peripheral resistance is estimated from the mean arterial pressure divided by the mean blood volume flow. Measurement of systemic arterial compliance is based on theoretical models containing many assumptions, and, as yet, there is no evidence that it holds independent predictive value for cardiovascular disease events.

Augmentation index (AIx) The AIx is generally accepted as a composite measure of arterial stiffness and wave reflections. As discussed earlier, the arterial waveform is composed of both a forward (incident) and backward (reflected)

wave, and an increase in arterial stiffness will cause the reflected wave to return to the ascending aorta and summate with the incident wave, earlier in the cardiac cycle. This has the effect of augmenting or increasing the aortic systolic pressure. The augmentation index expresses this level of pressure augmentation relative to the aortic pulse pressure. Hence an AIx of 33% indicates that approximately one third of the aortic pulse pressure is due to the summative effect of the reflected pressure wave. Although not a direct measure of arterial stiffness, the AIx is independently related to future cardiovascular disease events [18].

19.6 How to Treat Arterial Stiffness

The key points to note are that the majority of studies in humans have focused on the effect of different classes of antihypertensive agents, all of which lower arterial stiffness via a passive effect of lowering mean arterial pressure, rather than any direct effect on the arterial wall per se. There is currently an unmet need for novel de-stiffening agents which are ready to be tested in humans. Ultimately, these will require well-designed, adequately powered intervention studies.

Conclusions and Clinical Perspectives

- Arterial stiffness increases exponentially with age and blood pressure in almost all populations worldwide. Nevertheless, it appears to increase much less rapidly in truly rural or indigenous populations, suggesting that a major part of age-related stiffening is *pathological* and should not, therefore, be considered an inevitable part of the ageing process.
- Strategies to reduce or prevent arterial stiffening and its adverse clinical consequences are likely to have a significant impact on cardiovascular health in the longer term.
- The widespread availability of simple-to-use, non-invasive measurement devices makes arterial stiffness a prime candidate for introduction into the routine clinical setting.

Gaps in Knowledge

- How to treat? Further research is required to identify the precise mechanisms underlying pathological arterial stiffening and what the ideal therapeutic or prevention strategies should be.
- When to treat? Appropriate treatment thresholds have yet to be established. In this regard, age- and gender-specific reference values or thresholds for risk estimation may not be helpful or appropriate.
- Widespread adoption of arterial stiffness measurements into routine clinical practice is possible but will ultimately depend on development of appropriate guidelines on which clinical decisions should be based.

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Atherosclerosis

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_20

Key Concepts
- Atherosclerotic plaque rupture
- Mitochondrial energetic dysfunction
- Energetic switching in vascular smooth muscle cells

20.1 Introduction

Cardiovascular disease remains the commonest form of mortality and morbidity in the Western world. According to the World Health Organization (WHO), it accounts for more deaths than the combined incidence of all cancers. There is an urgency to understand and identify therapies that can be translated to reduce the effects of this disease and its associated comorbidities.

Within the umbrella term of cardiovascular mortality, atherosclerotic disease accounts for over two thirds of all deaths. Arterial vessel wall plaques are fibrofatty lesions that rupture into the blood stream causing occluding thrombus or emboli that can block the fine arteries of the heart and brain. Plaque rupture is due to loss of integrity of the overlaying vascular smooth muscle cell (VSMC) plaque cap. The atheromatous plaques contain a heterogeneous pool of different cell types. While inflammatory cells try to resolve and ultimately drive plaque progression, it is the vascular smooth muscle cells (VSMCs) which supply the structural stability to the plaque. They are a source of extracellular matrix, proteoglycans and collagens that are responsible for maintaining the plaque's tensile strength. Research shows us plaques rupture at sites of accelerated ageing with high levels of DNA damage and mitochondrial dysfunction. It is the loss of viability and vitality of these plaque smooth muscle cells that ultimately leads to plaque vulnerability and rupture (Fig. 20.1).

The mature plaque is a toxic environment of oxidised lipids, inflammatory cells and cytokines that promote ageing and cell death. Excess reactive species (RS) from defective metabolism at the mitochondria and phagocytes through NADPH oxidases are known to damage DNA and compromise VSMC viability. Recent work has extended this paradigm to particularly the VSMC's ATP-generating organelle, the mitochondria. The mitochondria lack protective DNA histones and repair enzymes and are more susceptible to DNA, lipid and protein damage. This compromises energy synthesis and promotes apoptotic cell death and cell senescence in the plaque.

Reducing risk factors which slow vascular ageing and dysfunction in vulnerable regions of the vasculature are predicted to improve plaque phenotypes. Slowing cellular ageing or finding alternative energy sources by reprogramming vascular smooth muscle cells are alternative research-based approaches that are hoped to prevent plaque rupture in the future.

20.1.1 History of Atherosclerosis Research and Anichkov

Atherosclerosis is the pathological condition that underlies key cardiovascular diseases and disorders, including coronary artery disease, cerebrovascular stroke and other associated conditions such as peripheral vasculature disease. It is also the major contributor to the collection of diseases termed metabolic syndrome which includes hypertension, obesity and diabetes. Historically, evidence would suggest that atherosclerosis has been part of the human condition since the beginning of early

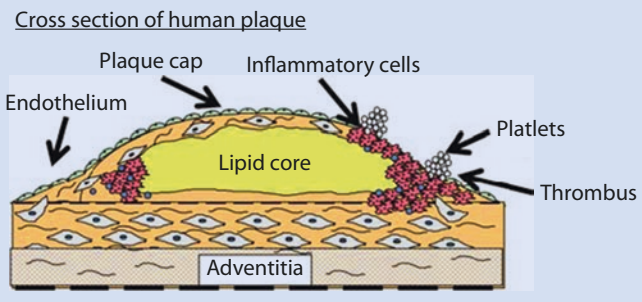


Fig. 20.1 Cross section of an idealised human atherosclerotic plaque [1]

human civilisations [2]. However, it has only been in the last 100 years or so that advancements have been made in understanding its aetiology.

It was the German pathologist Felix Marchand (*1846–1929*) who first introduced the term atherosclerosis – from the Greek (ἀθήρα) for gruel or porridge, 'athero', and (σκλήρωση) 'sclerosis' meaning hardening – to describe arterial plaques. While others had identified cholesterol in plaques, it was a Russian doctor Nikolai Anichkov (*1885–1964*) during World War I that developed the first accepted theory behind what causes atherosclerosis. In his seminal work often regarded as one of the greatest discoveries of the twentieth century, he was able to link excess dietary cholesterol to the development of the disease. In 1913, Anichkov had the clever idea of using hen's eggs as a high-fat diet to test generating atherosclerotic lesions in rabbits. When these experiments proved successful, he had the intuition of separating the cholesterol-rich yolks from the cholesterol-free white and repeating his experiments to identify the causative component.

In doing so, he developed the first in vivo model of atherosclerosis, linking excess cholesterol to atherogenesis.

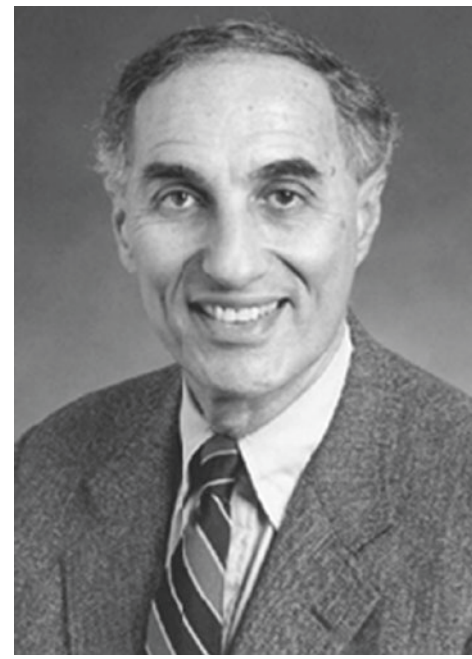
Nicolai Anichkov

Cholesterol is a biosynthetic lipid available from variable dietary sources (15–50%), with the residual being produced by animal hepatocytes from the Kreb's cycle precursor acetyl-CoA. It is an essential lipid component of all cellular membranes and is required to maintain flexibility and a dynamic cellular shape. It also serves as a precursor to steroid hormones such as glucocorticoids mineralocorticoids and androgen and oestrogens.

The polar nature of the blood requires the liver to repackage lipids received as chylomicrons from the gut with protein carriers such as low-density lipoproteins (LDLs) to release into the blood.

In excess, these particles are known to adhere to the insides or luminal surface of damaged blood vessels and promote a thickening of the vessel wall termed the neointima. Vascular damage can come from the circulation in the form of toxins, such as by-products of smoking or from hemodynamic and inflammatory reaction in which free radicals are released from endothelial cells, smooth muscle cells and circulatory cells such as phagocytes. These radicals oxidise LDL to produce a rancid oxLDL which is toxic to cells. The recruitment of white blood cells, such as monocytes and macrophages, take up these particles, but this often results in them becoming trapped and being unable to egress from the lesion. These cells subsequently die to form foam cells (first identified by Anichkov), and a fatty streak underneath the endothelial layer of the vessel is formed. This is the first sign of visible atherosclerosis in the vessel wall, which over time will develop into an atherosclerotic plaque.

20.1.2 Russell Ross' Response to Injury Hypothesis (1929–1999)



Russell Ross

The initiation of atherosclerosis as a response to injury to the endothelium was developed by Russell Ross [3]. As a dentist he developed an interest in wound healing which he applied to the atherosclerotic vessel wall. He was the first to identify and link vascular wall insults, such as Anichkov's hypocholesterolaemia, cigarette, smoke, hypertension and diabetes to wall injury. He developed methods to culture VSMC from the walls of arteries, and he identified platelet-derived growth factor (PDGF) as a key substance produced in response to injury that provokes smooth muscle cell proliferation and migration. He also contributed to another important concept in atherosclerosis, the hyper-proliferative wound response, term restenosis – or re-narrowing of vessels. He correctly identified low shear regions, where turbid flow reduced endothelial polarisation, such as at vessel branch points in the aortic arch and coronary arteries. These regions are then especially prone to develop atherosclerosis. His work went on to explain the vascular benefits of exercise to endothelial health to delay atherosclerosis.

Despite atherosclerosis having an image as an 'old persons' or disease of the elderly, the foundations for atherosclerosis do occur much earlier in life. Indeed, the work of Renu Virmani has shown that young Korean and Vietnam war veterans suffered from blocked arteries at an average age of only 21 years [4]. More recent work suggests that prenatal programming and maternal diet may also play a role in the genetic development of disease [5]. With the advent of sequencing technology, genome-wide association studies (GWAS) have attempted to explain an individual's cardiovascular phenotype or risk by identifying genetic predisposition termed quantitative traits (QTs). To date, over 150 separate studies have been performed on different diseases. Assessing candidate genes and single nucleotide polymorphisms (SNPs) has identified hundreds of genetic elements but with small individual effects. Causal variants that explain the heritability of CVD risk which could be used for diagnostic and predictive treatments have so far failed to be realised [6]. In addition, external environmental factors such as pollution and internal biological factors such as diet, exercise and stress are all thought to combine to increase or decrease an individual's relative risk of developing disease; these will be explored next, but it is fundamentally a lipid-driven process.

20.2 Risk Factors for Atherosclerosis

Initiation of atherosclerotic disease occurs in childhood, with substantial numbers of children presenting fatty streak formation and plaques in their aorta. While plaque development is primarily driven by excess cholesterol, progression and development of the disease is regulated and primed by number of systemic risk factors (Table 20.1).

Table 20.1 Risk factors for atherosclerosis

Nonmodifiable	Age
	Male sex
	Family history of premature atherosclerosis
Modifiable, established	Dyslipidaemias (hypercholesterolaemia; high LDL level, low HDL)
	Diabetes mellitus
	Hypertension
	Tobacco smoking
	Obesity or the metabolic syndrome
Emerging/under study	High CRP level
	High level of small, dense LDL
	High lipoprotein (a) level
	Hyperhomocysteinemia
	Hyperinsulinemia
	Hypertriglyceridemia
	Prothrombotic states
	Air pollution
Socioeconomic	Alcohol intake (other than moderate)
	Socioeconomic status
	Psychosocial factors (type A personality, depression, anxiety)
	Renal insufficiency
	Sedentary lifestyle

Based on Merck manual; Lam et al.

20.3 Development of Atherosclerosis: A Brief Primer

Atherosclerotic plaque development is characterised by accumulation of pathologically activated cells and subsequently atherosclerotic plaque tissues (cholesterol, extracellular matrix, etc.) in the vessel wall. It is increasingly suggested that this accumulation is occurring throughout the ageing process and the vasculature may act as sink or depot for excess fat and lipids. Plaque accumulation is diffuse within the vasculature, with as many as 50% plaques at autopsy not impinging the blood flow but have the potential to rupture. It is the focal rupture of lesions that cause acute pathological symptoms. The cellular players include endothelial cells, immune cells, lipid-laden cells (foam cells), vascular smooth muscle cells as well as vascular fibroblasts. All of these participate in the creation of atherosclerotic plaque lipid core. More evidences have accumulated that atherosclerosis is also modulated by bone marrow-derived progenitor cells and circulating cell-derived microparticles.

20.3.1 Oxidative Stress, Inflammation and Endothelial Dysfunction

The early pathological event in plaque formation is endothelial dysfunction. Inflammation and oxidative stress act in concert to drive the pathology within the vessel wall which is mediated by accumulation of monocyte/macrophages that also transdifferentiate into lipid-filled foam cells. Systemic risk factors for atherosclerosis discussed above cause endothelial dysfunction, which in turn is greatly caused by oxidative stress. The primary insult to the vessel wall is loss of nitric oxide (NO) bioavailability which leads to the upregulation of endothelial adhesion molecule and loss of endothelial anti-inflammatory properties. This enables adhesion and vascular recruitment of inflammatory cells such as T cells and monocytes to the vessel wall. This occurs initially within the perivascular space and subsequently in the developing atherosclerotic plaque. These immune cells modify the local microenvironment and when exposed to lipid molecules (oxidised and native LDL) undergo activation, releasing cytokines and chemokines which perpetuate vascular inflammation and lead to generation of neointima. This manifests already in subclinical atherosclerosis and can be monitored by ultrasound as increased intima-media thickness (IMT). Recruited monocytes are transdifferentiated to become vascular macrophages, which in concert with resident cells phagocytose lipids accumulating in the slowly forming neointimal space. A number of receptors are involved in this process including macrophage scavenger receptors (SCRA1-5) as well as toll-like receptors (TLR1-10). Such lipid-laden macrophages become foam cells, named because they contain large lipid droplets giving them 'foamy look'. While this process is initiated in order to clear the lipids from vascular wall, foam cells are highly activated and perpetuate the development of pathology. They also undergo apoptosis and cell death becoming trapped within the lesion. Cholesterol released forms crystal clefts which are often visible inside the lipid core as crescent shapes. This process continues in a vicious cycle, while the atherosclerotic plaque grows. The structure of the plaque is strengthened by collagen produced by fibroblasts, fibrocytes and VSMCs in the plaque.

This leads to the formation of so-called fibrous plaque and thin-capped fibroatheroma (TCFA). Plaque growth limits the vascular lumen and thus affects ability to increase blood supply in the conditions of increased metabolic needs, leading to clinical symptoms of exercise-induced angina.

Continued inflammation promotes accumulation of macrophages and other immune cells, which in concert with smooth muscle cells produce metalloproteinases (MMPs). These enzymes degrade extracellular matrix rendering the plaque unstable. Such plaque instability has been termed 'vulnerable plaque', as it is prone to rupture. Rupture in turn exposes strongly pro-thrombotic plaque core to flowing blood. This results in rapid thrombus formation with occlusion of the vascular lumen and disturbance of blood supply. Clinical consequences of these events depend on the location of ruptured atherosclerotic plaque and include myocardial infarction or ischemic stroke.

The key question remains – why, if all of these listed risk factors are systemic, do atherosclerotic plaques develop locally, in specifically prone sites? Why are areas of arterial system more prone for

this pathology? This is clearly the case in relation to arterial bifurcations and areas of disturbed flow. The answer is that in these areas oxidative stress, inflammation and endothelial dysfunction are particularly high with loss of shear depolarizing endothelial cells which enables the initiation of atherosclerotic plaque development, but in reality the entire vasculature is affected; it is just that regions are more affected and disease prone than others.

20.4 Endothelial Dysfunction in Initiating Atherosclerosis

Dysfunction of the endothelial cell lining is one of the primary events in the development of atherosclerotic plaques. In clinical studies sites within coronary arteries which had developed local endothelial dysfunction in subsequent years demonstrated significant atherosclerotic plaque development. Specifically these areas formed the culprit lesions leading to myocardial infarction. Endothelial cell activation occurs from activation of toll-like receptors, cytokines that lead to reactive oxygen species production. While mitochondrial derived ROS are responsible for a significant portion of intracellular ROS they are also generated from activation of the NADPH oxidase system. Pathogen-associated molecular pattern molecules (PAMPS) and in contrast damage-associated molecular patterns molecules (DAMPS) are cell-derived compounds which initiate and perpetuate immunity responses in the vasculature. Master transcription factors, such as NF-kB and AP-1, also perpetuate the response to endothelial cell dysfunction. Cytokines and chemokines, as well as hemodynamic factors, can drive additional epigenetic modifications that facilitate loss of endothelial function and help the development of the so-called fatty streak.

Alternations of various protective functions of the endothelium (anti-inflammatory, antiproliferative, vasorelaxant, anti-platelet aggregation) are also referred to as endothelial cell dysfunctions and have been shown to occur in both experimental animal models and in humans. The key characteristics of this condition are the loss of NO bioavailability as NO may modulate all of these functions. This condition has been found in the setting of hypertension, hypercholesterolaemia, diabetes atherosclerosis itself, as well as heart failure. Studies of vascular function in humans are conducted in vivo using flow-mediated dilation, forearm plethysmography or during angiography or ex vivo by means of organ bath studies.

Significant impairment of vasorelaxation has been shown in humans in association with atherosclerosis as well as presence of clinical risk factors of atherosclerosis. In patients with coronary artery disease, even angiographically normal coronary segments show paradoxical constriction to acetylcholine. The degree of endothelial dysfunction in coronary vasculature is also associated with the presence of clinical risk factors for atherosclerosis.

20.4.1 Plaque Maturation

Over time expansion of the lesion occurs with increased infiltration of inflammatory cells and lipids. Some of the inflammatory cells are thought to become trapped within the lesion and are unable to egress leading to acellular areas which eventually become calcified. As the lesion grows, the vessel wall compensates by expanding in a process termed positive remodelling. This reaches a maximum when the lesion starts to encroach into the lumen, in which negative remodelling predominates. For reasons unclear, possibly due to calcification, some plaques can stabilise at this stage or they may continue to expand. The cap is under significant tensile strain, and through repeated cardiac cycles, the extracellular material of the cap is thought to fatigue [8]. When the plaque ruptures, the thrombogenic core of the plaque initiates the coagulation cascade, blocking the vessel. If this occurs in the fine coronary arteries of the heart, it will produce a heart attack. The higher up the coronary tree, e.g. left anterior descending (LAD) coronary artery, the more ischemic damage will be done to the heart. Peripheral lesions may erode rather than rupture, such as those of the carotid arteries of the neck rupture. Rapid thrombosis will ensue when the circulation is exposed to tissue factors on the eroded plaque surface. Later, plaque and platelet aggregation occurs that can cause emboli to lodge in the fine arteries of the brain and cause stroke.

Work has shown that plaque VSMCs are more aged than surrounding vessel wall cells, and they have shortened telomeres [9] and higher levels of reactive oxygen species (ROS)

and DNA damage [10]. Shortened telomere can initiate a DNA damage response that drives replicative senescence and plaque ageing. ROS can be generated by intracellular enzymes such as NADPH oxidase, but they are also a natural by-product of energy generation by the mitochondrial respiratory chain. Their activity is normally counterbalanced by antioxidants with compounds such as glutathione. Glutathione has also been shown to be upregulated in response to pentose phosphate pathway activity and ensures excess radicals are quenched. However, production of glutathione other reducing agents that are themselves oxidised in the process of their activity also require energy to be produced. At some point the capacity of the plaque to maintain this balance is diminished, and damage from reactive species (RS) starts to predominate.

When more radicals are produced than quenched, this can easily be detected as excess DNA damage, such as in plaque-derived cells [10]. Plaques are known to have higher rates of senescent and apoptotic cells [9], and while the fibrous cap is important to plaque stability without VSMCs to produce matrix and collagen, the plaque is doomed to rupture. Indeed, evidence confirms that human plaque rupture occurs at sites of where there are fewest VSMC. While this clearly has implications for understanding the energy production within the cells, it also opens a new potential therapeutic target of intervention.

20.5 Plaque Rupture and Consequences of Disease

Maturation of the fatty streak to an advanced atherosclerotic plaque (Fig. 20.2) usually takes many years but in exceptions such as in cases of genetic hyperlipidaemias can also be surprisingly rapid [7]. During the development of the early lesion, VSMCs proliferate, migrate and secrete matrix proteins required for vessel wall repair.

In particular metalloproteinases (MMPs) (zinc finger proteases) in the plaque stability provide vascular remodelling of the extracellular environment (ECM). While growth factors, cytokines and hormones promote their activity, transforming growth factor β (TGF-β) and corticosteroids inhibit their activity, with MMP-2- and MMP-9-dependent degradation promoting early lesion formation and MMP-1/8/13 activity found in mature plaque. The transcriptional inhibitors of MMPs are tissue inhibitors of matrix metalloproteinases (TIMPs). Activation of MMPs and inactivation of TIMPs have been linked to susceptibility to a range of CVDs including aneurysm formation and atherosclerosis. Trials investigating MMP inhibitors may be useful therapies [17]. Indeed some clinical trials have already observed that statin therapy is able to reduce MMPs, which could increase the plaque stability. The inflammatory response also plays an important role in VSMC activation and plaque stability.

Ultimately the tensile strength of the plaque caps exceeds the mechanical forces both internal derived from the expanding plaque and from the shear forces of blood flow. Rupture of the plaques initiates the thrombotic cascade, platelet activation that results in fibrin deposition and clot accumulation. Blocking essential arteries to the heart restricts blood flow and induces acute ischemia. If flow is not restored quickly, the tissue dies and necroses and heart function is lost. If emboli are released from the clot, they can block the fine arteries of the cerebral circulation and promote stroke.

20.6 Clinical Developments, Technology and Treatments

The understanding of these processes of natural atherosclerotic plaque pathobiology has created numerous therapeutic attempts aimed at:

(i) Preventing development of atherosclerotic plaque
(ii) Treatments that lead to regression of atherosclerosis
(iii) Treatment to prevent plaque rupture

While numerous drugs have been tested and shown moderate effectiveness, the most efficient way to prevent development of atherosclerotic plaque are those that lead to plaque stabilisation by lipid reduction. This approach explains the success of statins. Stains are HMG-CoA-reductase inhibitors that block the biosynthesis of cholesterol when used in combination with risk factor reduction treatment such as lifestyle modification including exercise; they are the most effective method at reducing all causes of mortality.

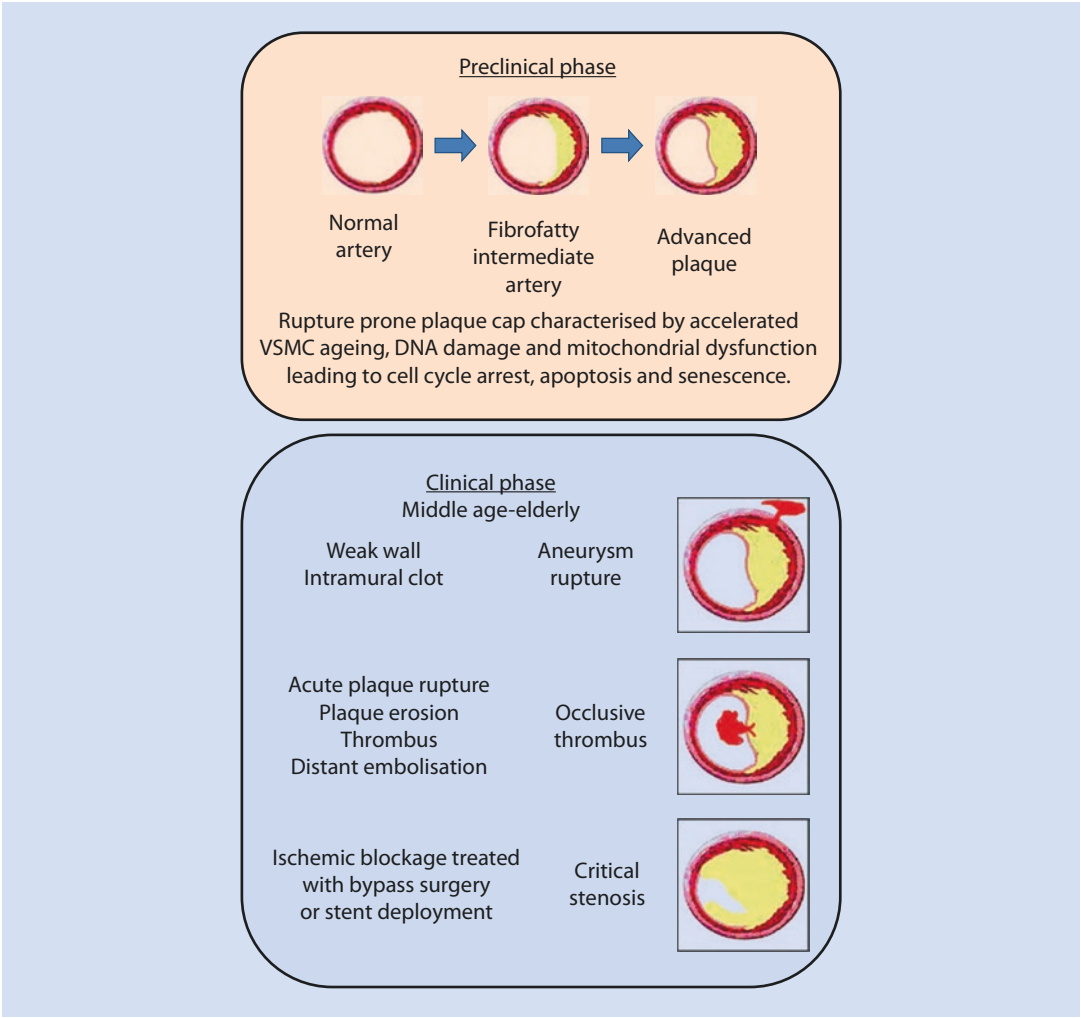


Fig. 20.2 Stages of the natural course of development of atherosclerosis with preclinical plaque progression characterised by accumulation of lipid- and inflammation-driven plaque expansion. This leads to accelerated VSMC plaque cap ageing and predisposition to plaque rupture. Clinical phase includes intramural weakness leading to aneurysm formation, acute plaque rupture and critical stenosis of the artery requiring bypass surgery or angioplasty for delivery of stents

Other clinical developments include attempts to limit atherosclerosis through rapid lipid lowering such as apheresis where lipids fractions such as Lp-a are removed from the blood. While this approach has been disputed, several studies have shown a significant degree of inhibition of atherosclerosis progression or even low degree of reversal of plaques. The role of rapid lipid lowering in atherosclerosis treatment/reversal will likely become evident from PCSK9 inhibitor trials, as these novel lipid-lowering monoclonal antibodies exceed in their effectiveness; any other drugs currently available at lowering circulating LDL (low-density lipoproteins) levels and these strategies are discussed below.

20.7 Atherosclerosis Therapies

20.7.1 Lowering Circulating Cholesterol Levels

Statins are very commonly prescribed. Statins, or HMG-CoA reductase inhibitors, target the rate-limiting step in cholesterol production. Targeting the biosynthesis of cholesterol and low-density lipoprotein cholesterol (LDL-C) is significantly reduced. This reduction of circulating cholesterol lowers the probability of lipoprotein particle entry into the artery wall, endothelial dysfunction and biogenesis of fatty streak formation and in turn

plaque formation. Furthermore it has been discovered that statins have inhibitory effects on VSMC migration and proliferation, as well as cytokine release providing additional benefits to limiting plaque progression.

20.7.2 Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)

PCSK9 is a serine protease, produced in the liver, which regulates levels of LDL by promoting degradation of hepatic LDL receptors after LDL binding, hence lowering the number of receptors present. Loss of function gives the converse effects: hypocholesterolaemia and decreased risk of atherosclerosis and CVD. Several inhibitors of PCSK9 have been discovered, and some have been taken through clinical such as a humanised monoclonal antibody (mAb), evolocumab. A placebo-controlled trial of evolocumab was carried out in 2014 by Blom et al.; this phase 3 trial gave a 57% reduction in LDL cholesterol levels, relative to the reduction in the placebo group. This reduction was resultant of administering patients 420 mg every 4 weeks over a 52-week period.

20.7.3 Lowering Blood Pressure (BP)

Decreasing BP, there is less strain on the artery wall, protection of the endothelium and therefore less chance of damage of atherosclerosis developing. The three main drug classes utilised to target hypertension in atherosclerosis are angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers (CCBs) and diuretics. A combination of each of these drug classes is often used in order to lower blood pressure and, in turn, lessen one's chances of developing atherosclerosis.

20.7.4 Anti-inflammatory Treatments

Several studies have tested the role of anti-inflammatories. These were initiated to test the hypothesis that interventions aiming to limit chronic low grade inflammation would be effective in treating atherosclerosis. These included both systemic drugs and the use of anti-inflammatory compounds on coated stents. These have had limited success, and interventional therapy remains the most efficient way of prevention and treatment of complications of the disease.

To be successful and intervene at relatively early stages of the disease, we need to develop biomarkers of atherosclerosis. Numerous biomarkers were proposed, but the European Society of Cardiology (ESC) guidelines only recommend the use of high-sensitivity troponin for the diagnosis and prognosis in the management of acute coronary syndromes (ACS), along with the assessment of lipid profile, creatinine and glycaemia. Similarly, in other forms of atherosclerotic disease such as stable coronary artery disease, these guidelines do not recommend testing any biomarkers beyond lipids, creatinine, glycaemia and glycated haemoglobin, adding B-type natriuretic peptide (BNP) or natriuretic peptide proBNP (NT-proBNP) only if heart failure is suspected. Importantly, in spite of clear pathogenetic importance of inflammation in atherosclerosis, the use of high-sensitivity C-reactive protein (hsCRP) or any other novel biomarker is not recommended. Future novel biomarkers that are being discussed are likely to include microparticles, nanoparticles, HDL, microRNA and possibly novel biomarker sets established by proteomic and metabolomics approaches.

20.8 Models of Disease and Research Perspective

Atherosclerotic research relies on testing cell and tissue behaviour under defined conditions. These models rely on either isolated cells for in vitro work which are often derived from primary tissue explants or the use in vivo models of disease. For atherosclerosis research, the modern mainstay of transgenic work is murine models of the disease. The apolipoprotein E (ApoE) mouse lacks the ApoE gene required for the correct uptake of LDL particles from the circulation. These mice develop rampant atherosclerosis with 'human-like' lesions over a short duration of weeks rather than the process which occurs in humans over many years and most likely even decades. The model can be crossed with other transgenic models of interest to generate

double or triple genetic knockouts in which the genes of interest can be ablated in a particular cellular lineage and tracked with a reporter gene product. These genetic rearrangements can be performed with minute amounts of natural compounds such as taxol, derived from the bark of the pacific yew tree which is added to normal or high-fat diets to activate enzymes that splice out the gene of interest. Newer in vivo gene editing technologies such as CRISPR and TARGAAT will be superseding some for these more traditional approaches, reducing the amount of time required to develop these models. These new technological approaches to atherosclerosis are now outlined with emphasis on the role of VSMC DNA damage and mitochondrial dysfunction:

20.9 Mitochondrial Dysfunction in Atherosclerosis: Selected Atherosclerosis Research Highlights

There is currently a large number of areas in which novel cardiovascular research is being performed; these include work on new anti-lipid therapies, immunotherapies and combination therapies such as the polypill. Here we focus on novel VSMC therapies to improve VSMC longevity that could prevent or delay plaque rupture. We emphasise progress in mitochondrial dysfunction as a novel mechanism of improving VSMC health. Other translational research proposals including endothelial dysfunction research, oxidative stress or vascular inflammation therapies are discussed in separate chapters.

20.9.1 The Mitochondria

Mitochondria (Mt) are intracellular organelles of bacterial origin with their own 16 kb maternally inherited genome. High levels of mitochondrial dysfunction are observed in both human atherosclerotic disease and animal models. Arguably the three most important homeostatic functions of the mitochondria are:

1. A primary sources of ATP and ROS
2. Calcium homeostasis required to regulate protein and enzymatic functions
3. Regulation of the intrinsic apoptosis cascade

ATP is the energy currency of the cell. It is required for all exergonic reactions, from transcription and translation to DNA repair and antioxidant synthesis. The loss of this energy generation capacity has significant implications for disease and VSMC survival. Finding ways to improve mitochondria function beyond the cell's own capacity is challenging. The majority of faulty mitochondria undergo mitophagy, a form of phagocytic engulfment and proteosomal degradation, rather than rely on mitochondrial DNA (mtDNA) repair [11, 12]. Yet improving energy capacity in VSMCs is predicted to improve cell survival.

20.9.2 Therapeutic Approaches

Intervening early during the disease process is intuitively what research focusses on in order to reduce the pathological effects of 'mutant' mitochondria. Many of the approaches investigated to improve mitochondria function rely on altering the abundance of pathogenic mutations of the mitochondria which co-exist with wild-type DNA molecules, in a state known as heteroplasmy.

20.9.3 Heteroplasmic Shifting

Depopulating the endogenous mitochondria can be used to provide a rapid and established replacement for the cell. This work was extended by others to selectively delete mitochondrial genomes which express unique restriction sites. While these are rare, they have been shown to be successful in vitro for both murine and human cell lines. Recently tumour cells depleted of mitochondria have been observed to hijack mitochondria from healthy cells. Endogenous formation of nanotubules and transfer of mtDNA have been proposed as a potential mechanism in which aged and depleted cells recover mitochondrial function [13].

20.9.4 mtDNA Therapy

Antigenomic treatment is a method by which the replication of mutated mtDNA is inhibited, thereby preventing its propagation into daughter cells, increasing wild-type genomes and removing faulty or mutant mtDNA genomes to re-establish

respiratory chain activity. Targeting the Mt genome with peptide nucleic acids (PNAs) has had some success in vitro [14].

20.9.5 Cell Therapy

Some groups have trialled approaches with progenitor cells. These cells have a lower susceptibility to mtDNA damage but still retain the capacity to differentiate into the host tissue as a method to decrease in the impact of mtDNA damage. Recently stem cell technology has advanced to generate de novo VSMC from human pluripotent stem cells (hPSCs). Using a novel chemically defined protocol, Cheung and Sinha in 2012 and followed up in 2016 were able to induce origin-specific VSMC subtypes. It is anticipated that technological approach will have broad applications in modelling origin-dependent disease susceptibility and also in developing bioengineered vascular grafts for translational and regenerative medicine.

20.9.6 Mitochondrial Drug Therapy

Mitochondrial targeted drugs and probes are a relatively recent development to tackle mitochondrial dysfunction. The antioxidant MitoQ accumulates specifically in the mitochondria and decreases oxidative damage. MitoQ has been proven beneficial in cardiac hypertrophy and ischaemic-reperfusion injury. A variety of Mito compounds have been developed in this series and included probes such as MitoB, MitoSOX, and MitoPerox. MitoSNO, an *S*-nitrosothiol and NO generator, has been shown to protect against ischemia-reperfusion injury [15]. The mechanism has been eloquently described by the same authors to act by S-nitrosation of mitochondrial complex 1, the entry point for electrons from NADH into the respiratory chain. Murphy et al. now show that the reversible S-nitrosation of complex 1 slows the reactivation of respiratory chain during the initial few minutes of the re-oxygenation of ischemic tissue, thereby decreasing ROS production, oxidative damage and tissue necrosis. More advanced delivery approaches are already on the horizon with the advent of biodegradable nanocarriers that can be targeted to specific tissues and have the potential to deliver drug and gene editing cargos.

20.9.7 Exercise-Induced Gene Shifting

The lack of exercise and a sedentary lifestyle are regarded as major risk factors for cardiovascular disease and atherosclerosis. Research has shown that by mobilising proliferation in target tissues, it is possible to have mitochondrial biogenesis. Both resistance and endurance training have been studied which show an increase in mitochondrial biogenesis and a decreased mtDNA damage. This also correlated with improved respiratory chain capacity and a partial rescue of the pathology with reduced apoptosis which was observed in multiple tissues. While exercise no doubt has significant benefits for a whole range of diseases, it is not easily targeted to particular sites of interest, such as regions of the vasculature. Additionally, calorie restriction has been shown to reduce mitochondrial respiratory chain activity and ROS generation and may also be useful adjunct.

20.9.8 Alternatives: Metabolic Reprogramming

While mitochondrial oxidative phosphorylation is the preferred method of generating energy, it does have the drawback of generating reactive species. Cells can use glycolysis and the pentose phosphate pathway (PPP) as alternatives, so-called aerobic glycolysis. T cells and induced pluripotent stem cells and cancer cells all undergo this metabolic shift during rapid growth. Although less efficient, it has been suggested that the shift also helps to clear reactive oxygen species by decreasing dependence on oxygen and provide a way for cells to reroute their energetic demands.

This energetic reprogramming is feasible and can be enhanced by transgenic manipulation of key pathways. AMP-activated protein kinases (AMPK) is a potential target and is regarded as the master regulator of the bioenergetic capacity of the cell. AMPK has been associated with lifespan extension and favourably changed in several disease models including cardiovascular diseases. AMPK can invoke a switch in fuel utilisation during periods of acute energetic stress. For example, during mitochondrial demise, the PTEN-induced putative kinase 1 (Pink-1) flags the loss of mitochondrial membrane potential ($m\Delta\Psi$) and appears to promote energetic reprogramming to

promote glycolysis in plaque VSMCs [16]. The process is complex and may involve stabilisation of hexokinase II at the outer mitochondrial envelope and other import glycolytic intermediates including IGF activation of the prosurvival kinase Akt which along with AMPK can induce the hypoxia-inducing factor (HIF-1). HIF upregulates glucose transporters and intermediates that favour glycolysis but can also invoke AMPK during ATP depletion when ADP levels increase. The speeds of these dynamic fluxes are almost immediate and thought to occur in seconds to minutes before transcription regulation can occur. However, transcriptional effects are ultimately required for these changes to become permanent.

Conclusions: Basic Science to Clinical Perspective

Atherosclerosis is clearly a multifactorial disease of the vasculature and is driven by excess lipids and the ensuing complications of vessel wall inflammation. Current clinical diagnostic, medicines and interventional therapies have made a profound contribution to improve patient mortality and morbidity. Yet novel VSMC atherosclerosis research attempts to go beyond this and strives for further improvement.

At the cellular level, VSMC survival is critical to preventing plaque rupture. The plaque VSMC phenotype is inextricably linked to mitochondria health which has been shown to be crucial in the development of disease. Not only are they the sources of reactive species (RS) that damage the nDNA and mtDNA of the cells involved but also appear to promote vessel wall ageing that ultimately promotes vascular disease.

This damage effects respiratory chain proteins required for oxidative phosphorylation and thereby reduces ATP synthesis. The immediate consequence for the cell is less free energy for normal function. In trying to understand the cell and molecular defects such as those in energy metabolism that occur during disease, the hope is that bespoke patient treatments could be developed. Modifying the way the cells use energy to hopefully extend VSMC lifespan is one such goal. Key goals are to improve cell viability and proliferation and reverse the loss of matrix and collagen synthetic capacity, while also delaying apoptotic death and senescence, which are all features of vulnerable atherosclerotic plaques.

By elucidating those critical pathways required to maximise energy production in the cell, it may be possible to provide a clinical solution that is applicable to not only atherosclerosis but perhaps a number of cardiovascular diseases where energy generation is compromised. Understanding these complex pathways and then targeting the vasculature may reveal a new avenue to extend the host cell lifespan and ultimately delay features of disease. It is these novel therapies that will drive new translational and targeted clinical therapies of the future.

Gaps in Knowledge

Cardiovascular disease and atherosclerosis is a widespread problem not only in the UK but worldwide. The current therapies and drugs prescribed are not entirely effective and, in the most part, are used to treat symptoms of the disease rather than address or prevent its underlying cause and development. The novel therapeutics discussed here target specific parts of the atherosclerosis disease model rather than target the peripheral effects. However, there are novel and emerging therapies identified here, but all are not without their own issues.

In the most part, lack of targeting and efficacy limits their more widespread use. With an ever-growing obesity problem in the UK, the number of patients affected by atherosclerosis will only rise. The success of statins shows us that limiting the circulating levels of LDL cholesterol, we could limit one's chances of developing atherosclerosis and in turn CVD. However these classes of drug are not perfect and non-compliance is a significant issue. Future work on PCSK9 suggests vaccinating high-risk patients, e.g. those with familial hypercholesterolaemia could effectively trump the use of statins. While current evidence suggests trial vaccine studies did not give a large decrease in circulating LDL cholesterol, focussing more resources on understanding the mechanism and engineering the vaccine itself may be a plausible therapy for the future.

Atherosclerosis research has come a long way since the time of Anichkov, but there remain gaps in our understanding, e.g. effective ways to reduce DNA damage

and ageing. Controlling these fundamental processes will directly impact the cells' longevity and response to injury and its capacity to repair. Future therapies should focus on improving our models of atherosclerosis, including imaging technologies, and refining the tools needed to reprogram metabolism to improve the vessel's wall response to injury.

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General Reading

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Inflammation and Immunity in Vascular Diseases

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_21

Key Concepts

- Inflammatory and immune mechanisms play a key role in the pathogenesis of atherosclerosis, hypertension and other vascular pathologies.
- Studies in animal models have been greatly useful in identifying specific immune pathways, which could be targeted for therapeutic utility in vascular disease.
- Inflammation ultimately dictates the vulnerability of atherosclerotic plaques to rupture and cause potentially fatal clinical outcomes, i.e. myocardial infarction or stroke.
- Clinical targeting of vascular inflammation, although in its infancy, may represent a future viable approach for the control of vascular pathologies.

21.1 Introduction

Of the approximately 17.6 million global deaths from cardiovascular disease recorded in 2016, 85% were attributed to ischaemic heart disease and cerebrovascular disease both of which are largely atherosclerosis-driven conditions [1]. For decades this disease has been known to have a prominent inflammatory component, which has been extensively described in the literature. Furthermore, it has been demonstrated that the extent of systemic and vascular inflammation can improve our ability to predict clinical manifestations such as myocardial infarction (MI) or stroke. A great deal of current research is focused in improving our understanding of immune and inflammatory processes driving cardiovascular diseases (CVD) and how this knowledge can be harnessed for diagnostic and therapeutic utility [2].

The immune system is the body's defence against injury, infectious organisms and other invaders. It can be split into the innate and adaptive immune responses. Innate immunity is the first line of defence against infection or injury, and the cells involved are continuously patrolling various tissues to identify potential hazards. Some innate cells, such as macrophages and neutrophils, express pattern recognition receptors (Toll-like receptors; TLRs) which grant them a limited but broad and rapid capacity for recognising damage- or pathogen-associated molecular patterns (DAMPs or PAMPS, respectively), allowing them to initiate the immune response and inflammatory cascade. In addition, antigen-presenting cells (APCs) such as dendritic cells (DCs) are continuously scavenging for antigens, which they display on their surface to cells of the adaptive immune system within the secondary lymphoid organs (the spleen and lymph nodes). Adaptive (also known as acquired) immunity is specific and can recognise a much larger repertoire of antigens, and each cell within the adaptive immune system is able to recognise and respond to one individual antigen. There are two main cell types involved in adaptive immunity, B and T lymphocytes (or B and T cells). When presented with their specific antigen in the correct environmental context, these cells become activated and expand to fight the pathogen. B lymphocytes produce antibodies which recognise the specific antigen of that B cell, targeting that antigen for removal by other immune cells; this process is often referred to as the humoral response. T lymphocytes are split into cytotoxic or helper T cells based on their expression of surface proteins CD8 or CD4, respectively. As suggested by their names, cytotoxic T cells function by killing infected cells, while helper T cells work to help other immune cells fight infection. Helper T cells are of particular interest to immunologists due to their involvement in almost all stages of the adaptive response. The adaptive response has evolved so that responses to harmless antigens, such as self-antigen, induce immune tolerance. This is mediated mainly by regulatory T cells (Tregs) to prevent potentially damaging and unnecessary immune system activation. Immune cells produce various small proteins called cytokines, to communicate with other cells to induce, maintain or suppress the inflammatory process.

Research has found inflammation to be involved in several vascular diseases, such as atherosclerosis, hypertension and vasculitis [3, 4].

In this chapter we will focus largely on the immune and inflammatory processes involved in the pathology of atherosclerosis as this is well established within the literature and, as indicated above, accounts for a large proportion of cardiovascular deaths worldwide.

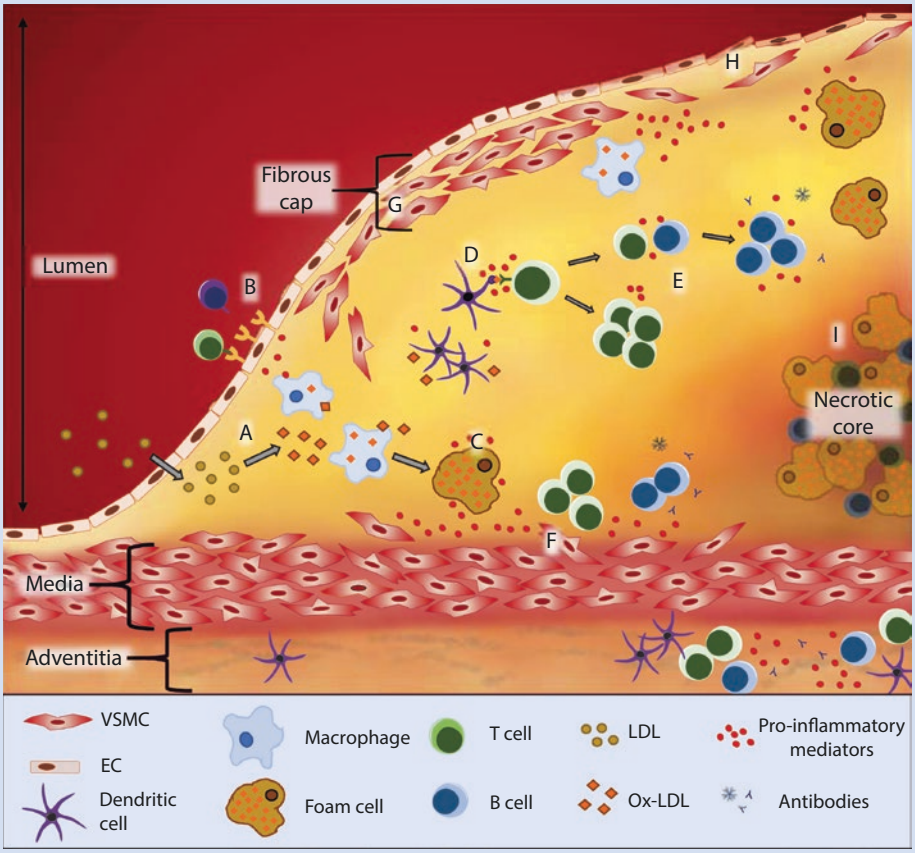

Fig. 21.1 Immune cells and inflammation in atherosclerotic plaque development **a** Circulating cholesterol bound to LDL diffuses through the endothelial layer of arterial vessel walls. Here it accumulates and can be modified through oxidation to become ox-LDL. **b** Ox-LDL activates tissue-resident innate immune cells, such as macrophages, which then secrete pro-inflammatory mediators and chemokines to recruit more immune cells into the vessel. **c** The macrophages continue to phagocytose ox-LDL until they become filled with cholesterol and are unable to continue efferocytosis or leave the vessel wall. These are referred to as foam cells, which continue to secrete pro-inflammatory cytokines. **d** Dendritic cells, which reside in the vessel wall, or have been recruited in due to the inflammation, uptake vascular antigens on the surface of their major histocompatibility complex (MHC) class I or II. **e** T cells able to recognise MHC-bound antigens will become activated resulting in their proliferation, secretion of various cytokines and activation of B cells, which are able to secrete antibodies. **f** The secretion of these pro-inflammatory mediators by various immune cells activates the VSMCs of the medial layer, which then migrate and proliferate in the intimal layer of the vessel, forming the fibrous cap **g** around the plaque. The thickness of the fibrous cap and subsequent stability of the plaque are largely dependent on the number of pro-inflammatory immune cells and the inflammatory mediators being secreted. **h** Some inflammatory cells secrete digestive enzymes, which degrade the fibrous cap causing it to become thin, unstable and prone to rupture. **i** Inefficient clearing of dead cells and debris within the plaque results in the formation of a necrotic core. This is a potent source of pro-inflammatory mediators, which are secreted from dying cells, and contributes to the overall size and stability of the plaque

21.2 Vascular Inflammation in the Atherosclerotic Plaque

Atherosclerosis has a multifaceted pathology, made evident by the complex composition of the plaques forming in the blood vessels. The immune system is known to play an extensive and complex role from the pathology onset to the advanced stages of the disease (Fig. 21.1).

The initiation of pathology occurs when there are high blood concentrations of low-density lipoprotein (LDL) which results in increased occur-

rence of LDL uptake and retention by the arterial wall. This is particularly prevalent at sites where there is lower shear stress, such as branching sites or curvatures of the vessel. Modification of LDL in this environment through oxidation results in the accumulation of oxidised LDL (ox-LDL), which acquire properties of DAMPs, resulting in stimulation of the innate immune system. A complex process follows involving activation of adaptive immune cells, endothelial cells and smooth muscle cells which in turn facilitate the exacerbation of the inflammatory process with influx of more immune cells, including monocytes that can then differentiate into macrophages and DCs [3]. Very advanced plaques can progress to a number of stages based on their composition. Some plaques develop a predominance of inflammation and accumulation of dead cells and LDL in a 'necrotic core', whereas others become fibrous and calcified, even developing bone-like tissue. Plaque calcification is influenced by inflammatory mechanisms and may influence both plaque stability and vulnerability. It is now recognised that the content of a plaque is as important as the amount it occludes the vessel lumen to how likely it is to cause acute cardiovascular events (heart attack or strokes). The role of the immune system in this divergence is discussed more below.

It has long been believed that LDL-mediated damage to the endothelial and smooth muscle cells of susceptible sites in the vessel triggers lesion formation and recruitment of immune cells. However, it is now recognised that resident innate immune cells within the vasculature are present all the time and may also be critical in the earliest stages. The consequent inflammatory processes within the vasculature at the site of the developing plaque ultimately drive pathology through to the advanced stages of the disease. During the advanced stages of pathology, there is evidence for the formation of structures termed artery tertiary lymphoid organs (ATLOs) within the adventitia proximal to plaques in mice. Interestingly, these ATLOs have been found to help to regulate the adaptive immune responses and have been shown to have a protective role in advanced atherosclerosis [5], highlighting opposing roles for the immune system at different stages of pathology.

It should be mentioned that a broad range of immune cell subsets are also present in the healthy vessels, although the proportion, activation and absolute content of these cells very significantly increase in pathology such as atherosclerosis.

21.2.1 Macrophages

Macrophages are well characterised in the context of atherosclerosis as they actively participate in all stages of the pathology. When ox-LDL is present within the blood vessel wall, it is phagocytosed by macrophages attempting to clear it. Through scavenging of ox-LDL in the vessel, macrophages will become activated towards an inflammatory phenotype, releasing pro-inflammatory cytokines like tumour necrosis factor (TNF)-alpha, interleukin (IL)-6 or IL-12, which lead to increased local inflammation. Overtime, this process results in the macrophages themselves becoming full of cholesterol, at which point they are unable to function as phagocytes or leave the vessel and become what are known as foam cells. Accumulation of these foam cells within the vessel wall to the point of being visible in a dissected artery is referred to as a fatty streak. Interestingly, these lesions can be found within the aorta and coronary arteries as early as childhood and adolescence. Due to the inability of foam cells to leave the vessel, there is no resolution of the inflammatory response; instead it is maintained by them continuing to secrete pro-inflammatory cytokines.

Recent data indicates that the increased number of macrophages observed in the atherosclerotic vessels depends predominantly on local macrophage proliferation rather than recruitment of circulating monocytes [6]. When these cells dye by apoptosis, they remain within the lesion and contribute to the formation of the above-mentioned necrotic core, a structure within the plaque composed of dying, necrotic cells and tissue debris from the surrounding, damaged vessel layers. The extent of macrophage apoptosis and ineffective clearance of the necrotic core is thought to be one of the main factors contributing to the plaque instability in advanced atherosclerosis [7].

Resolving macrophages function to resolve inflammation through phagocytosis of apoptotic bodies and debris; secretion of anti-inflammatory cytokines, such as IL-10; and release of factors able to promote tissue repair or stabilise the fibrous cap of the plaque, such as collagen [3]. The mechanism behind the polarisation of macrophages towards different functions within the plaque remains largely unknown. One hypothesis suggests that a population of tissue-resident macrophages, originating from the embryonic yolk sac, proliferate to seed the resolving

population, whereas inflammatory macrophages differentiate from infiltrating monocytes. However, a recent study found evidence that pro-resolving macrophages may also be derived from recruited monocytes [8].

21.2.2 Dendritic Cells

The contribution of DCs is also key to driving the inflammatory pathology of atherosclerosis. However, this remains less well understood. This is mainly due to the fact that the precise identity of vascular myeloid cell subsets is poorly characterised as they have been defined by the expression of a limited number of markers. Recent technologies such as cytometry by time of flight [9] or single-cell RNA sequencing (scRNA-Seq) are now helping to classify aortic immune cell heterogeneity at the single-cell level [10].

As professional antigen-presenting cells, DCs are key in linking innate and adaptive immunity, and several DC subsets have been shown to play a key role in atherosclerosis. For example, studies in mice have found that selective deficiency in molecules required for antigen presentation (major histocompatibility complex (MHC) class II) in a specific subset of DCs called plasmacytoid DCs ultimately protects from proatherogenic T cell immunity [11]. Furthermore, mature DCs have been found to accumulate within plaques during lesion development, as shown in mouse and human studies. This would suggest that both antigen presentation and DC maturation are involved in driving atherogenesis. However, there is evidence which indicates that DC activation of T effector cells may not be the only mechanism by which DCs influence pathology. DC migration from peripheral tissues to the lymphoid organs is severely impaired during hypercholesterolemic conditions. Therefore, their inability to leave the vessel could have similar implications to that of macrophages, such as continuous secretion of pro-inflammatory cytokines and consequent recruitment of more immune cells [12].

In humans, DCs have been shown to form clusters at rupture-prone regions of plaques, where they can be seen making frequent DC-T cell contacts [13]. The nature of antigen underlying such interactions remains unknown, and various candidates have been proposed although without unequivocal identification.

21.2.3 T Cells

Several T cell subsets are identified in the atherosclerotic plaque, and through the release *in situ* of a broad range of cytokines, they modulate the development of the pathology and importantly regulate plaque stability and risk of rupture. A large number of the T cells identified within human lesions display an activated T effector phenotype, around two thirds of these are T helper cells, the other third being cytotoxic T cells [14]. T helper cells can be further subdivided into smaller subsets based on the type of cytokines they secrete and consequent immune response they elicit. Traditionally, T helper 1 (Th1) cells secrete a cocktail of cytokines including interferon (IFN)-gamma, IL-2 and TNF-alpha which serve to drive the cell-mediated immune response and are predominant in atherosclerosis. Th2 cells mount responses to extracellular parasites through release of several interleukins, including IL-4, IL-5 and IL-13, which promotes humoral antibody-mediated immunity. This is not believed to play a prevalent role in vascular disease as the Th1 phenotype. Finally, the most recently described subsets are Th17 cells which are phenotypically similar to Th1 cells but are characterised by their secretion of the cytokine IL-17. These cells are also highly increased under atherosclerotic conditions. The Th1 and Th17 bias in vascular inflammation is also observed when there is a breach in immune tolerance in autoimmune disorders.

T cells are one of the earliest immune cells to be recruited into the developing lesion and continue their influx well into the advanced stages of pathology. This suggests there is a chronic antigen-driven immune response, which is further supported by evidence that groups of T effector cells isolated from plaques possess monoclonal T cell receptors, meaning they all recognise one specific antigen. The specific antigen that drives the T cell response remains unclear; however, it is believed to be an epitope (small peptide fragment) of native and modified LDL [15].

During the immune response, the burden of inflammation can be dictated by the proportion of T effector cells, particularly Th1 and Th17, to immunosuppressive Treg cells. Treg cells have been shown to inhibit the formation of foam cells, as well as drive the anti-inflammatory macrophage phenotype. The mechanisms controlling this ratio in the context of atherosclerosis are not well understood [3, 14].

21.2.4 B Cells

B cells can be broadly separated into two distinct cell subsets, B1 and B2 cells. B1 cells have a protective role in atherosclerosis development, whereas B2 cells are thought to be detrimental for the pathology, although this is disputed by some studies. The role for B cells in atherosclerosis was first highlighted by a study which found removal of the spleen (the site of B cell maturation) in mice aggravates the disease process. Subsequent transfer of mature B lymphocytes which had been exposed to the pathology was sufficient to not only rescue the phenotype but inferred enhanced protection over the sham group. Specific transfer of B1 cells could also achieve this reversal, and this subset is now believed to carry the most potent protective influence. The overall protective role of B cells was then challenged by studies in which depletion of B2 cells resulted in decreased lesion size, potentially because the B cells influence pathogenic helper T cells they interact with. This has led to the model that is now widely accepted of opposing roles of the different B cell subsets, similar to what is seen for other immune cells such as macrophages [14, 16].

21.3 Inflammation and Plaque Stability

The fibrous cap is the structure which covers and protects an atherosclerotic plaque, preventing its contents from mixing with circulating blood. It is comprised of fibrous connective tissue, the thickness and composition of which determines plaque stability and vulnerability to rupture. In the scenario of plaque rupture, the content of the atheroma leaks out into the blood stream, leading to the formation of a blood clot called a thrombus. These can cause blockage in blood vessels, stopping blood flow and delivery of oxygen to downstream tissues; this process is called ischaemia. If ischaemia occurs in coronary or cerebral arteries – stopping blood flow to cardiac or brain tissue – this clinically manifests as a myocardial infarction or stroke, respectively. Distinct biological features of vulnerable plaques include the presence of a thin fibrous cap (thin-cap fibroatheromas; TCFA) and a core rich in macrophages and T lymphocytes [17]. Culprit lesions in acute coronary syndromes (ACS) show particularly strong immune activation and elevated numbers of macrophages [3, 7]. Matrix metalloproteinases (MMPs) are tissue-degrading enzymes released by activated macrophages and foam cells, contributing to plaque instability through digestion of the fibrous cap. Immune cells in unstable plaque also produce reactive oxygen species (ROS), which are able to activate MMPs and exacerbate endothelial dysfunction.

Mast cells have also recently gained attention in atherosclerosis. These are innate immune cells normally associated with defence against parasites or allergies, which release histamines during inflammatory responses to antigen (such as pollen). They have been identified especially at sites of plaque erosion and rupture. Pro-inflammatory cytokines, histamine, leukotrienes, thromboxane and proteases such as tryptases and chymases produced by mast cells can induce MMPs causing plaque destabilisation and/or intraplaque haemorrhage.

Importantly, other arterial layers, different from the plaque, have recently been shown to play a pivotal role in vascular inflammation. In particular, the spotlight is now on the adventitia and perivascular adipose tissue (PVAT).

Importantly, circulating biomarkers are not able to identify vulnerable plaques, and also routinely used invasive and non-invasive diagnostic approaches are not able to work at the molecular resolution required to fully discriminate stable from unstable plaques. Recent advances in the understanding of the role of vascular inflammation in the processes leading to plaque rupture will pave the way to more progress in this field [18].

21.4 Experimental Models of Atherosclerosis

Inflammatory and immune mechanisms in atherosclerosis have been extensively studied in experimental murine models [19]. Several different models have been utilised by researchers to determine the pathological processes spanning plaque development; however no single one mimics the entire complexity of the human pathology.

21.4.1 Altered Diet

Early mouse studies employed modified diet highly enriched in saturated fat, cholesterol and cholate to generate pathology. Interestingly, these

studies found C57BL/6 inbred mice to be more susceptible to developing lesions than BALB/c and C3H, due to their genetic predisposition towards a pro-inflammatory response to oxidised lipids. Despite this, on modified diet C57BL/6 mice will only develop small early-stage lesions, and so further genetic modifications were introduced in this strain.

21.4.2 Genetic Modification

The two most common genetic modifications used are global ablation in apolipoprotein E (*Apoe*)$^{-/-}$ or low-density lipoprotein receptor (*Ldlr*)$^{-/-}$. *Apoe*$^{-/-}$ mice are spontaneously hypercholesterolemic and therefore will develop lesions during feeding of both normal and high-fat diets, whereas *Ldlr*$^{-/-}$ mice require a high-fat diet. Both develop early foam cell-rich fatty streak lesions that over time will develop into more advanced plaques with further immune cell infiltration and fibrous cap formation.

The classical *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ murine models have been key in helping us understand various pathological and inflammatory processes, which occur during lesion formation and development, particularly through combining them with other genetic mutations. For example, early studies using immunodeficient mice crossed onto the *Apoe*$^{-/-}$ background found them to have reduced pathology. This effect can be reversed when T cells are transferred into these mice causing an aggravated pathology, clearly demonstrating a causative role for the immune system in the disease process [14].

21.4.3 Genetic Backcrossing and Chimaeras

Genetic knockouts are useful tools for dissecting the role of particular genes, or the cell type that gene regulates, in disease development. In atherosclerosis research, this has been achieved through either backcrossing of mainly *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice with mice deficient in a gene of interest or studying chimaeric mice. To generate chimaeras for atherosclerosis studies, the haematopoietic cells of an *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mouse are ablated by lethal irradiation and then replaced by that of another genetically modified mouse through a bone marrow transfer (BMT), thus generating an atherosclerosis-susceptible mouse which possesses a genetically modified immune system to study specifically how immune cell function affects pathology.

21.4.4 Inducible Atherosclerosis

In recent years a new method for inducing hypercholesterolaemia and atherosclerosis in murine models without genetic engineering has been developed [20]. Overexpression of plasma protein convertase subtilisin/kexin type 9 (PCSK9) in mice via adeno-associated viral (AAV) vector gene delivery results in the degradation of hepatic LDL receptors and consequent hypercholesterolaemia, giving rise to the atherosclerotic pathology when combined with high-fat diet [20]. This methodology allows testing of the genetic interaction of several mutations without the need for difficult and time-consuming backcrosses with genetically modified hyperlipidaemic mice.

The main limitation of all models of atherosclerosis is the absence of a clinical event, such as occurring in humans. While some studies have claimed that mouse plaques can rupture, this is the subject of considerable debate [19].

21.5 Inflammation and Immunity in Hypertension

Inflammation in vascular disease does not solely refer to atherosclerosis; other vascular pathologies also possess a prominent immune component, such as vasculitis, abdominal aortic aneurysm and hypertension. These disorders share the pathological process of oxidative stress, which occurs due to an imbalance in a system's production of reactive oxygen species (ROS) and the inability to detoxify reactive species and repair the damage they cause. Overproduction of ROS and reactive nitrogen species within the vasculature permeabilises the endothelial layer of blood vessels, increasing the influx of lipoproteins such as LDL which will subsequently be modified to ox-LDL in the oxidising environment, leading to stimulation of the innate immune system as discussed previously. Inhibition of the ROS-producing enzyme, NADPH oxidase, attenuates macrophage accumulation in a hypertensive rat model [4]. Furthermore,

immunosuppression reduced hypertension in rats, while transfer of immune cells from hypertensive animals could induce pathology in normal rats. These data demonstrate that an immune response is associated with hypertensive pathology. A key study published in 2007 by Guzik et al. [21] found that RAG1 immunocompromised mice lacking both mature B and T cells did not develop high blood pressure after the induction of hypertension using two different experimental methods. Interestingly, the transfer of T cells and not B cells restored pathology in these mice. This pathological role for T cells in hypertension was shown to be dependent on the hormone angiotensin II, a component of the renin-angiotensin system, which is a key mediator of hypertension. Similar to atherosclerosis, the specific antigen(s) which elicit the adaptive immune response in hypertension remain unknown.

21.6 Circulating Biomarkers of Inflammation in Vascular Disease

Given the significant contribution of immunoinflammatory responses in vascular pathologies, inflammatory biomarkers are routinely used for the assessment of CV risk.

21.6.1 C-Reactive Protein

C-reactive protein (CRP) is a marker of inflammation produced by the liver that is regularly measured in medical blood tests for patient diagnosis, disease management or as a prognostic biomarker. CRP levels have been widely demonstrated to associate with increased risk of cardiovascular incidence, and CRP was suggested to play a functional role in atherogenesis itself. It can be found within the atherosclerotic lesion, bound to LDL and colocalised with various immune cells. However, CRP has been difficult to study in murine atherosclerosis, as this is not an acute-phase protein in mice. When human CRP is overexpressed in *Apoe*$^{-/-}$ mice, pathology was increased, albeit modestly and confined to male mice. Despite this, Mendelian randomisation studies have found that CRP is not causative in cardiovascular disease, demonstrating the potential unreliability of murine studies in identifying contributing factors of pathology [2], and that biomarkers are not necessarily causally involved. CRP alone is not an effective biomarker for diagnosis or prognosis of unstable plaques, primarily due to its non-specificity; expression of CRP can be triggered by any acute systemic inflammation, such as pathogen infection. For this reason, it is useful only when used in combination with other more specific disease characteristics to establish patient risk.

21.6.2 Cytokines and Chemokines

In both mice and humans, several circulating cytokines – mediators and controllers of immune responses – are detectable and have been tested as biomarkers. Pro-inflammatory Th1-related cytokines (i.e. IFN-gamma, IL-18, IL-12, etc.) are known to promote disease development, whereas anti-inflammatory Treg-secreted cytokines (i.e. IL-10) exert anti-atherogenic activities. Decreased levels of IL-6 are associated with better clinical outcomes in coronary heart disease (CHD). This is supported by Mendelian randomisation data, which demonstrated that genetic variations resulting in higher circulation of IL-6 receptor are protective in CHD [2]. This is backed by experimental evidence which revealed that IL-6 is secreted by pro-inflammatory macrophages following stimulation with ox-LDL, as well as smooth muscle cells responding to inflammation and injury, resulting in increased expression of vessel wall adhesion molecules, facilitating influx of immune cells. However, this is disputed by evidence from an *Apoe*/*Il6* double knockout model, in which there was a decrease in plaque stability. The contribution of chemokines to vascular inflammation has been extensively reviewed [22]. Unfortunately, not all pro-inflammatory mediators observed in the plaque can be detected in peripheral blood.

21.6.3 Immune Cells

It has been reported for many years now that ACS are associated with increased counts of total circulating leucocytes, particularly an increased proportion of T lymphocytes and monocytes [23]. As would be expected, activated Th1 effector cells are detected at higher levels in peripheral blood of ACS patients, while Tregs are decreased. Th17 cells are also increased in patients with advanced

atherosclerosis and ACS, although studies on the role of Th17 in atherosclerosis development and plaque stability led to conflicting results, as one cohort of patients showed increased risk of cardiovascular incidents associated with low IL-17, the signature cytokine of Th17 cells. Therefore, a clearer understanding of the distinct roles of T cell subsets is required for their use as biomarkers of cardiovascular risk to be effective. Monocyte subpopulations in ACS appear to be altered as well.

21.6.4 Summary

In general, murine studies support a role for circulating biomarkers such as cytokines and acute-phase proteins as indicators of pathology in atherosclerosis development. Additionally, various indicators of systemic inflammation are generally accepted to provide clues for risk stratification in human atherosclerosis [2]. A focus on local immune events within the vascular wall, unstable plaque or coronary thrombus may be of more use than the systemic cues.

Conclusions and Clinical Perspectives

Importantly, in September 2017 the CANTOS trial (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) was published [24]. CANTOS is a large (10,061 participants in 39 countries), randomised, double-blinded, placebo-controlled clinical trial using a monoclonal antibody against IL-1beta (the first-identified inflammatory cytokine) for secondary prevention in patients previously affected by myocardial infarction and with high levels of CRP. Patients treated with canakinumab showed significantly reduced rate of secondary cardiovascular events, however, without reduced all-cause mortality. This trial finally confirms the viability of targeting inflammation in CVD. However, more research is still required in this area with the aim to develop new, more selective and better-tolerated immunomodulatory therapies for atherosclerosis and to develop better methods for early diagnosis of vascular inflammation and monitoring of disease progression.

In conclusion:

- The immune system plays a complex and crucial role in vascular pathology; despite this it remains unaddressed by routine clinical therapeutics and diagnostics.
- The stability of atherosclerotic plaques is largely determined by its inflammatory profile. However, using current clinical diagnostic tools, we are unable to easily distinguish stable versus unstable plaques.
- Mouse models have and remain to be very useful tools for dissecting out specific inflammatory pathways which have a large effect on overall disease burden, serving as indication of effective potential targets for therapy.

Gaps in Knowledge

- We still need to understand in-depth immune mechanisms in atherosclerosis with the aim to identify potential new and selective therapeutic targets.
- The exact process by which antigen-specific T cells become activated in CVD is poorly understood. Further research is required to explain how recognition of epitopes within the vessel wall drives adaptive immune responses. This knowledge would lead to the development of new potential vaccination strategies.
- As the importance of examining and targeting inflammation gains more traction in the clinical setting, it is crucial that we are better armed with conclusive evidence surrounding the effective use of anti-inflammatory therapies in CVD.

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Diagnostic and Therapeutic Targeting of Inflammation

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_22

Key Concepts

- Inflammation plays a key role in vascular function by mediating endothelial dysfunction and atherothrombosis.
- Systemically, links between C-reactive protein, IL-6 or TNFα and cardiovascular risk have been demonstrated. In particular IL-6 seems to be linked causally as shown by Mendelian randomization.
- Atherosclerosis and atherothrombosis are mainly localized; therefore, targeting inflammation using FDG-PET and more recently utilizing nanomolecular targeting of immune cells have been used to identify inflamed, unstable plaques. Moreover, perivascular fat CT attenuation has been recently used to monitor perivascular response to vascular inflammation.
- Two major randomized trials were concluded showing that targeted immune interventions aimed at IL-1β (using canakinumab in CANTOS trial) are effective in alleviating risk of myocardial infarctions, but such effect is not observed in unselected general population treated with methotrexate (in CIRT).
- Majority of studies show effectiveness of immune-targeted interventions on vascular risk and dysfunction in patients with autoimmune diseases such as SLE or arthritis.
- Intensive treatment of periodontitis removes not only local periodontal inflammation but improves vascular dysfunction.
- In summary, immune targeting can be used in a myriad of diagnostic and therapeutic approaches which will likely be unravelling in the forthcoming months.

22.1 Introduction

Increasing understanding of the causal link between inflammation and the pathogenesis of vascular disease leads to urgent need for therapeutic and diagnostic use of these important discoveries. Indeed, while numerous genetic and pharmacologic manipulations have led to alleviation of vascular disease in models of atherosclerosis, heart failure, hypertension as well as diabetes, translating these findings to clinical use poses a significant challenge that will be discussed in this chapter.

22.2 Assessing Inflammation in Vascular Pathology Diagnosis

Since vascular inflammatory infiltrates have been found by pathology studies in atherosclerotic vessels, a quest continues to understand the links between local inflammation in the vessel wall and development of pathology. This has been strongest shown in relation to unstable atherosclerotic plaque, but inflammatory infiltrates have been identified at early stages of atherosclerotic pathology as well as hypertension in perivascular adipose tissue.

Initial ideas were focusing on systemic levels of biomarkers such as C-reactive protein (CRP), hs-CRP, IL-6 or TNFα. Indeed, all of these have been shown to be associated with cardiovascular risk as well as hypertension development [1]. While initially substantial focus was placed on predictive value of CRP levels [2], recent particularly strong evidence from several meta-analyses as well as Mendelian randomization approaches point to IL-6 as a key circulating biomarker potentially causal in vascular disease. However a key disadvantage of circulating biomarkers is that they are relatively unspecific and may reflect inflammatory burden elsewhere in the organism (TNFα, IL-6) or even be affected by organ, e.g. liver dysfunction (CRP).

Therefore, intensive efforts have been undertaken to identify immune cells within the vessel wall, whether it is in atherosclerotic plaques or in the perivascular fat and adventitia in the earlier stages of vascular inflammation. This has been achieved through detecting high metabolism (high glycolytic rates) of immune cells within the vessel wall that leads to accumulation of fluorodeoxyglucose (FDG)-positron emission tomography (PET) or using specific magnetic nanoparticles in MRI detection. More recently, more specific techniques have been proposed combining nanoparticles with surface-enhanced Raman spectroscopy (SERS) detection. FDG-PET imaging is currently

used to detect high metabolically neoplastic cells and associated inflammation. While initially quite inaccurate, introduction of positron emission tomography-computed tomography (PET-CT) and PET-cardiac magnetic resonance (CMR) has strengthened its clinical utility.

FDG-PET was first introduced to imaging of vascular inflammation more than 15 years ago but, in contrast to cancer, still remains primarily a research tool rather than a useful diagnostic utility in vascular medicine [3]. FDG uptake by the vessels reflects their infiltration with macrophages in the plaque which corresponds to plaque instability. Vascular inflammation measured using this utility is an independent predictor of cardiovascular risk and can be reversed by therapies inhibiting vascular inflammation such as pleiotropically acting statins, although prospective data are limited. Other possibly more specific agents have been tested in the context of detection of vascular inflammation. This includes 18F-sodium fluoride (NaF), which corresponds not only to inflammation but also calcification [3].

Magnetic resonance-based techniques for detection of inflammation are based to large extent on the fact that magnetic nanoparticles have propensity to be engulfed by macrophages or can be coupled with antibodies targeting immune-specific molecules or cells. Such signals can be with high resolution visualized by magnetic resonance imaging (MRI) or PET. Various radiotracers are being developed to visualize inflammation in the vascular wall using an integrative PET with MRI approaches. Nanobody radiotracers targeted to different pro-inflammatory molecules have been developed including nanobodies targeting vascular cell adhesion molecule (VCAM)-1, lectin-like oxidized low-density lipoprotein receptor (LOX)-1 or macrophage mannose receptor (MMR) radiolabeled with copper-64 or gallium 68 and used to visualize inflammation in the aorta of hyperlipidemic apolipoprotein E (ApoE)$^{-/-}$ mice and atherosclerotic rabbits using a combination of in vivo PET/MRI [4]. The use of such short-lived and clinically relevant radioisotopes which are already widely used as a contrast agent for PET/MRI opens the possibility for quick translation.

The development of SERS creates a unique modality to detect unique and characteristic Raman signals enhanced by noble metal nanoparticles targeted to immune cells or pro-inflammatory adhesion molecules such as VCAM-1 or ICAM-1. Initial attempts have been described of fingerprint-like Raman signals from reporter molecules on the NP surface able to detect ICAM-1, VCAM-1 or P-selectin in inflamed arteries in animal models and human coronary arteries [5] providing proof of concept for utility of this method in detecting vascular inflammation. Unique characteristics and heterogeneity of such signals make this method potentially useful for detecting multiple inflammatory molecules simultaneously.

Very recently, a well-known phenomenon of the effect of inflammation on adipocyte morphology and function has been turned into a simple and potentially very valuable technique. A new computed tomography angiography analysis method was developed to detect vascular inflammation through assessment of perivascular fat attenuation. This technique uses the simple fact that vascular inflammation results in reduction of lipid accumulation in perivascular adipose tissue (PVAT)-derived preadipocytes. This approach has been successfully used in cardiovascular risk prediction making it a potentially important and cheap option in noninvasive detection of plaque instability due to vascular inflammation [6].

In summary, diagnostically, both systemic and local inflammations were targeted. Systemically, simple approaches were undertaken to show links between C-reactive protein, IL-6 or TNFα as indices of systemic inflammation and their links to cardiovascular risk. However, vascular diseases such as atherosclerosis and atherothrombosis are mainly local pathologies. Therefore, targeting inflammation using FDG-PET and more recently utilizing nanomedical approach have been developed. Moreover, recent observations that vascular inflammation changes perivascular fat properties created a simple opportunity to measure perivascular fat CT attenuation in the detection of vascular inflammation. All of these may be essential for selecting patients for therapeutic interventions aimed on inhibiting inflammation.

22.3 Therapeutic Strategies for Targeting Vascular Inflammation

Having identified that inflammation is an inherent part of the development of vascular dysfunction that can be clinically visualized in patients with vascular disease, therapeutic strategies have

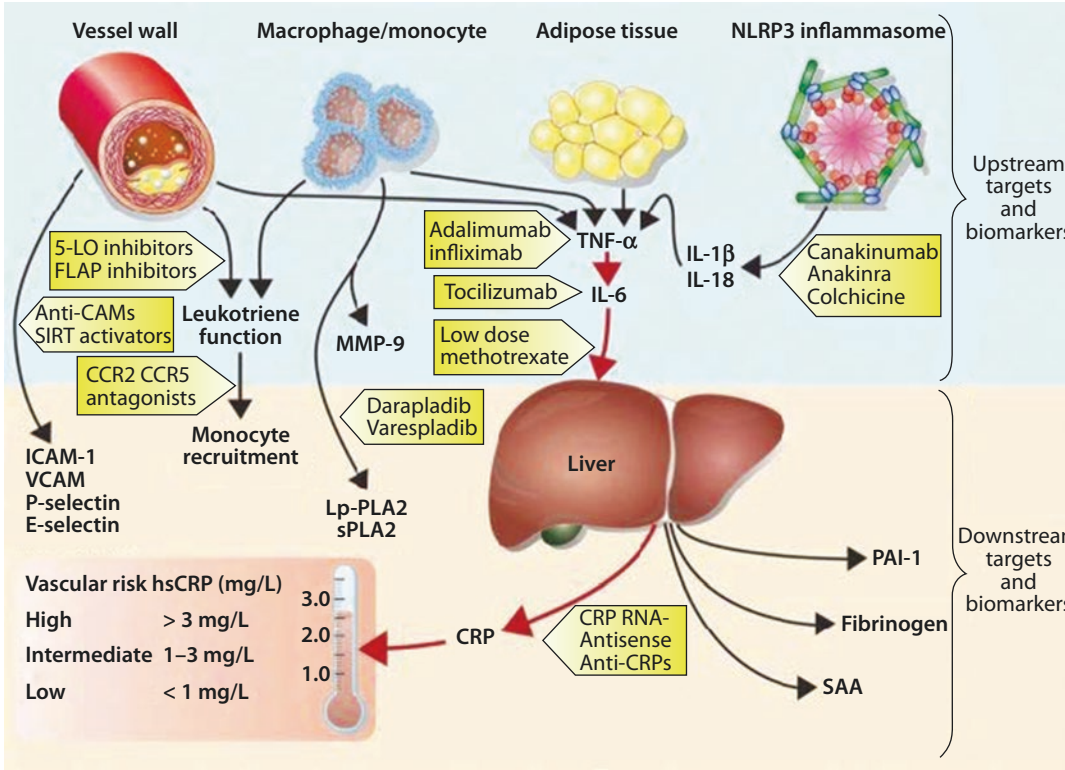

Fig. 22.1 Targeting specific immune pathways in vascular biology. (Taken from Ridker, Luscher, 2015, *Eur Heart J* [7, 8] ➔ NEED LICENSE)

been developed to target vascular inflammation (Fig. 22.1) [7, 8]. These range from unspecific anti-inflammatory approaches through the use of moderately specific compounds acting on selected arms of immunity (innate/adaptive, etc.) leading up to the most recent studies of specific targeting of individual cytokines and chemokines, implicated in the pathogenesis of vascular dysfunction by mechanistic studies.

22.3.1 Anti-inflammatory Effects of Classical Vascular Pharmacotherapy

HMG-CoA reductase inhibitors (statins) are most established in cardiovascular prevention. Through multiple mechanisms, they prevent the development of vascular dysfunction. While being potent lipid-lowering compounds, statins have strong anti-inflammatory properties. These are mediated by their effects on adhesion molecule expression in the vessel wall, nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activation in a wide range of vascular and inflammatory cells, as well as macrophage content and M1/M2 polarization in neointima or plaque. Statins inhibit T cell and macrophage cytokine release and prevent upregulation of chemokines such as RANTES in models of atherosclerosis. The JUPITER trial showed for the first time in a randomized and double-blind fashion that statins can reduce major cardiovascular events even in subjects without hyperlipidaemia but with elevated high-sensitivity CRP levels [9]. Concomitant reduction of low-density lipoprotein (LDL) cholesterol and CRP leads to maximal benefit showing that both effects of statin appear to be additive in cardiovascular prevention [10].

Statins are however not the only medications with significant anti-inflammatory effects. For example, angiotensin-converting enzyme (ACE) inhibitors may have immunomodulatory effects shown initially in the kidneys but then extended to vascular inflammation and endothelial dysfunction observed in subjects with autoimmune pathologies. This includes ramipril [11] as well as angiotensin II receptor blockers (ARBs) [12].

The anti-proteinuria effect of ARBs in systemic lupus erythematosus (SLE) has been supported in another small study [13]. These small studies are hampered by a lack of randomization and a suitable control group, concurrent use of immunosuppressive therapy and the short duration of treatment.

22.3.2 Immunometabolic Reprogramming in Vascular Inflammation

Understanding immunometabolism led to the development of approaches targeting fatty acid oxidation and glycolysis within immune cells for alleviating vascular inflammation. Classic drugs like metformin, an activator of 5′ AMP-activated protein kinase (AMPK), alter T-cell differentiation inhibiting Th1 and Th17 cells while enhancing T regulatory cells [14]. The key concept in this therapeutic approach is that it may allow immune cell metabolic reprogramming. For example, inhibiting glycolysis through pyruvate kinase can reprogram macrophages towards an M2 phenotype, while inhibition of glycolysis (using, e.g. 2-deoxyglucose) can lead to switching T-cell profile from Th17 to T regulatory [15], well known for their antiatherogenic effect.

22.3.3 Targeting Vascular Inflammation in Autoimmune Pathologies

Anti-inflammatory treatments that are traditionally used to treat autoimmune diseases can also reduce vascular inflammation ranging from vasculitis to atherosclerosis or hypertension-associated vascular immune cell infiltration. Effects of these medications on vascular inflammation that accompanies hypertension are a good model to look at this problem, as the prevalence of hypertension is very significantly increased in a myriad of autoimmune disease. For example, mycophenolate mofetil (MMF) has been shown to reduce vascular and renal inflammation in patients affected by psoriatic arthritis and rheumatoid arthritis (RA), with essential hypertension. This also results with significant alleviation of hypertension [16]. Similar effects were reported for other medications such as lornoxicam in patients with RA [17], and more recently evidence has accumulated that specific anti-TNF treatments lead to an improvement of vascular function, in particular beneficial effects on endothelial function and reduced aortic stiffness [18, 19]. Moreover, these effects are also observed in microvasculature as demonstrated recently in patients with spondyloarthropathies. Similar effects inhibiting vascular inflammation have been shown for tocilizumab, a humanized monoclonal antibody for the IL-6 receptor (IL-6R) in RA patients [20], although metabolic effects of these treatments on lipoproteins should be taken into account [20–22]. While based on majority of evidence regarding the role of immune cells in vascular dysfunction, T cells and macrophages are typical targets, targeting B cells, e.g. using rituximab, may inhibit atherosclerotic or hypertensive vascular dysfunction and remodelling [23].

22.3.4 Targeting Vascular Inflammation in Cardiovascular Disease

As discussed in previous chapters, genetic and pharmacologic targeting of vascular inflammation in animal models of vascular disease has led to an in-depth understanding of inflammatory mechanisms of atherosclerosis, hypertension and vascular dysfunction [24–26]. It has also provided preclinical evidence supporting the use of immune-targeted therapies in alleviating vascular dysfunction. For example, targeting cytokines such as IL-1β, TNFα and chemokines like CCL-2 or RANTES leads to prevention of endothelial dysfunction in a range of animal models [24–27]. Targeting key inflammatory mechanisms has also been shown to alleviate vascular remodelling in models of hypertension and atherosclerosis. For example, targeting of vascular T-cell-derived cytokine IL-17A inhibits deposition of collagen in the aortic adventitia and media [28], subsequently preventing aortic stiffening. Other cytokines such as TNFα act synergistically to further promote inflammatory responses [28]. Etanercept, a TNFα antagonist, reduces endothelial dysfunction and pulse wave velocity and aortic stiffness in patients with rheumatoid arthritis and in mice with hypertension.

Accumulation of such basic evidence has led to clinical trials in which the hypothesis has been tested that targeting inflammation may reduce cardiovascular risk.

Two recent major outcome trials have addressed the important question of the utility of targeting inflammation in general in patients with cardiovascular risk. The first of these, the Canakinumab Anti-inflammatory Thrombosis Outcomes Trial (CANTOS), investigated specific inhibition of IL-1β using a monoclonal antibody, canakinumab, which can reduce recurrent vascular events [29]. While the choice of this specific intervention has been widely discussed, the selection of this compound was primarily justified by the fact that in previous studies, it has shown very significant dose-dependent reductions in IL-6 and CRP. This is important as IL-1β producing nod-like receptor pyrin domain 3 (NLRP-3) inflammasome has been implicated in the pathogenesis of atherosclerosis, hypertension as well as vascular dysfunction. In the second study, the Cardiovascular Inflammation Reduction Trial (CIRT), a much less specific intervention was tested using low dose of methotrexate (15–20 mg per week).

While low-dose methotrexate, at least in arthritis patients, has also been shown to reduce CRP, IL-6 and TNFα [30], both studies have shown very different results shedding clear light on perspectives and future directions of targeting of vascular inflammation.

In summary, while targeted immune intervention aimed at IL-1β (in CANTOS trial) was effective in alleviating risk of myocardial infarctions, such effect is not observed in unselected general population treated with methotrexate (in CIRT). It is important to note that while in CANTOS, IL-6, CRP and TNFα were all reduced by the treatment, low-dose methotrexate failed to affect these biomarkers in patients with stable cardiovascular disease. This is important, as it helps to explain the discrepancy between CIRT and smaller studies showing effectiveness of immune-targeted interventions on vascular risk and dysfunction in patients with autoimmune diseases such as SLE or arthritis. The CIRT was performed in subjects with stable coronary artery disease. Thus, when the plaque is stable, low-dose methotrexate does not achieve clinical effect.

In summary, it is essential to emphasize the need to recognize the diversity of possible inflammatory mechanisms in vascular disease that can be targeted. The evidence suggests that targeting the IL-1β-IL-6 pathways is of primary importance which indicates a critical role of innate immunity linked to NLRP3. Thus, ongoing trials using either colchicine or direct inhibitors of NLRP3 may also bring additional valuable information. Colchicine was indeed tested recently in Low-Dose Colchicine (0.5 mg/day; LoDoCo trial) trial in subjects with stable coronary artery disease (CAD) and led to the reduction in the primary end point of recurrent acute cardiovascular events [31].

22.3.5 Targeting Chronic Systemic Inflammation

An important aspect of targeting systemic immune activation in sites of chronic inflammation may be a viable approach. For example, intensive treatment of periodontitis removes not only local periodontal inflammation but improves vascular endothelial dysfunction [32], and the degree of improvement is associated with improvement in measures of periodontal disease and reduction of key systemic pro-inflammatory biomarkers such as CRP and IL-6 [32]. This provides proof of concept for the use of such therapies in alleviating residual inflammatory cardiovascular risk.

22.3.6 Local Targeting of Vascular Inflammation

On an opposite, but much less clinically translated end of the spectrum of vascular inflammation, targeting is the concept that while vascular/endothelial dysfunction is observed systemically, atherosclerotic plaques develop locally, and local targeting of vascular inflammation is needed in the future. The development of specific nanoparticles targeting local cardiovascular inflammation in the management of CVD is ongoing and will allow the targeted delivery of atheroprotective, anti-inflammatory or thrombolytic drugs. Cell labelling with nanoparticles for cell-based therapies can also be envisioned to enhance regenerative medicine approaches [33]. A recent study provided evidence that atherosclerotic plaque macrophages can be targeted using a nanomedicine cargo in which prednisolone was encapsulated into liposomal

nanoparticles. After systemic intravenous infusion, nanoparticles localized in the macrophages isolated from atherosclerotic plaques [34]. The effect on pathology development is yet to be determined, but the proof of concept of such local targeting is clear.

Conclusions and Clinical Perspectives

In summary, immune targeting can be used in a myriad of diagnostic and therapeutic approaches, and clinical utility of which will likely be unravelling in the forthcoming months and years. The first proof-of-concept trials clearly show that specific targeting of the immune system may find a place in vascular medicine, although much more work using various approaches is needed to identify the most feasible and safest approaches.

Gaps in Knowledge

A number of key questions remain, as the area of vascular inflammation targeting is in its decisive moment:

- Which modality of vascular inflammation imaging will prove most clinically useful and predictive of cardiovascular events?
- Which immune cell should be targeted diagnostically and therapeutically; will it be the same in different cardiovascular disorders?
- How specific level of therapeutic targeting needs to be?
- What will be the role of patient stratification/precision medicine in designing and introducing vascular inflammation targeting to practice?
- What will be the role of immune checkpoint inhibitors?
- How to improve safety profile of immune-targeted therapies?

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Perivascular Adipose Tissue

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_23

23

Key Concepts

- While traditionally viewed as little more than a simple lipid reserve, perivascular adipose tissue (PVAT) boasts an extensive biological arsenal which when mobilized can modulate vascular function.
- PVAT dysfunction in disease is associated with alteration to its secretory profile which can create a contractile and pro-inflammatory effect.
- PVAT dysfunction may be responsible for the comorbidity and mortality seen in various disease states, particularly the development of hypertension in obesity – now one of the world's leading causes of mortality.
- Recently, it has been discovered that PVAT has a role as a sensor of coronary inflammation. This can be detected using a novel CT-derived metric, representing the first attempt at clinical translation of PVAT biology.

23.1 Introduction

Obesity is a major global pandemic effecting over 650 million people globally. Defined as a body mass index greater than 30 kg per m^2, its prevalence is rising at an alarming rate [1, 2]. This high prevalence in obesity has myriad consequences, but the most concerning are the rising rates of cardiovascular and metabolic diseases, which together conspire to drive the premature mortality in obese individuals [3].

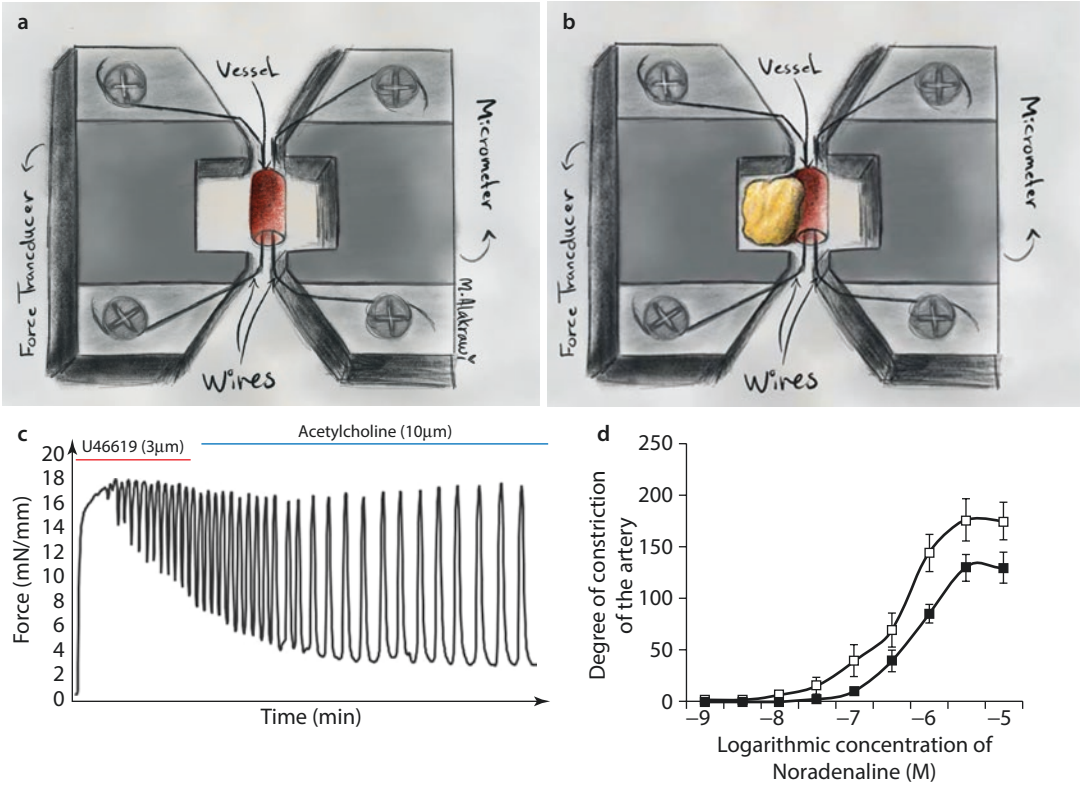
Obesity is characterized by pervasive increase in fat depots throughout the body. The traditional view of adipose tissue as a connective tissue dominated by adipocytes and a site for the storage and mobilization of energy has now evolved [4]: we now know that adipose tissue is highly active metabolically and indeed dynamic, capable of producing numerous physiologically active molecules known as adipokines, which influence organ function through paracrine and endocrine signalling [5, 6]. The ubiquity of adipose stores around the body, including abdominal fat packed between organs in the abdomen, epicardial fat located around the heart and coronary arteries, and subcutaneous fat found in the hypodermis, reflects their diverse roles in human physiology.

The high prevalence of hypertension in obesity has been suggested to account for over 64% of incident hypertension [7]. However, clarification of the precise cellular mechanisms underlying the rise in blood pressure as a result of weight gain remains elusive. From a CNS perspective, there is an increased sympathetic discharge coupled with reduced parasympathetic activity [8, 9]. The increased sympathetic activity transmitted throughout the vasculature results in an increased tone of small arteries, and this increase in peripheral resistance raises central pressures. Once blood pressures are elevated, there is a consistent hemodynamic profile characterized by a low cardiac index-high resistance pattern, which is now recognized as a hallmark of early hypertension [10].

In addition to central mechanisms, obesity also increases the contractility of small arteries via local effects. These local effects include dysfunction of the endothelium, alterations in calcium (Ca^{2+}) signalling within the vascular smooth muscle, and inflammation in the fat surrounding small arteries within the circulatory system. In this chapter, we concentrate on the mechanisms by which the fat which surrounds the small artery – hereafter referred to as perivascular adipose tissue (PVAT) – regulates arterial contractility. The studies described almost exclusively relate to white, rather than brown, adipose tissue. In health, PVAT, rather than merely encasing the artery in an inert manner, in fact exerts a vasodilatory effect on small arteries. In other words, the fat which surrounds the small arteries possesses inherent properties which keep the adjacent arteries themselves slightly dilated. However, in a series of studies, we have discovered that in human obesity, this vasodilatory capacity of the PVAT is lost. From a cardiovascular perspective, the vasodilatory effect of PVAT surrounding small arteries and the subsequent loss thereof in obesity are highly significant. Small artery contractility is a major determinant of peripheral resistance and therefore blood pressure. Thus, viewed collectively, the body's adipose tissue in health keeps small arteries vasodilated and so maintains blood pressure at a low level. However, in obesity, the loss of this vasodilating effect results in greater constriction of resistance arteries. At a central circulatory level, this leads to elevations in the peripheral resistance and thus a rise in blood pressure. Furthermore, the vasoactive influence of PVAT is not limited to small resistance arteries.

In a series of seminal studies from the Antoniades group at the University of Oxford, an intricate channel of bidirectional communication, vital for vascular health, was discovered between adipose tissue and adjacent coronary arteries. The group subsequently developed a novel clinical methodology for the routine assessment of PVAT inflammation which is discussed at length in this chapter.

In the laboratory, the predominant methodology for the study of PVAT vasodilatory function is wire myography. This is illustrated in ◘ Fig. 23.1 which shows two adjacent segments of artery from ex vivo sample – one studied without PVAT and the other studied with PVAT intact. The accompanying cumulative dose-response curves indicate that the artery with PVAT intact constricts less than the artery where the PVAT was left intact. These dynamic properties of PVAT challenge the traditional view that holds PVAT as little more than structural support to the blood vessel [11]. As attention shifts to the actions of adipose tissue on vascular function, our knowledge of PVAT is going through a renaissance of its own. This has been spurred by the accruing evidence that this outer layer of the vessel wall, namely, the adventitia and PVAT, releases a broad range of vasoactive substances, known as adipocyte- or adventitial-derived relaxing factors (ADRFs) [12, 13]. Thus, analogous to the endothelium, a healthy and functional adventitia may



◘ **Fig. 23.1** A cartoon illustrating the technique of wire myography. To study arteries in this manner, thin wires are threaded through the artery, and a degree of artificial tension is then applied by drawing the wires apart. When the artery constricts, the wires sense the increase in tension, and thus the biological functions of the arterial segment can be studied. In order to study the effect of PVAT on arteries, the adipose tissue is removed from one section of an artery **a** but left intact on an adjacent section **b**. The arterial segments are then mounted on the wire myograph. A representative tracing of tension detected is shown in **c**. Here, the tension rises when a constricting agent (U46119) is applied (the upstroke of the line). Following this, a vasodilator (acetylcholine) is applied, and the artery gradually loses its tension, although in the case shown, the artery exhibits a see-saw motion known as "vasomotion." **d** shows the cumulative dose-response curves to a contractile agonist known as noradrenaline. The clear boxes are the tension induced in the arterial segments which do not have PVAT, and the black segments are derived from the segment which has PVAT intact. It can be deduced from this graph that the artery with PVAT doesn't constrict as much to noradrenaline as the artery without PVAT – i.e. the PVAT exerts an "anti-contractile" effect

be involved in maintaining vascular homeostasis, and similarly, a compromise in its actions – "adventitial dysfunction" – may contribute to disease. Further evidence for this adventitial dysfunction arises from the observed derangement in the structural and functional properties of PVAT depending on the metabolic and vascular phenotype of the subject. Here we aim to conceptualize PVAT as a physiologically important component of the vessel wall, summarizing key developments and highlighting outstanding areas on which future work might focus.

23.2 PVAT and Adipokines

Various adipokines have been studied to elucidate their vasoregulatory properties and have been the subject of a recent review [14]. Figure 23.1 illustrates the complex interactions between the vessel wall and various ADRF (adventitium-derived relaxing factors) released from the adventitia and PVAT (Fig. 23.2).

23.2.1 Adiponectin

Adiponectin, the most abundant of the adipokines, is a 28-kDa protein released exclusively by the adipose tissue [15]. Several different forms [16] have been identified, including a full-length form and a shorter-length globular fragment, that increase nitric oxide synthase activity through endothelial receptors. The shorter form acts via the AdipoR1 receptor, while the full-length form acts via AdipoR2 receptors, both of which are ubiquitously distributed although AdipoR1 levels are around 15 times higher than those of AdipoR2 in humans [15]. In rat aorta samples, adiponectin exhibits anti-contractile effects by reducing 5-hydroxytryptamine (5-HT)-mediated constriction of blood vessels. It has been previously demonstrated [17] that adiponectin is secreted from murine and human PVAT and influences vascular contractility, possibly by increasing nitric oxide bioavailability. Notably, this vasodilatory effect is attenuated in obesity by the development of adipocyte hypertrophy, leading to hypoxia, inflammation, and

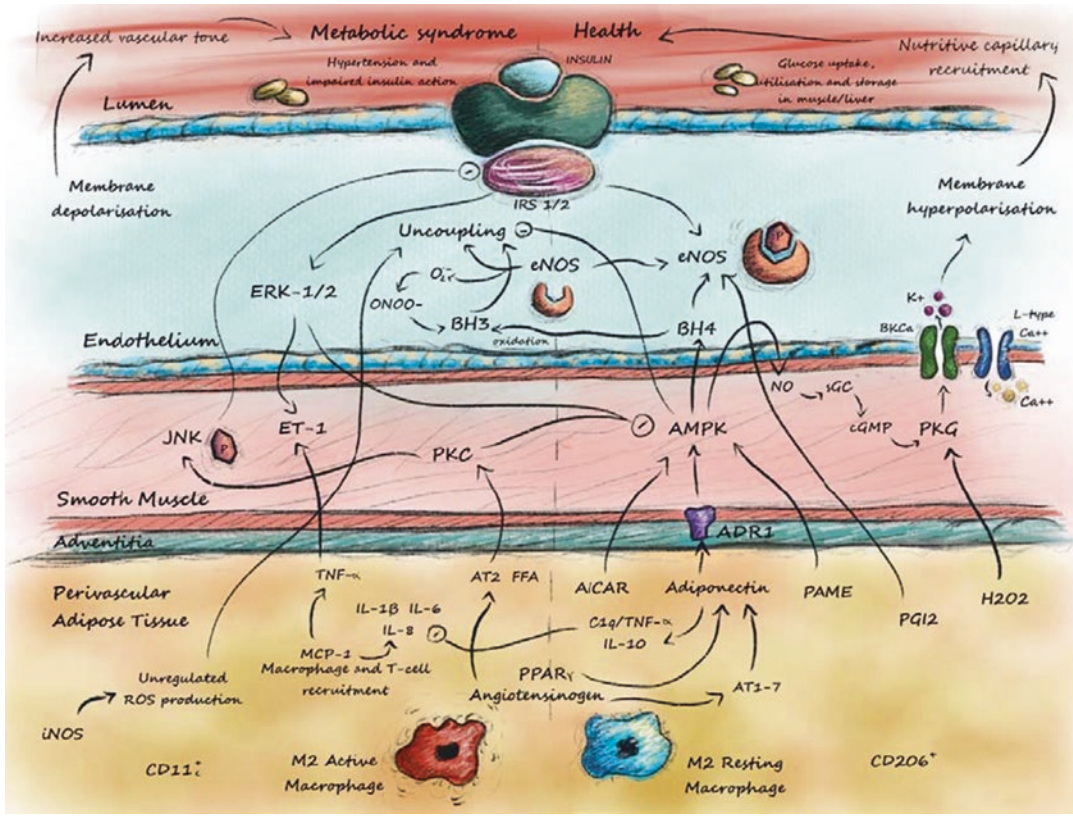


Fig. 23.2 A schema illustrating the complex relationships between ADRFs and the vessel wall in health and its disruption in "metabolic syndrome"

oxidative stress. While the contribution of adiponectin to the functional properties of PVAT is undoubtedly important, the precise mechanisms by which its effects are manifest remain unclear. For example, directly applied adiponectin does not influence the activity of key small artery vasodilatory ion channels, small artery calcium signalling, or indeed the diameter of small arteries constricted in response to physiological intraluminal pressure (myogenic constriction) [18]. Nevertheless, obesity is characterized by reduction in adiponectin levels, and this strongly associates with reductions in the vasodilatory capacity of PVAT [14].

23.2.2 Leptin

Leptin has significant vasoregulatory actions also, with anti-contractile effects on both the human forearm [19] and rabbit aorta [20]. Released from WAT, leptin acts through an endothelium- and NO-dependent mechanism as well as exerting its effects centrally on the hypothalamus to regulate appetite and activate the sympathetic nervous system (SNS) [21]. Interestingly, a brain-adipose axis has been proposed whereby, as well as acting centrally to regulate food intake, leptin-induced activation of the SNS nerve endings in PVAT may cause peripheral vasodilation [22]. Others [23] have demonstrated that leptin may increase blood pressure (BP) in murine models, even though, paradoxically, an in vivo infusion of leptin is associated with a drop in blood pressure. Research from canine models [24] suggests that high leptin levels are associated with endothelial dysfunction. Similar findings were reported by Payne et al. [25] who found that leptin impairs coronary endothelial function via a protein kinase C-β-dependent pathway in a swine model of metabolic syndrome. Given that leptin, a pro-inflammatory adipokine, has vasodilator and cardioprotective effects [26], the role of elevated levels of leptin in obesity, a pro-inflammatory state with leptin resistance, remains unclear.

23.2.3 Adrenomedullin

This protein has been found to be released from a number of sites including WAT [27]. Early studies reported that adrenomedullin exerts a potent dose-dependent vasodilator effect on rodent vessels [28]. Subsequent research [29] in humans has provided further evidence for the vasodilatatory role of adrenomedullin in pulmonary hypertension. However, although adrenomedullin has been found to reduce reactive oxygen species (ROS) levels in vascular smooth muscle cell [30], its role in regulating vascular tone is not yet completely understood.

23.2.4 Other Factors

Adipokines with less well-defined functions, including resistin, might also play a role in the anti-contractile effects of PVAT. Although poorly investigated, resistin [31] has been shown to play an indirect effect by altering protein expression and cytokine and macrophage recruitment [31]. As neither a specific receptor nor a source has been identified, further research into this protein would be beneficial. Hydrogen sulphide has also been identified as a factor released from PVAT which can modulate adjacent arterial contractility.

23.3 PVAT and Bidirectional Communication

As the leading global cause of death, atherosclerosis is under intense investigation for mechanisms underlying its pathogenesis. Traditionally, the pathogenesis of vascular disease has been considered as an "inside-out" process where inflammation is initiated at the luminal surface and proceeds outwards. Recently however there is increasing appreciation that factors operating outside the coronary arteries are capable of inducing luminal changes inside the arteries. While the link between atherosclerosis and PVAT has not been studied directly, the adventitia has been implicated in atherogenesis through a proposed outside-in signalling mechanism [32]. This theory proposes that the secretion of mediators from PVAT that encourage inflammation and chemotaxis leads to differentiation and infiltration of macrophages and other lymphocytes, in turn causing endothelial dysfunction and plaque formation. Early experiments have also demonstrated the development of atherosclerotic plaques and neointimal formation [33] from coronary artery injury [34]. Two studies have shown that oxidative stress within the adventitia counteracts the vasodilatory effect of NO [35, 36]. Further evidence for an outside-in mechanism comes from studies examining epicardial adipose

23

tissue (EAT). Mazurek et al. [37] examined subcutaneous fat from the leg and EAT in 42 patients with multiple cardiovascular risk factors undergoing coronary artery bypass grafting. They reported that EAT had a more pro-inflammatory profile than subcutaneous fat with increased expression of tumour necrosis factor-α, MCP-I, IL-1β, and IL-6 mRNA, in addition to inflammatory cell infiltration. In another study, de Vos and colleagues [38] examined the relationship between pericoronary EAT and cardiovascular risk factors in postmenopausal women. They reported a significant statistical relationship between cardiovascular risk factors and EAT as measured using multidetector computerized tomography. Similarly, a relationship between EAT and coronary calcification was also found. The authors proposed that adipose tissue acts in a paracrine or endocrine manner in order to induce local atherosclerotic lesions.

Following on from these insights, attention focused on the imaging of coronary and epicardial PVAT for the purposes of risk stratification in cardiovascular disease. A number of different imaging techniques have been trialled including transoesophageal echocardiography, cardiac CT, and MR imaging. Most promisingly, coronary PVAT imaging through routine CT angiograms has been used as a surrogate marker of coronary inflammation and vascular disease [39]. Underpinning this is the observation that the release of pro-inflammatory mediators from the blood vessels themselves into the surrounding PVAT inhibits the transformation of perivascular pre-adipocytes into mature, lipid-laden cells. The technique relies on using an algorithmically derived fat attenuation index (FAI) from CT coronary angiograms to demonstrate the degree of lipid accumulation in mature adipocytes surrounding coronary vessels. This FAI has been validated extensively and shown to be increased in patients with coronary artery disease (CAD) compared to healthy subjects. Interestingly, FAI was also higher around culprit lesions in patients with acute myocardial infarction compared to patients with stable coronary artery disease. Figure 23.3 provides an overview of this approach to PVAT imaging in identifying patients with increased cardiovascular risk.

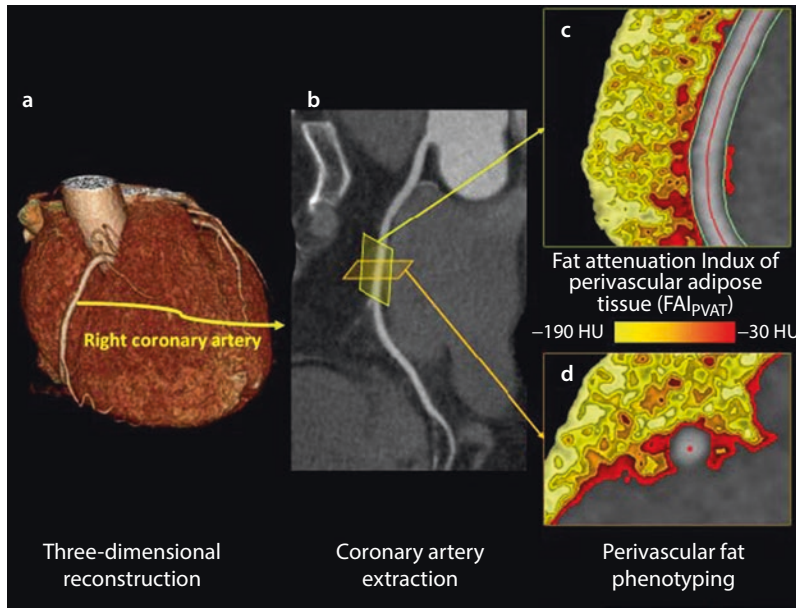


Fig. 23.3 Computed tomography phenotyping of perivascular adipose tissue for detection of coronary inflammation. In the presence of coronary inflammation, the release of inflammatory mediators from the vascular wall results in impaired adipocyte differentiation and intracellular lipid accumulation in the perivascular space. These phenotypic changes can now be detected by a novel computed tomography (CT)-based technology that first tracks the coronary vessels in standard coronary CT angiograms **a**, **b** and subsequently segments the perivascular space by applying prespecified attenuation thresholds (−190 to −30 Hounsfield units [HU]). The calculated fat attenuation index (FAI$_{PVAT}$), a metric of perivascular fat attenuation, can be used as a marker of vascular inflammation, with inflamed vessels demonstrating higher FAI$_{PVAT}$ values close to the vascular wall (**c**, **d** red colour) compared to the non-perivascular fat just a few centimetres away (**c**, **d** yellow colour). (Images courtesy of Antonopoulos and colleagues at the University of Oxford – used with permission)

23.4 Adventitial Dysfunction and Disease

23.4.1 Atherosclerosis

Although PVAT has been shown to influence atherosclerotic plaque formation, studies evaluating its exact role are scarce. In one postmortem study [40], PVAT mass and the degree of macrophage accumulation directly correlated with atherosclerotic plaque size. A pro-chemotactic effect has been described in both humans and rat models in atherosclerosis [41], and it is interesting to note that portions of coronary arteries that lack PVAT are more likely to undergo pro-atherosclerotic change [42]. Further evidence comes from Ohman et al. [43] who found that transplanting dysfunctional WAT into carotid arteries of apo-E knockout mice encouraged atherosclerotic plaque development. These results were not replicated with healthy WAT. In contrast, Chang et al. [44] reported that PVAT activity is increased at low temperatures, creating an anti-atherosclerotic effect. Mice kept at 16 °C had decreased plaque formation, which the authors argue is due to increased PVAT-induced lipid clearance as illustrated by reduced triglyceride levels at this temperature. Moreover, at this temperature, the expression pattern of PVAT alters more closely to resemble that of BAT. Given that aortic PVAT has phenotypic similarities to BAT [45], and one of the major functions of BAT is to provide adaptive thermogenesis [46], it is possible that heat generation-influenced PVAT has a role to play in its effect on vascular physiology. Thus, PVAT may have both pro-atherosclerotic properties, mediated by inflammation, and protective anti-atherosclerotic properties.

23.4.2 Hypertension

Given that PVAT plays a major role in vasoregulation, it follows that PVAT function may be altered in hypertensive states. However, only a few studies have examined the role of PVAT in hypertension, but these have consistently demonstrated a decrease in PVAT mass and loss of vasodilatory effect. Indeed, PVAT mass and adipocyte size were decreased in spontaneously induced [47], AngII-induced [48], and DOCA salt-induced rat models of hypertension [49]. Galvez-Prieto et al. [50] have shown that these findings were coupled with vasoconstriction and PVAT impairment in the spontaneously hypertensive rat. The authors also discovered a decrease in PVAT-derived leptin secretion, seen as a fall in mRNA and protein expression and a corresponding reduction in NO release. Ruan et al. [49] reported that an abundance of PVAT-derived complement 3 (C3), a pivotal component of the complement-dependent immune response pathways, resulted in fibroblast migration and adventitial remodelling. In one intervention study, treatment with ACE inhibitors in hypertensive rats improved PVAT-mediated vasoregulation. However, it should be noted that PVAT has mostly been studied in the context of salt-induced or genetic hypertension models, while in many cases hypertension can often co-exist with obesity and insulin resistance. Further, in some hypertensive models, the anti-contractile effects of PVAT are actually intact [47]. Therefore, while hypertension may have similar effects to obesity in local vasoregulation and vascular remodelling, perhaps, via PVAT, this mechanism has not yet been completely established.

Arterial stiffness has been strongly associated with hypertension, although its exact pathophysiological mechanism remains unknown [51]. Arterial stiffening is partly due to age-related changes in vessel structure, namely, the replacement of compliant elastin with stiffer collagen. This has been suggested to increase blood pressure, resulting in vascular remodelling to alleviate wall stress. Fleenor and colleagues [52] have recently tested the hypothesis that PVAT contributes to large artery stiffness associated with ageing. They transplanted PVAT from young and old donor mice in the abdominal aortas of young recipient mice. The old PVAT recipient mice had greater arterial stiffness with increased superoxide ion production.

23.4.3 Obesity

Obesity is characterized by energy excess whereby energy intake is not balanced by expenditure [53]. This results in an increase in adipose tissue mass by adipocyte expansion and proliferation in order to meet storage demand. In addition to an expansion in mass, complex changes occur, including changes to the composition of lipid droplets and

remodelling of extracellular matrix as well as immune cell infiltration [54, 55]. In obesity, adipose tissue is characterized by persistent inflammation, oxidative stress, hypoxia, and insulin resistance [56, 57]. Thus these changes have motivated research into the impact of obesity on PVAT structure and function, especially because aortic PVAT mass directly correlates with hypertension, diabetes, and blood vessel calcification as seen in the Framingham Heart Study [58].

An increase in PVAT mass and adipocyte size in human obesity, and associated hypoxia and inflammation, is coupled with an impaired anti-contractile effect [17]. These inhibitory effects were mimicked by the application of IL-6 and TNF-α to PVAT and were attenuated by TNF- α antagonists and superoxide dismutase (SOD). Further [59], experimentally induced hypoxia produced macrophage activation and recruitment, in turn increasing free radical production and resulting in a diminished vasodilatory effect of PVAT. In another study in obese mice [60], endothelial dysfunction in high-fat diet (HFD) was attenuated by removal of PVAT or treatment with ROS scavengers or apocynin, an NADPH oxidase inhibitor. Similarly, PVAT from New Zealand obese mice demonstrated increased production of ROS, excess macrophage recruitment, and reduced SOD activity [61]. Additionally, adipokines from PVAT are also implicated in the anti-contractile effect of obesity. The normal vasodilation and calcium inhibitory effect of leptin, which encourages NO release, was lost in the obese Zucker rat, resulting in vasoconstriction [62]. On the other hand, obesity is characterized by impaired release of adiponectin, which possesses anti-inflammatory and vasodilatory properties [63].

Dysfunctional PVAT has been shown to stimulate proliferation of vascular smooth muscle in Wistar-Kyoto rats and HFD-induced obese rats [64], suggesting that PVAT in obesity may cause thickening of the arterial wall. Although the mechanism is not fully understood, these changes may be due to alterations in PVAT adipokine release, such as leptin, resistin, visfatin, and adiponectin. Indeed, adiponectin deficiency has been shown to exacerbate the neointimal growth [65]. Furthermore, visfatin, which is preferentially expressed in PVAT from rat and monkey aorta, stimulates proliferation of vascular smooth muscle cells though an insulin-dependent nicotinamide mononucleotide pathway [66]. Leptin and resistin have also been shown to possess proliferative properties [31, 63]. In contrast, adiponectin is known to exert an anti-proliferative influence on smooth muscle cell proliferation, an effect that may be impaired when adiponectin levels are reduced such as in obesity [67].

Overall, the studies suggest that the composition of adipose tissue changes in obesity and this leads to structural and functional changes to the arteries within the fat. These changes are associated with the development of hypertension and other microvascular diseases. As such, understanding the cellular and molecular mechanisms by which obesity changes adipose tissue may lead to new more targeted therapies to overcome obesity-related cardiovascular disease.

23.4.4 Insulin Resistance and Diabetes

Insulin has long been known to exert effects on small artery diameter. While the precise mechanism underpinning these effects is not entirely clear, the metabolic enzyme AMP kinase, which causes vasodilation, has been proposed. Evidence suggests that in obesity, the microcirculation of subjects with insulin resistance is characterized by impaired NO production due to the reduced phosphorylation of Akt and eNOS [68] tilting the balance towards insulin-mediated vasoconstriction. This shift from vasodilation to vasoconstriction may be due to dysfunction of the normal insulin substrate signalling mechanisms.

Consistent with this, an elegant work from the Eringa group has demonstrated that PVAT is involved in insulin-mediated vasoregulation [69, 70]. Thus, studies have shown that intact PVAT is required for the vasodilatory effect of insulin in healthy small arteries and this insulin-mediated vasodilation is enhanced by adiponectin exposure. However, when isolated arteries were incubated with PVAT from HFD-induced obese and diabetic mice, this vasodilatory effect of insulin was lost. Further murine studies have also lent credence to the argument that PVAT controls insulin-dependent vasoregulation in the microvascular circulation. Interestingly, in health PVAT may cause vasodilation through

AMPKα2 activation in the vessel wall [71]. The vasodilatory effect of insulin in muscle resistance arteries was antagonized by an alternative inflammatory pathway mediated by the inflammatory kinase Jun NH2-terminal kinase (JNK). Consistent with this, vasodilation of resistance arteries was inhibited by PVAT from obese mice, but vasodilation was restored by inhibition of the JNK pathway, suggesting that inflammation of PVAT may play a role in determining vasodilation in type 2 diabetes and obesity. The reduction in vasodilatory adipokines, such as adiponectin, released by PVAT [72], and an increase in pro-inflammatory cytokines including TNF*a* [73] in obesity may underpin this anti-contractile-to-contractile shift in the vasoregulatory effect of insulin.

It has also previously been proposed that insulin modifies vascular tone in order to modulate a postprandial increase in nutritive flow in lean subjects [13]. However, accumulation of PVAT around arterioles interferes with insulin's vasoregulatory properties, diminishing its vasodilatory effect and stimulating vasoconstriction [74]. In this scenario, PVAT may exist as a defence mechanism to guard against muscle injury in the face of a chronically elevated energy supply. However, the overproduction of cytokines from excessive PVAT accumulation may lead to endothelial dysfunction, inflammation, and atherosclerosis [37].

Conclusion and Clinical Perspectives

Due to its close proximity to the cardiovascular system, perivascular adipose tissue is of particular interest in cardiovascular sciences. While traditionally viewed as little more than a simple lipid reserve, our understanding of perivascular tissue is undergoing a paradigm shift. It is clear that PVAT boasts an impressive arsenal of adipokines, cytokines, and other biological secretions that play a complex role in modulating vascular function in health. However, PVAT dysfunction in disease is associated with alteration to its secretory profiles which can create a contractile and pro-inflammatory effect. We now also understand that PVAT has a role as a sensor of coronary inflammation. This can be detected using a novel CT-derived metric, representing the first attempt at clinical translation of our ever-increasing knowledge of PVAT biology.

Gaps in Knowledge

- The sequence of events that leads to these changes in obesity, hypertension, and atherosclerosis is unclear, and further elucidating the underlying processes would allow the development of therapeutic strategies that target PVAT function.
- The communication between the intima and adventitia is evident, but the mechanisms and networks which permit transport between the outer and inner layers of the vessel wall remain uncertain.
- While PVAT imaging represents an attractive development in clinically translating PVAT biology, its role has yet to be validated in prospective cohorts or integrated fully in clinical risk stratification.

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Vascular Biology of Cancer Chemotherapeutic Drugs

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_24

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Key Concepts

- Developments in anticancer therapy have substantially improved the prognosis for patients with cancer.
- Adverse vascular effects of anticancer therapy include hypertension, arterial and venous thromboses, and proteinuria.
- Adverse cardiovascular effects of anticancer drugs have become more relevant as patients live longer.
- Newer anticancer therapies have presented new challenges with a range of acute vascular toxicities that were initially unexpected.

24.1 Introduction

Advances in the treatment of cancer have improved the prognosis of patients with a range of malignancies, primarily due to the development of novel anticancer drugs [1]. These drugs alter cellular pathways critical for cancer cell growth and survival [2]. Such pathways often overlap with normal, healthy cell signalling, and this can lead to unwanted and sometimes unanticipated side effects, particularly affecting the cardiovascular system (Fig. 24.1).

Angiogenesis, the process of new blood vessel formation, is central to cancer cell growth and metastasis, and anti-angiogenic agents represent a key development in anticancer therapy. Angiogenesis is also critical for healthy blood vessel formation, and partly as a result of this biological overlap, these agents are associated with a broad range of cardiovascular complications, including left ventricular dysfunction, heart failure, hypertension, myocardial infarction, and thromboembolism. More established chemotherapy drugs, including alkylating agents, antimetabolites, and anticancer antibiotics, are well known to be associated with cardiotoxic effects, particularly left ventricular dysfunction and heart failure (Table 24.1). These limitations can

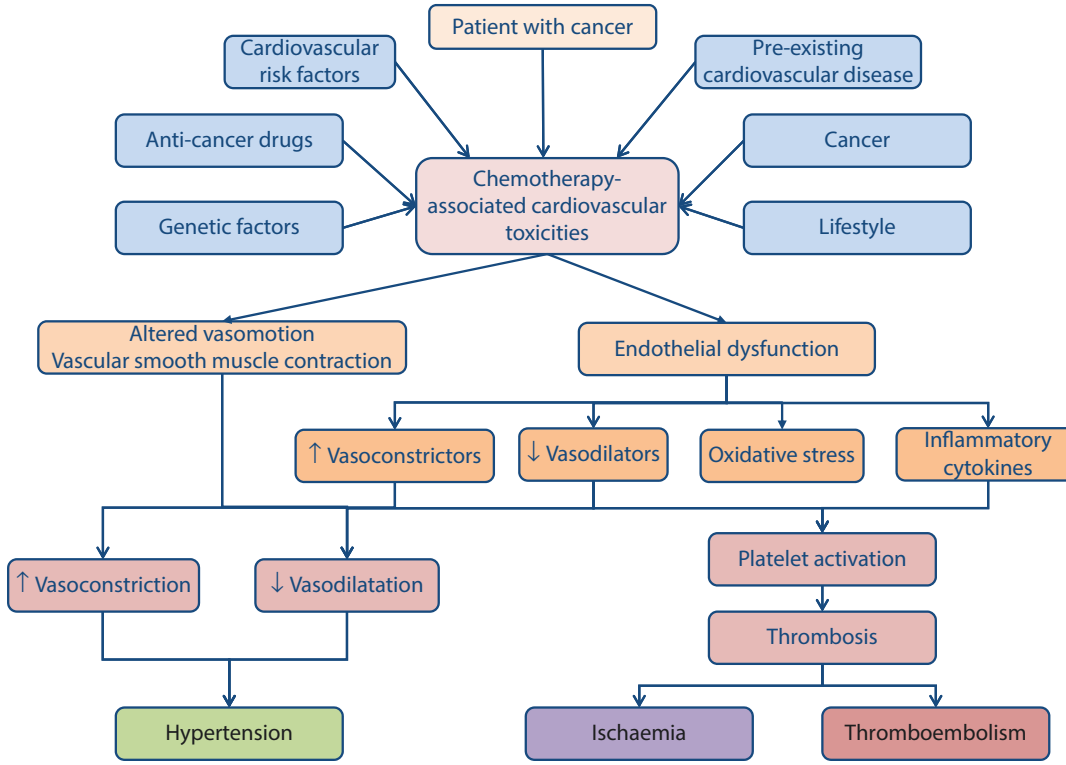

Fig. 24.1 Factors contributing to chemotherapy-associated vascular toxicities. Multiple potential stimuli, such as baseline cardiovascular risk factors, pre-existing cardiovascular disease, genetic and lifestyle factors, cancer itself, and anticancer drugs, influence vascular function and arterial structure leading to altered vascular tone, impaired endothelial function, and platelet activation. These processes contribute to cardiovascular diseases that include hypertension, myocardial ischaemia, and thromboembolism, which may be facilitated and aggravated by anticancer chemotherapy drugs

Table 24.1 Chemotherapy agents with principal cardiovascular complications, common cancer indications, and potential mechanisms

Chemotherapy drug class	Chemotherapy agents	Indications	Principal cardiovascular complications		Potential mechanisms
VEGF signalling pathway inhibitors					
	Bevacizumab	Colorectal cancer	Hypertension	++++	Endothelial dysfunction
	Sunitinib	Renal cell carcinoma	Proteinuria		↓ NO signalling
	Sorafenib	Hepatocellular carcinoma			↑ ET signalling
					Capillary rarefaction
					Vascular remodelling
					Oxidative stress
			Ischaemia	+	Platelet activation
			Thromboembolism		↓ NO and PGI_2 signalling
Tyrosine kinase inhibitors for haematological malignancy					
	Ponatinib	Chronic myeloid leukaemia	Ischaemia	+++	Acute arterial thrombosis
	Nilotinib				
	Dasatinib	Acute lymphoblastic leukaemia			
Alkylating agents					
	Cisplatin	Testicular cancer	Hypertension	++	Endothelial dysfunction
		Lung cancer	Ischaemia	+++	Platelet activation
		Cervical cancer	Thromboembolism		↓ NO and PGI_2 signalling
		Ovarian cancer			Vasospasm
			Nephrotoxicity	++++	Tubular injury
Antimetabolites					
	5-Fluorouracil	Colorectal cancer	Ischaemia	+++	Vasospasm
		Breast cancer			
Anthracyclines					
	Doxorubicin	Breast cancer	Cardiotoxicity	+++	Myocyte apoptosis
	Epirubicin	Acute leukaemias			
		Lymphomas			

VEGF vascular endothelial growth factor, *NO* nitric oxide, *ET* endothelin, *PGI_2* prostacyclin

restrict treatment options and may negatively impact on the management of patients with cancer. In recent years there has been increasing focus on collaboration between oncologists and cardiovascular specialists, to maintain improvements in cancer-related survival whilst ensuring this is not at the expense of increased cardiovascular side effects [1].

This chapter provides an overview of the vascular effects of common chemotherapy drugs and discusses potential mechanisms through which these drugs induce vascular toxicity. The clinical significance of this is highlighted.

24.2 Growth Factor Signalling Pathways

24.2.1 Vascular Endothelial Growth Factor (VEGF) Inhibitors

Vascular endothelial growth factor (VEGF) is among the most important growth factors involved in angiogenesis. This 45-kDa glycoprotein is produced by many cell types, including endothelial progenitor cells, endothelial cells, renal epithelial cells, fibroblasts, macrophages, and some tumours. The VEGF gene undergoes alternative splicing to multiple isoforms: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Fig. 24.2a). VEGF-A is the best characterized and binds to three tyrosine kinase receptors (VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR), and VEGFR3 (Flt4)). VEGFR1 and VEGFR2 are predominantly expressed in endothelial cells, and VEGF-A binding to VEGFR-2 has the major vascular effects [3].

Activation of VEGFR-2 stimulates pathways that include PI3K/AKT/protein kinase B-mammalian target of rapamycin (mTOR), endothelial nitric oxide synthase (eNOS), and prostacyclin (PGI_2) that regulate vasodilation and inflammatory responses (Fig. 24.2b) [3]. VEGF also signals through phospholipase C (PLC), Raf-1, and mitogen-activated protein (MAP) kinases, which regulate endothelial cell survival, proliferation, migration, and permeability.

Whilst VEGFIs interrupt VEGF signalling directly, this also occurs as a secondary effect of 'classical' cytotoxic drugs, including antimetabolites, taxanes, anthracyclines, and alkylating agents [2].

VEGFI now forms the basis of anticancer therapy for a wide range of solid tumours and haematological malignancies. There are four main groups of VEGFIs:

1. *Monoclonal antibodies against VEGF* (e.g. bevacizumab). This class of agents selectively binds VEGF and inhibits VEGF/VEGFR interaction.
2. *Small molecule inhibitors of intracellular tyrosine kinases* (e.g. sunitinib, sorafenib). These agents are not VEGFR-2-specific and also inhibit other receptor tyrosine kinases, including platelet-derived growth factor (PDGF) and c-Kit signalling, which are implicated in tumour angiogenesis. This increases anticancer efficacy but also contributes to increased adverse cardiovascular effects [4].
3. *VEGF 'trap'* (e.g. aflibercept). This is a recombinant fusion protein that comprises VEGF-binding regions of VEGFR-1 and VEGFR-2.
4. *Monoclonal antibodies against VEGFR* (e.g. ramucirumab). These target VEGFR2 receptors, to prevent VEGF binding [5].

Interruption of VEGF signalling is associated with adverse cardiovascular effects and clinical sequelae that include hypertension, left ventricular systolic dysfunction, myocardial infarction, stroke, and venous thromboembolism [2].

24.2.1.1 Hypertension

Hypertension is by far the most common cardiovascular complication associated with VEGFIs. An absolute rise in blood pressure is an almost universal finding in patients receiving these drugs, and up to 80% of patients develop hypertension. VEGFI-associated hypertension is often severe and difficult to treat [3]. The mechanisms underlying VEGFI-associated hypertension remain incompletely defined but include endothelial dysfunction, reduced nitric oxide (NO) generation, increased endothelin-1 (ET-1) bioavailability, capillary rarefaction (reduced capillary density), vascular remodelling, and oxidative stress (Fig. 24.3) [3]. An acute, dose-dependent increase in blood pressure occurs within hours to days of starting treatment [3], and this effect reverses when the drug is withdrawn. Hypertension associated with VEGFI is a marker of oncological response to treatment [6], supporting the hypothesis that hypertension is at least partly an on-target effect. The risk of hypertension is particularly high in patients with a history of hypertension and in those treated with a combination of VEGFIs [3].

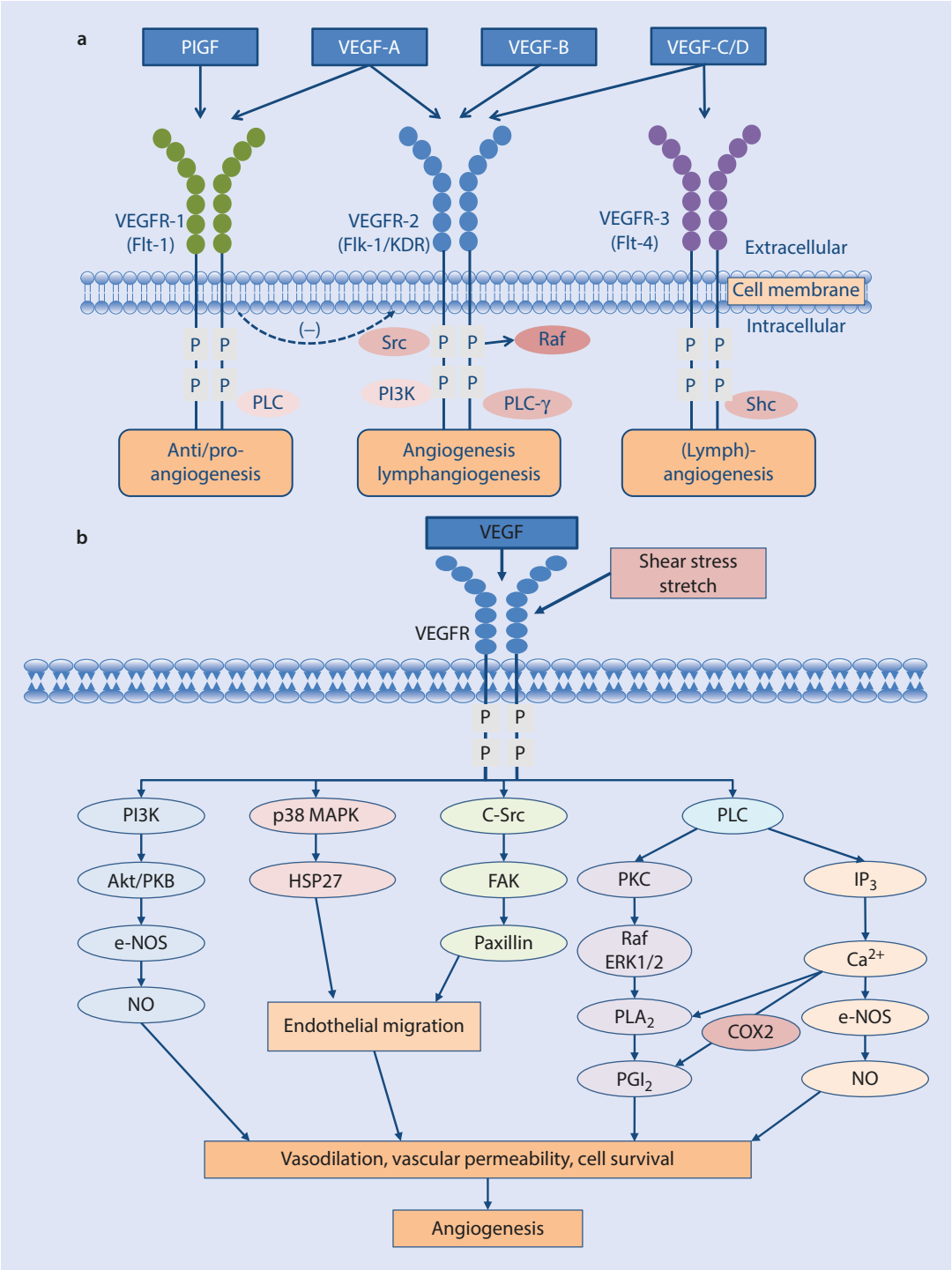

Fig. 24.2 VEGF signalling pathways **a** vascular endothelial growth factor ligands (VEGF-A/VEGF-B/VEGF-C/VEGF-D) interact with VEGF receptors (VEGFR-1/VEGFR-2/VEGFR-3), resulting in angiogenesis and/or lymphangiogenesis. VEGFR1 is believed to negatively regulate VEGFR2. *Flt-1* Fms-like tyrosine kinase 1, *Flk-1* fetal liver kinase 1, *KDR* kinase domain receptor. **b** Pathways through which VEGF/VEGFR signalling via tyrosine kinase-dependent phosphorylation pathways contribute to angiogenesis. Signalling pathways activated by ligand binding to VEGFRs include PI3K/Akt/PKB, PLC, and the RAF/ERK cascade. This results in increased eNOS and COX-2 activity with increased production of NO and PGI2, which are important regulators of vascular tone and function

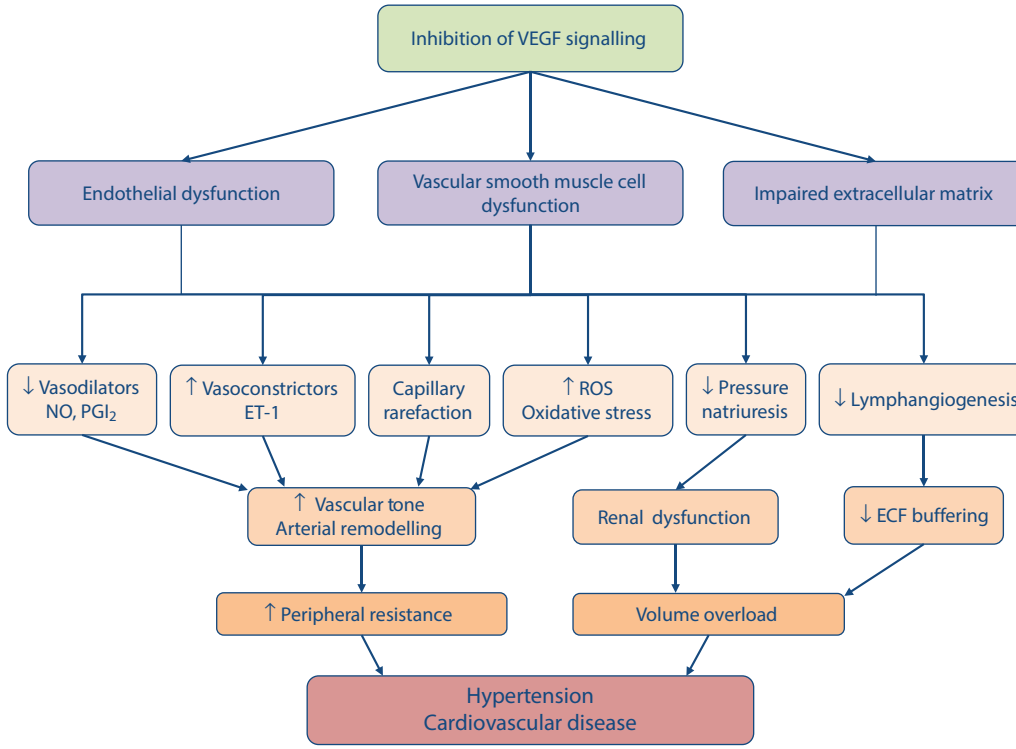

Fig. 24.3 Mechanisms through which VEGFIs may contribute to the development of hypertension. These are primarily related to endothelial dysfunction with reduced vasodilators (NO and PGI2), increased vasoconstrictors (ET-1), oxidative stress, and capillary rarefaction. The consequence of these is increased total peripheral resistance, whilst reduced pressure natriuresis and impaired lymphatic function contribute to volume overload, which also promote blood pressure elevation. ECF extracellular fluid, ROS reactive oxygen species

Proteinuria also occurs as a dose-dependent side effect in up to 60% of patients treated with VEGFI, and, whilst the majority of cases are not severe, grade 3 or 4 proteinuria occurs in up to 6% [7, 8]. Renal thrombotic microangiopathy has been associated with VEGFI and, when it occurs, can have major adverse clinical implications [9].

24.2.1.2 Thrombosis

VEGFIs are associated with both thrombotic and haemorrhagic side effects, but the pro-thrombotic effects predominate. Whilst both venous and arterial thromboses are reported in association with VEGFI, the risk of arterial thrombosis appears greater [5]. A 3.5-fold increased risk of myocardial infarction and 1.8-fold increased risk of arterial thrombosis associated with VEGF inhibitor therapy have been demonstrated [6].

A number of VEGFIs, such as sunitinib and sorafenib, target small-molecule receptor tyrosine kinases and are associated with cardiovascular side effects that include heart failure, hypertension, thrombosis, and thromboembolism. Other multi-targeted tyrosine kinase inhibitors (TKIs) used to treat haematological malignancies, such as ponatinib, nilotinib, and dasatinib, are associated with a high incidence of arterial thrombosis [1]. Ponatinib is a potent multi-targeted TKI against the oncogenic fusion gene, Bcr-abl, used to treat chronic myeloid leukaemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukaemia resistant to or intolerant of traditional TKIs [10]. Ponatinib is associated with an almost 12% incidence of arterial thrombotic events at 2 years [10]. There is also a 3% incidence of venous thrombosis at 2 years [10].

The underlying reason or mechanisms explaining the high incidence of acute arterial events associated with ponatinib and nilotinib remain unclear. Notably, however, not all anti-Bcr-Abl TKIs are associated with such high risk, and, over 10 years, nilotinib is associated with a 14-fold greater risk of progressive peripheral arterial disease than the prototype

anti-Bcr-Abl TKI, imatinib. It is likely that this variability reflects as yet unidentified off-target effects [10].

24.3 Chemotherapy Targeting Oestrogen Receptor Signalling

Oestrogen receptor (ER) signalling is involved in the growth and development of breast cancers which are oestrogen receptor positive, and this group represents up to 80% of patients with breast cancer [11]. Chemotherapy drugs which target ER signalling, and the aromatase enzyme responsible for the early steps in oestrogen synthesis, are therefore used to treat patients with breast cancers that are ER positive.

Tamoxifen is an antioestrogen drug used for over 30 years and has had a major impact on improving disease-free and overall survival in patients with breast cancer. Anastrozole and letrozole are aromatase inhibitors that are also used to treat patients with ER-positive breast cancers and have also had a major impact on improving the prognosis of this group of patients [12]. Interruption of ER signalling with agents such as tamoxifen and the aromatase inhibitors has also been associated with increased risk of thromboembolism and hypertension.

24.4 Alkylating Agents

24.4.1 Platinum-Based Compounds

Cisplatin is associated with adverse cardiovascular effects, including hypertension, myocardial ischaemia and infarction, stroke, and thromboembolism [1, 2, 13].

24.4.1.1 Thrombosis

Venous and arterial thromboembolism represents the most concerning of vascular toxicities associated with cisplatin-based chemotherapy. Cisplatin provokes endothelial dysfunction and an associated hypercoagulable state with platelet activation and increased von Willebrand factor. Impaired nitric oxide (NO) bioavailability appears to be a key contributing mechanism. In addition to the potentially devastating effects of arterial thromboembolism, vascular complications may also occur via thrombosis *in situ* as a consequence of endothelial dysfunction [2].

In addition to the major issues regarding the acute vascular toxicity of cisplatin-based therapy, there is concern about the propensity to premature cardiovascular disease which may present years after the index cancer therapy. Cisplatin is associated with a sevenfold increased risk of major cardiac event over 14 years (6% of patients) in patients treated for metastatic testicular cancer [13]. Patients treated with platinum-containing drugs display persistent adverse cardiovascular risk profiles, including hypertension and hyperlipidaemia [13]. However, whilst a large population-based study recently demonstrated an almost fivefold increased risk of cardiovascular mortality in the first year after cisplatin treatment [14], the risk of thrombotic complications fell after 1 year. Furthermore, when assessed using brachial artery flow-mediated dilatation (FMD), cisplatin-associated endothelial dysfunction has not been demonstrated consistently over time. No change in FMD was observed when assessed within 10 weeks of platinum-based chemotherapy [15], whilst marked decreases are seen immediately following treatment [16] and at 1 year [17]. The time course of endothelial impairment therefore remains incompletely defined. There may be a biphasic response with 'hyperacute' and partially reversible endothelial toxicity in the immediate peri-treatment period followed by a subsequent decline as a result of a persistent adverse cardiovascular risk profile [1].

24.4.1.2 Hypertension

Hypertension is frequently reported in association with cisplatin-based chemotherapy. The reported incidence of cisplatin-associated hypertension is variable with one group reporting that 53% of patients developed hypertension over a median follow-up of 11 years (OR 2.3; 95% CI 1.5–3.7) [18]. Endothelial cell damage and dysfunction are believed to be important contributing factors [2].

24.4.1.3 Nephrotoxicity

Cisplatin has long been associated with dose-dependent nephrotoxicity. Cisplatin-induced acute kidney injury can require interruption of the chemotherapy regimen and a reduction in subsequent dose of cisplatin. Endothelial dysfunction appears central in the pathophysiological

process, and microalbuminuria occurs in up to 22% of patients 10 years after completing cisplatin-based chemotherapy [13].

24.4.2 Cyclophosphamide

Cyclophosphamide acts as an alkylating agent to exert its anticancer effects. It is associated with vascular complications including hypertension, myocardial infarction, stroke, venous thrombosis, and Raynaud's phenomenon [1]. Notably, the administration of continuous low-dose cyclophosphamide results in reduced circulating concentrations of VEGF. This may explain at least some of the overlap between vascular toxicities seen in association with cyclophosphamide and those seen with VEGFI. Cyclophosphamide is also associated with interstitial pneumonitis and pulmonary fibrosis. Lung biopsy from affected patients demonstrates vascular sclerosis and signs of pulmonary hypertension [1, 2]. This may be a consequence of neutrophil and monocyte adhesion to damaged pulmonary vascular endothelium with co-located platelet accumulation [1, 2].

24.5 Antimetabolites

24.5.1 5-Fluorouracil

5-Fluorouracil (5-FU) and its orally available prodrug, capecitabine, are associated with myocardial ischaemia, which most likely reflects coronary artery spasm, although thrombosis or endothelial dysfunction may also contribute. Myocardial ischaemia may present from asymptomatic ST segment changes on electrocardiogram through angina, myocardial infarction, and sudden cardiac death [19]. The risk for ischaemia is greatest when these agents are administered as a continuous infusion (5-FU) and at high dose. Direct endothelial toxic effects of these drugs reduce endothelial nitric oxide synthase (eNOS) activity to promote increased arterial tone/spasm. Furthermore, endothelium-independent vasoconstriction is mediated by effects upon protein kinase C [2]. The coronary endothelium is particularly susceptible to these effects. Red blood cell viscosity is also increased by 5-FU, and the consequent reduction in blood flow velocity may predispose to thrombus formation [1]. However, 5-FU has not been associated with the development of accelerated coronary atherosclerosis [1].

Pre-existing coronary artery disease is the primary risk factor for 5-FU-related coronary ischaemia, and this is compatible with the observation that vasospasm tends to occur at sites of thrombus and plaque formation [20]. Unfortunately, repeated 'challenge' with 5-FU or capecitabine tends to result in recurrent symptoms, and alternative agents should be used if this occurs [1].

24.6 Anticancer Antibiotics

24.6.1 Anthracyclines

Anthracyclines such as doxorubicin and epirubicin are well-established and potent chemotherapy agents used for effective treatment of solid tumours (primarily breast and sarcoma) and haematological malignancies (leukaemia and lymphoma). Their principal unwanted cardiovascular effect is left ventricular dysfunction. However, this is primarily a direct dose-related cardiotoxic effect rather than a secondary consequence of vascular toxicity such as systemic hypertension and arterial or venous thrombosis [5] athough endothelial toxicity is increasingly recognised in the aetiology.

24.6.2 Bleomycin

Bleomycin disrupts the cellular cytoskeleton and damages DNA. In doing so it offers anticancer properties but also causes an important reduction in endothelial cell growth and induction of apoptosis. Associated cardiovascular complications include myocardial ischaemia and infarction, thrombosis and thromboembolism, pulmonary fibrosis, and Raynaud's phenomenon, which are at least partly mediated by these endothelial toxic effects [1, 2].

24.7 Microtubule-Targeted Agents (Taxanes and Vinca Alkaloids)

Microtubule-targeted agents include the taxanes (e.g. paclitaxel, docetaxel) and the Vinca alkaloids (e.g. vincristine and vinblastine). These taxanes disrupt the cytoskeleton and have substantial anti-angiogenic properties [2]. Their effects are dose-

dependent, and, at lower doses, they interrupt critical signalling pathways and prevent cellular motility and intercellular interactions [2]. At higher doses, they cause endothelial cell detachment and apoptosis as well as microtubule deficiency [2]. Paclitaxel is associated with selective activation of c-Jun kinase (JNK) which enhances tissue factor expression and may further contribute to thrombotic complications [2]. Taxane-induced capillary leakage contributes to the development of peripheral oedema as well as pleural and pericardial effusions.

Vincristine and vinblastine exert their anticancer effects by binding to tubulin to precipitate cell death. They are principally used in the treatment of leukaemia and lymphoma, and their main cardiovascular side effects are myocardial ischaemia and infarction. These phenomena tend to occur during or shortly after therapy and may be a reflection of cellular hypoxia-induced coronary artery vasospasm [1].

Conclusion and Clinical Perspective

Patients treated for malignant disease have benefitted enormously from the rapid expansion and development of anticancer drugs. Whilst survival from cancer has increased, this has been in association with a greater prevalence of treatment-related vascular toxicities and accelerated vascular disease. Acute vascular toxic effects of newer anticancer agents, initially under-appreciated and remaining incompletely understood, have become a major source of concern. Continued anticancer drug development must now become more mindful to the potential for cardiovascular toxicity and clinical trial design should incorporate cardiovascular end points and data collection. Efficient treatment with as little compromise between anticancer effects and vascular toxicity requires a clear understanding of the crossover between tumour biology, vascular biology, and the 'on-target' and 'off-target' effects of medicines. This highlights the critical role for cross-specialty work between clinical disciplines involving specialists in cardiovascular medicine, oncology, and other related fields.

There are very few clinical guidelines on the management of cardiovascular disease in patients with cancer although the European Society of Cardiology has recently published a position statement [21]. It is important to perform a careful baseline assessment of cardiovascular risk factors prior to commencing anticancer therapies that are associated with cardiovascular toxicities. This allows for patients at increased risk to be identified and modifiable cardiovascular risk factors to be addressed. Furthermore, it also allows appropriate interpretation of subsequent results or changes that may develop during chemotherapy. We must continue efforts to unravel mechanisms whereby anticancer drugs cause cardiovascular disease. There is an urgent need for better identification of therapeutic strategies to ensure a good oncologic response to treatment does not come at an unacceptable cardiovascular price.

Gaps in Knowledge

Despite the recent rapid growth in the discipline of Cardiovascular-Oncology and advances in our understanding of the mechanisms contributing to anticancer drug-associated cardiovascular diseases, as well as approaches to the detection and treatment of cardiovascular complications, there are gaps in our knowledge that warrant attention.

Progress in anticancer therapy continues at pace, and immune checkpoint inhibitors have been introduced rapidly and are used for treatment of a quickly expanding range of cancer types, often in combination with drugs that have their own vascular toxic side effects. These immunotherapies have been associated with a small but important incidence of myocarditis (often fatal) [21], but the longer-term vascular effects remain undefined as does the impact of combination with other therapeutic classes, such as VEGFI.

The detection of coronary artery disease in patients who may receive or are being treated with anticancer drugs that can induce myocardial ischaemia is an even more evidence-free zone. Indeed, most current pathways simply recommend using diagnostic algorithms that are applied to patients without cancer. It remains unclear whether this approach is appropriate or if screening for coronary artery disease in asymptomatic patients receiving drugs

associated with myocardial ischaemia may prevent the development of potentially devastating cardiac events. The latter approach may lead to unnecessary or premature discontinuation of treatment with important anticancer drugs [22].

The underlying mechanisms contributing to VEGFI-associated hypertension remain incompletely defined. However, there are clear differences in the aetiology when compared with those mechanisms involved in the development of systemic hypertension. Indeed, the renin-angiotensin-aldosterone system does not appear critically involved in the development of VEGFI-induced hypertension. Reduced NO-mediated vasodilatation and/or increased endothelin-1-mediated vasoconstriction appears to be of central importance. Future research should focus on clearly elucidating mechanisms contributing to VEGFI-induced hypertension to allow the development of more efficacious and targeted approaches for the treatment and prevention of blood pressure elevations that occur in the context of VEGFI [22].

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Coronary Microvascular Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_25

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Key Concepts

- Coronary microvascular disease (CMD) is illustrated by the inadequate increase in coronary blood flow during stress due to impaired vasodilatation of the arterioles or increased resistance in the coronary microvasculature.
- CMD is measured by coronary flow reserve (CFR, which is the vasodilator capacity of the epicardial artery and its microcirculation) and myocardial perfusion reserve (MPR, which is the maximal possible increase in myocardial blood flow in response to exercise).
- The cause of CMD is unclear, but the proposed mechanisms include impaired endothelium-dependent vasodilation, impaired smooth muscle relaxation and enhanced vasoconstrictor activity in the coronary microcirculation.

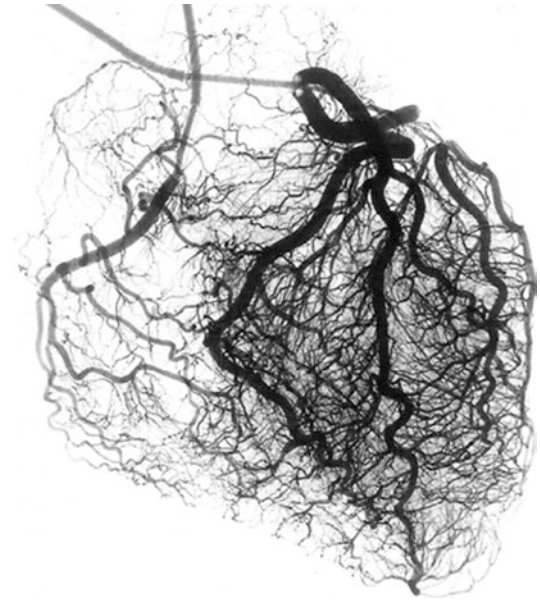

Fig. 25.1 Filling of the coronary microvasculature in a normal post-mortem human heart by immersion radiography [31] (© Copyright, University of Glasgow)

25.1 Introduction

In 1963, William Fulton demonstrated the existence of the coronary microcirculation (Fig. 25.1) using a pathoanatomic imaging technique developed to differentiate between vascular overlay and true connections between adjacent parts of the circulation [1]. These coronary anastomoses, which can be identified in autopsy specimens, are usually too small to be visible angiographically and can be as small as 30 μm. In the last two decades, we have learnt that abnormalities in the function and structure of the coronary microcirculation can occur in the absence of obstructive coronary artery disease (CAD) [2].

In clinical practice, a significant proportion of patients with chest pain do not have obstructive CAD, and yet they have a poorer prognosis, with higher rates of hospitalisation and adverse cardiovascular events [3]. Compared with population-matched controls, they have almost double the risk of death, myocardial infarction and stroke over a 7.5-year period. Approximately 30–50% of patients with chest pain but no obstructive CAD are believed to have coronary microvascular disease (CMD) [4–6]. CMD has significant social and economic impact on the healthcare system and is a problem of unmet need.

25.2 Definition and Diagnosis

Symptomatically, CMD manifests as microvascular angina, which is a distinct clinical entity from angina due to obstructive CAD. CMD is typically defined as an inadequate increase in coronary blood flow during stress due to impaired vasodilatation of the arterioles or increased resistance in the coronary microvasculature.

The diagnosis of CMD involves the assessment of microvascular function. This is determined quantitatively by measuring coronary flow reserve (CFR) and index of microvascular resistance (IMR). CFR reflects the vasodilator capacity of the epicardial artery and its microcirculation [7], whereas IMR is a measure of resistance in the microcirculation [8]. These are measured during invasive coronary angiography with intracoronary infusion of saline using a coronary catheter, a pressure-sensing coronary guidewire and thermodilution.

Myocardial perfusion reserve (MPR) is another means of diagnosing CMD, using positron emission tomography (PET) or cardiac magnetic resonance imaging (CMR) [9]. MPR is the maximal possible increase in myocardial blood flow in response to exercise [10]. It incorporates perfusion through both the epicardial coronary arteries and the microcirculation.

Although CFR and MPR are widely used as the diagnostic criteria for CMD, they are continuous variables and the threshold for defining dysfunction, and therefore the sensitivity and specificity can vary. Generally, a CFR and MPR of greater than 2 [11] and an IMR of lower than 25 [12] are considered normal.

25.3 Causes of CMD

25.3.1 Causal Factors

The causes of CMD can be heterogeneous, and among the many possible causes are:

- Endothelial and smooth muscle dysfunction
- Extravascular compressive forces
- Inappropriate sympathetic tone
- Microvascular atherosclerosis and inflammation [2]

Patients with CMD typically share the same vascular risk factors as those with obstructive CAD, such as hypertension, smoking and diabetes. However, in contrast, CMD is associated with the female sex [11].

25.3.2 Pathophysiology of CMD and Endothelin

Reduced nitric oxide (NO) release causing impaired endothelium-dependent vasodilation [13, 14] is one of the most commonly proposed mechanisms of CMD. This is illustrated by reduced coronary blood flow in response to acetylcholine.

However, reduced endothelium-dependent vasodilation does not fully account for CMD. Reduced coronary blood flow in response to endothelium-independent vasodilator, such as adenosine, has also been observed [13, 14]. This suggests a primary impairment of smooth muscle relaxation.

Enhanced vasoconstrictor activity in the coronary microcirculation has also been shown [13]. A reduction in coronary blood flow can be induced by acetylcholine despite the absence of epicardial vasoconstriction. The presence of slow flow in the epicardial coronary arteries during coronary angiography also suggests microvascular constriction [15].

Endothelin-1 (ET-1) is implicated in the pathophysiology of CMD. ET-1 is a 21-amino acid peptide that is released mainly by endothelial cells, but other cells such as vascular smooth muscle cells (VSMCs) are also sources [16]. ET-1 is a highly potent vasoconstrictor via its VSMC receptors (ET_A, ET_B), but it also has pleiotropic effects. ET-1 is mitogenic, pro-oxidant, pro-inflammatory and inotropic, and ET-1 also regulates renal fluid and electrolyte homoeostasis [16]. ET-1 augments vascular tone constitutively (i.e. flow-mediated) and under stress [17], and local ET-1 activity reflects bioavailable vasoconstrictor and vasodilator chemicals [18], especially those derived from the endothelium [19]. ET-1 is implicated in the pathogenesis and progression of CAD [20].

ET_A receptors mediate vasoconstriction [16]. ET_B receptors are located on endothelial and VSMCs, and ET_B has NO-dependent vasodilator effects in healthy blood vessels or vasoconstrictor effects if NO is deficient. In pulmonary resistance arteries, the constrictor response to ET-1 is biphasic and varies with ET-1 concentration [21]. Selective pharmacological antagonism of ET_A and ET_A/ET_B has confirmed that ET-1/ET_A/ET_B regulate resting and stimulated forearm blood flow in patients with CAD [22]. ET-1 enhances coronary vascular tone in vivo via ET_A activation, it contributes to coronary endothelial dysfunction [23], and its tonic effect on myocardial perfusion is related to the presence and extent of risk factors for atherosclerosis [24].

25.4 CMD in Clinical Practice

25.4.1 Myocardial Ischaemia and Angina

In clinical practice, the role of CMD as the cause of myocardial ischaemia and angina is not well understood and often doubted. This is because unlike disease in the epicardial arteries, disease in the microvasculature cannot be visualised by coronary angiography, and objective markers of myocardial ischaemia (such as stress-induced left ventricular wall motion abnormalities) are often undetectable. The latter could be explained by the scattered distribution of perfusion abnormalities in the microvasculature, in contrast to the homogenous distribution due to a diseased, narrowed epicardial artery.

25.4.2 Takotsubo Cardiomyopathy

The pathogenesis of Takotsubo cardiomyopathy, which is a transient systolic dysfunction of the mid- and apical segments of the myocardium in the absence of obstructive CAD, has remained unclear although several explanations have been proposed. One of the most commonly proposed explanations is coronary microvascular dysfunction [25]. This theory is supported by the observation of reduced coronary flow velocity in patients with Takotsubo cardiomyopathy [26]. Coronary microvascular dysfunction can lead to transient ischaemia and, subsequently, myocardial injury and cardiomyopathy.

25.4.3 Management

Management of CMD is empirical because of the paucity of data on its causes. Traditional vasodilators are the first step in treatment. β-blockers are reasonable when the predominant symptom is effort-related and have been shown to improve symptoms [27]. When symptoms persist despite first-line treatment with β-blockers, calcium channel antagonists and nitrates could be helpful although they have shown conflicting results in clinical trials [27].

Although not a traditional antianginal, angiotensin-converting enzyme inhibitors may improve microvascular function [28] by counteracting the vasoconstrictor and pro-oxidant effects of angiotensin II.

There are some other drugs which could be used in a selected population of patients. Studies have reported symptomatic improvement with statins [29] and hormone replacement therapy with oestrogen [30]. This improvement is likely mediated by improvement in endothelial function. Endothelin receptor antagonists are an established treatment for microvascular disease in the lung (pulmonary arterial hypertension) and present a possible therapeutic option for CMD which will require further research.

Conclusion and Clinical Perspective

- Patients with CMD have a poorer prognosis compared with population-matched controls.
- CMD presents a challenge for diagnosis and treatment.
- Current treatment for CMD constitutes secondary preventative drugs and antianginal therapy, similar to treatment for CAD.
- There is a paucity of data on CMD in general, and more research into this problem is needed.

Gaps in Knowledge

- The true extent of the problem is unknown, and more research into the contemporary epidemiology of CMD is required.
- There is a lack of evidence evaluating therapies to relieve angina in patients with CMD.

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Cerebral Small Vessel Disease and Vascular Cognitive Impairment: Preclinical Aspects

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_26

Key Concepts

- The regulation of cerebral blood flow is complex and governed by cerebral autoregulation, neurovascular coupling, and endothelial function.
- Impaired cerebrovascular function causes cerebral perfusion mismatches resulting in inadequate blood supply to metabolically active parts of the brain. Over a long period, these perfusion mismatches lead to the development of vascular dementia and most likely contribute to Alzheimer's disease.
- Cerebrovascular endothelial dysfunction leads to blood-brain barrier (BBB) breakdown and cerebral blood flow dysregulation.
- Chronic inflammation impairs cerebral blood flow regulation, which has been linked to detrimental effects of cerebral perivascular macrophages and peripheral T-helper cells that produce interleukin 17 (IL-17).

26.1 Introduction

Cerebral SVD causes approximately 25% of all strokes and up to 50% of dementia cases depending on the population studied [1]. In humans, cerebral SVD causes lacunar infarcts, white matter hyperintensities, microbleeds, enlarged perivascular spaces, and cerebral atrophy [2]. In the context of animal models, cerebral SVD is considered to include any impairment in the structure and/or function of small arteries, arterioles, capillaries, veins, and venules with lumen diameters smaller than 100 μm. This definition includes both the pial vessels on the surface of the brain and their downstream parenchymal vessels. The parenchymal arteries are notable anatomically in that while their origin is on the surface of the brain, they subsequently plunge deep into the brain parenchyma and thus are crucial for precise delivery of blood [3]. Therefore, cerebral SVD results in the vasculature failing to distribute oxygen and nutrients to the regions of the brain where they are required in order to service neuronal metabolic activity. This chronic mild cerebral hypoperfusion also impairs neuronal function and memory formation by reducing protein synthesis and synaptic plasticity. At present there are no effective treatments for cerebral SVD; this is a result of our lack of understanding of the pathogenesis of the condition and the difficulty in identifying the early stages of the disease when treating its progression would be most viable. Animal models of both sporadic and genetic forms of cerebral SVD have the potential to inform future drug discovery efforts.

There are several common risk factors for cerebral SVD. Aside from aging, hypertension is the major risk factor. Hypertension causes mild, but chronic, inflammation, and it is becoming increasingly clear that this inflammation detrimentally affects the cerebral vasculature. In this chapter we will describe the physiologic mechanisms by which the cerebral vasculature regulates cerebral perfusion, and for each of these mechanisms, we will describe the effects of cerebral SVD as it relates to hypertension. We will focus on rat and mouse models of hypertension. A great benefit of these models is the ability to experimentally isolate specific segments of the cerebral vasculature to allow investigators to interrogate the pathways associated with vascular function/dysfunction in a region-specific manner.

26.2 Models of Cerebral Small Vessel Disease and Vascular Cognitive Impairment

There are several hypertensive rat and mouse models that show one or more traits of cerebral SVD. Here, we will discuss the most commonly used and the best characterized models. The spontaneously hypertensive stroke-prone rat (SHRSP) is a polygenic and multifactorial model that mimics human essential hypertension. SHRSP develop hypertension early in life, and by 3 months of age, they are markedly hypertensive. The life expectancy of an SHRSP is variable (from 9 to 15 months) depending on the levels of salt and protein in the diet and the colony from which the rats were obtained. Some studies show that SHRSP have reduced cerebral perfusion by 5 months of age, leading to hypoxia and ischemia. The cerebral infarcts observed at this time point mimic those that occur in hypertensive humans. Additionally, the SHRSP develop white matter injury which is a key indicator of cerebral SVD [4]. Feeding SHRSP a high-salt and low-protein

diet exacerbates the hypertension and speeds up the development of cerebrovascular injury. This is the result of the extensive renal damage which makes the model less clinically relevant [4]. One concern about this model is the propensity of SHRSP to develop microhemorrhages and larger strokes at a higher rate than observed in humans with cerebral SVD. This concern can be alleviated by using spontaneously hypertensive rats (SHR) that develop less severe hypertension than SHRSP over the same age range. SHR develop many of the markers of cerebral SVD including cognitive dysfunction, a reduced brain volume, vascular injury, white mater injury, and astrogliosis [5]. The two-kidney two-clip model of renovascular hypertension also leads to the development of white matter injury and myelin loss. This is associated with a breakdown of the blood-brain barrier and vascular changes particularly in the small blood vessels that include an increase in the wall-to-lumen ratio of the arteries, increased collagen deposition, and vascular inflammation [4, 6].

There are also several mouse models of hypertension that are proving to be potentially useful for studying cerebral SVD and the associated cognitive decline. The BPH/2J genetically hypertensive mice have impaired cerebral blood flow regulation, and they exhibit evidence of cognitive decline [7]. Studies from our own lab show that 4 weeks of subcutaneous angiotensin II (AngII) treatment through a continuous "minipump" produces hypertensive mice with significant remodeling of their cerebral small vessels [8]. These mice also develop significant cognitive impairments and impaired cerebral artery dilation [9]. The benefit of the AngII model is that it can be applied to knockout mouse strains to identify the specific genes involved in the development of hypertensive cerebral SVD.

The hypertensive models of cerebral SVD described above are considered to be sporadic models of the disease. There are also genetic models of cerebral SVD that are useful in understanding disease pathogenesis. Mice that express a mutant form of collagen α1 have intracerebral hemorrhages and cerebral vessel defects that mimic those seen in humans with cerebral SVD. Mice with mutations in the *NOTCH3* gene have normal blood pressure but they develop the pre-symptomatic stages cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a monogenic form of cerebral SVD [2].

Large animal models of cerebral SVD include primates, sheep, and dogs. These models exhibit vascular injuries that result in cognitive decline; however they are not widely used because of their cost and the prolonged aging process. These models do have several advantages, not least of which is the fact that their brain anatomy is closer to that of a human. Primates have more advanced cognitive abilities than rodents, which, along with the improved resolution for neuroimaging, makes the use of these models advantageous particularly in preclinical drug testing [10].

26.3 Cerebrovascular Anatomy

Before describing the effects of cerebral SVD, it is important to pause and consider some of the unique features of the cerebral vasculature which prevent the extrapolation of scientific findings in peripheral blood vessels to the brain. The need to consider the cerebral vasculature as an independent entity is perhaps best highlighted by the fact that the genetic mutations responsible for CADASIL are found in all vascular smooth muscle cells, but only the cerebral arteries are functionally impacted [11]. The implication is that although small arteries throughout the body appear similar at first glance, their functionality from both cellular and physiological perspectives varies widely dependent on where in the body they are situated. Thus, small arteries in the brain will behave and respond to stimuli very differently from small arteries in the mesenteric or subcutaneous circulations.

Cerebral SVD occurs in both the arterial and venous segments of the circulation. In this chapter we will discuss only the arteries and arterioles because this segment of the vasculature has been the focus of the majority of the preclinical studies. Based on their basic structure, the cerebral arterial circulation can be divided into three distinct regions: the pial arteries/arterioles, parenchymal arterioles (PAs), and the capillary bed (Fig. 26.1a). The pial vessels and the capillary bed are similar in that their extensive subsidiary collateral artery and arteriolar networks allow for blood flow redistribution when an individual artery is injured or occluded; this reduces the risk of developing large cerebral infarcts. PAs are not connected by collateral vessels; therefore in the context of the perfusion of the cerebral capillaries

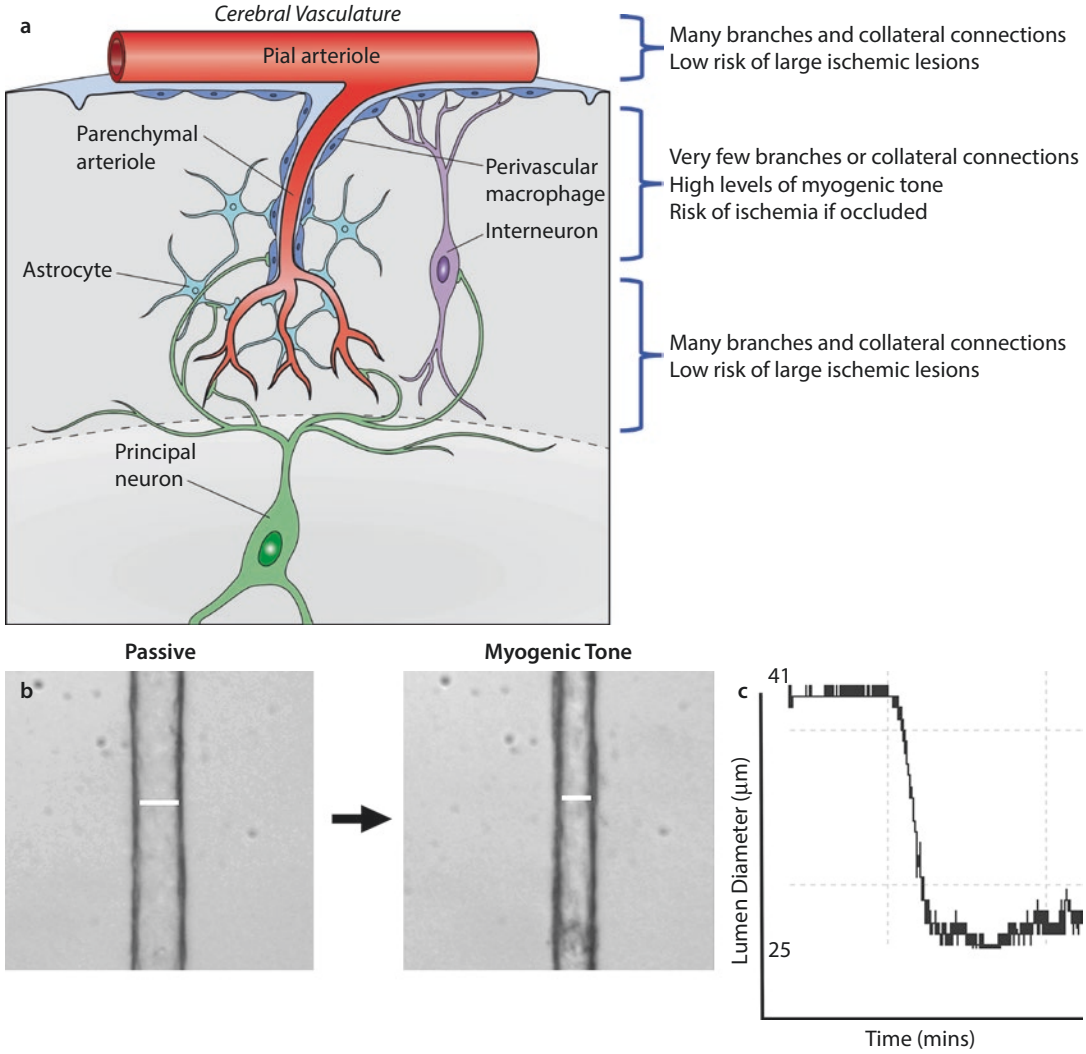

Fig. 26.1 Panel (**a**) A schematic diagram of the cerebral circulation. Panel (**b**) shows a mouse parenchymal arteriole under passive conditions and after the artery spontaneously constricts in response to an increase in the intraluminal pressure. This phenomenon is also known as "myogenic tone" which can be defined as the intrinsic ability of arteries and arterioles to maintain active contractile force in the smooth muscle cells. Factors contributing to the regulation of myogenic tone include the pressure inside the vessel, resting potassium conductance, calcium channel activity, and the sensitivity of the contractile pathways to calcium. In both images the white line represents the lumen diameter of the PA. Panel (**c**) shows an original tracing indicating the development of tone in the PA, as tone develops the artery constricts reducing the lumen diameter from ≈40 to ≈25 μm

where gas and nutrient exchange occurs, the PAs are the "weak link" or bottleneck. Importantly, occlusion of one PA initiates a cascade of events that results in the development of an infarct much larger than that predicted from the region perfused by the PA. This infarct expansion is the result of the constriction of capillaries and blockade of venules downstream from the originally occluded PA. The lack of venous efflux causes blood stagnation and eventual blockage of the PAs surrounding the originally occluded arteriole [12]. The pial arteries/arterioles and the PAs also differ in their innervation; the pial vessels have multiple layers of smooth muscle cells and are innervated by the peripheral nervous system. These nerves disappear as the PAs dive into the brain parenchyma. Here, occasional axon terminals or dendrites are found in close association with the PAs, but most of the arterioles are covered in astrocytic endfeet [13].

The interaction between the smooth muscle and endothelial cells in the vasculature with other cell types, including neurons, astrocytes, glia, pericytes, and perivascular macrophages, is unique to the brain. All of these extravascular cell types have the potential to impact blood vessel function and cerebral SVD progression (Fig. 26.1a). The neurons, astrocytes, and vascular cells (smooth muscle cells, endothelial cells, and pericytes) act in concert to regulate cerebral blood flow; this functional network of cells is known as the neurovascular unit. The composition of the neurovascular unit varies depending on the location of the blood vessels within the cerebrovascular tree [13].

The endothelial cells are central to the functioning of the neurovascular unit. The structure of the endothelial layer in the brain is different from elsewhere in the body. These endothelial cells are linked together with tight junction proteins, including claudins and occludins, to limit the entry of circulating cells and substances into the brain and form the blood-brain barrier. The myoendothelial gap junctions connect the endothelial cells to the vascular smooth muscle cells. These membrane microdomains are particularly important for the regulation of cerebral artery dilation [6].

26.4 Structural Mechanisms Regulating Blood Flow and Vascular Resistance

26.4.1 Artery Remodeling

Vascular resistance is the force that opposes blood flow; put simply, when vascular resistance is high, blood flow through that vessel is low. In peripheral vascular beds, the vast majority of the vascular resistance is carried by the arterioles. The site of areas of high resistance in the cerebral vasculature is a controversial subject. Current dogma states that under baseline conditions, the cerebral arteries upstream of the pial arterioles carry approximately 50% of the resistance; the other 50% is carried by the arterioles and venules in the brain parenchyma. In this scheme the PAs account for 30–40% of cerebrovascular resistance, and the capillaries contribute little resistance [3]. However, recent studies using advanced microscopy data with large-scale hemodynamic simulations suggest the capillaries contribute most of the cerebrovascular resistance [14]. Vascular resistance is regulated by changes in artery structure and by changes in artery constriction and dilation.

Hypertension causes chronic increases in cerebrovascular resistance via several mechanisms [3]. Artery remodeling is the term given to structural changes in the vasculature that are observed under passive conditions, when the vascular smooth muscle cells are deactivated to prevent constriction. Under experimental conditions this is measured by studying arteries at different intraluminal pressures in solutions devoid of calcium – thus preventing any constriction of the arteries in response to the pressure [6]. Hypertensive cerebral artery remodeling reduces the lumen diameter of the vessels and the area available for blood flow and subsequently increases vascular resistance. In some cases, the reduction in lumen diameter occurs with a relative increase in the artery wall thickness as a result of hypertrophy. This causes an increase in the wall-to-lumen ratio, which is recognized as a key marker of end-organ damage. This process, known as inward artery remodeling, has been observed in larger cerebral arteries, pial arteries, and arterioles and in the PAs from hypertensive rats and mice [6, 8, 15]. The reduction in the lumen diameter of cerebral arteries is particularly important in situations where the vessels become maximally dilated which occur during cerebral ischemia. The development of hypertensive artery remodeling actually begins as a protective process to reduce the wall stress that occurs when blood pressure is increased. The remodeling process also serves to protect the capillaries and venules from increases in pressure in an attempt to reduce the risk of microhemorrhage and blood-brain barrier breakdown. However, the remodeling also limits the ability of the arteries to accurately regulate cerebral blood flow [3].

Many mechanisms associated with hypertension are involved in the regulation of cerebral artery remodeling. Of these, activation of the renin-angiotensin-aldosterone system is the most well studied. It is well known that angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists, and mineralocorticoid receptor antagonists all prevent artery remodeling [6]. A diverse range of other potential mechanisms for artery remodeling have also been proposed. These include but are not limited to increased superoxide generation, matrix metalloproteinase activation,

epidermal growth factor receptor activation, and chloride channel activation [3, 6]. In terms of developing therapies for *cerebral* SVD, it is important to identify treatments that reverse artery remodeling. The mechanisms described above are all important for the development of remodeling, and very few of them have been tested for their ability to reverse the process. In patients with sustained or unstable hypertension, complete reversal of the remodeling process may even be detrimental because reducing the wall thickness in the face of elevated or fluctuating blood pressures could lead to the development of hemorrhages.

26.4.2 Artery Rarefaction

Loss of arterioles and capillaries through the process known as artery rarefaction also increases vascular resistance [6]. The vessels which are lost are often collateral vessels, and their loss limits the ability of the brain to respond to ischemic insults and vascular injury. While we know very little about the mechanisms responsible for the loss of blood vessels in the brain, it does appear that impaired nitric oxide (NO) production leads to collateral artery loss [3]. However, it is not clear at present if this process is reversible or if it can be stopped with treatment.

26.5 Active/Dynamic Mechanisms That Regulate Cerebral Blood Flow

Neurovascular coupling and cerebral autoregulation work in concert to regulate the perfusion of the brain parenchyma. Both mechanisms are impacted by cerebral SVD in hypertension in ways that reduce cerebral perfusion. The cerebral endothelium is also involved in the regulation of cerebral blood flow. It will be discussed separately from the other mechanisms that regulate flow because it has significant additional effects on cerebrovascular function.

26.5.1 Neurovascular Coupling

The term neurovascular coupling, or functional hyperemia, describes the process that links changes in the activity of neurons and glia to increased blood flow. This process ensures the delivery of sufficient oxygen and nutrients and the removal of waste products from the active brain regions. Neurovascular coupling is mediated by the coordinated efforts of the endothelial cells, neurons, and astrocytes as well as the other cells in the neurovascular unit. Active neurons can signal directly to the vasculature to increase cerebral blood flow, or they can utilize the associated astrocytes to relay messages to the blood vessels. Recent studies suggest that the involvement of astrocytes in neurovascular coupling differs along the length of the vascular tree. In arterioles the astrocytes act to modulate neurovascular coupling, while they have a more direct role in mediating coupling in the capillaries [16]. At present there is not a definitive list of the signaling molecules involved in the neurovascular coupling process, but potassium ions, neurotransmitters including GABA and acetylcholine, NO, adenosine, prostanoids, and other arachidonic acid metabolites have all been implicated in the process [3]. Pericytes, which are in close association with the vessels may also be involved in the regulation of neurovascular coupling, but this concept is somewhat controversial. Neurovascular coupling is generally accepted to be impaired in hypertension [17]. This impairment was exacerbated by the aging process and could not be prevented by treatments that lowered the blood pressure in hypertensive rats [18]. Similar impairments have been observed in mouse models of hypertension [19]. Impaired neurovascular coupling results in hypoperfusion of the active brain regions. If blood flow is to be increased by neurovascular coupling, the vessels upstream of the microcirculation must also dilate to ensure sufficient perfusion. This is driven by propagated dilation, which is impaired in hypertension, and this could potentially exacerbate the impairments that occur when neurovascular coupling is compromised [3]. The mechanisms responsible for impaired neurovascular coupling in hypertension have not been fully elucidated and are likely to involve several integrated processes including structural remodeling and impaired dilation.

26.5.2 Cerebral Autoregulation

Cerebral autoregulation is the process that allows the brain to maintain fairly constant cerebral blood flow in the face of fluctuating perfusion

pressures. Through this process, cerebral arterioles dilate in response to reduced intraluminal pressure and constrict when pressure increases; thus, blood flow remains constant. Both myogenic tone and myogenic reactivity contribute to the autoregulatory process. Myogenic tone refers to the spontaneous contractile force maintained in the vascular smooth muscle cells of arteries and arterioles (Fig. 26.1b). This is regulated by the sensitivity of the contractile machinery to calcium and the activity of the calcium channels. Intraluminal pressure and potassium channel conductance are also important regulators of tone [6]. It is generally accepted that myogenic tone generation is increased in the cerebral arteries from hypertensive models and that antihypertensive therapies prevent this [15]. Myogenic reactivity refers to the ability of arteries to change their tone in response to changes in intraluminal pressure. The mechanisms by which cerebral arteries sense the changes in intraluminal pressure have not been fully elucidated but are thought to include integrins, G proteins, ions channels, and kinases [6].

The autoregulatory curve for any given artery depicts the range of intraluminal pressures over which autoregulation occurs and where blood flow remains constant. At pressures above and below the autoregulatory range, blood flow is directly proportional to the pressure in the artery. In hypertension, the autoregulatory curve is shifted to the right which means the blood vessels autoregulate at higher pressures. This increases the risk of reduced perfusion in situations where the intraluminal pressure is significantly reduced, as might occur with the injury or occlusion of an upstream artery. Blood pressures above the autoregulatory range could lead to the development of hemorrhages as the elevated blood pressure and flow disrupt the microvessels. The cerebral endothelium modulates also myogenic reactivity. It does this by affecting dilation through the generation of NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). Importantly, all of these dilator pathways have been shown to be reduced by hypertension in a variety of vascular beds [6].

Loss of the autoregulatory capacity of cerebral arteries may be an indication of end-stage vascular disease. Feeding SHRSP a high-salt diet causes the large cerebral arteries, including the middle cerebral artery (MCA), to lose their ability to autoregulate. Although, the MCA is not considered to be a small vessel, this loss of the autoregulatory capacity could be important for the development of cerebral SVD. The increased blood flow that occurs when autoregulation fails will be transferred to the downstream arterioles along with the potential for fluctuations in blood pressure. This could cause the development of microhemorrhages [6].

26.6 Endothelial Function and Cerebral Small Vessel Disease

The endothelial cell layer is the common component in all vascular structures from the largest arteries to the smallest capillaries. The endothelial cells within the brain form a continuous layer of cells linked together by tight junction proteins. In many ways, the vascular endothelium can be viewed as the master regulator of cerebral SVD because it regulates many of the pathways central to the appropriate function of the cerebral arteries. Healthy endothelial cells are required for proper functioning of the blood-brain barrier (BBB) because they regulate the movement of cells and molecules between the blood and the brain. Disruption of the BBB plays a major role in the pathogenesis and inflammatory cascade in cerebral SVD. Aside from their role as gatekeeper to the brain, endothelial cells regulate a diverse array of functions not associated with nutrient exchange including immune function, thrombosis, angiogenesis (new capillary formation), arteriogenesis (remodeling of preexisting vascular anastomoses into functional arteries), collateral growth, and vascular rarefaction [20, 21].

Hypertension significantly impairs BBB function. This has the potential to increase neuroinflammation and to cause cerebral edema formation. Drugs which inhibit the actions of the renin-angiotensin system can reduce BBB breakdown in hypertensive models. Interestingly this seems to be a direct effect of AngII and not of the elevated blood pressure per se [3]. Recent studies have shown that AngII administration directly causes the BBB to open, allowing AngII access to the perivascular space [19]. BBB breakdown requires the activation of matrix metalloproteinase enzymes (MMPs) which have been shown to be important in the hypertensive remodeling process [6].

Endothelial cells play an increasingly well-recognized role in regulating neurovascular coupling through their ability to produce both vasoconstrictors (prostaglandin H2 and thromboxane A2) and vasodilators (NO, prostacyclin, carbon monoxide, and hydrogen sulfide). In the pial arteries, endothelium-dependent dilation is largely mediated by NO production, whereas in the PAs, NO-independent dilation is also important [3]. Endothelial cells can cause direct vasorelaxation by mediating endothelium-dependent hyperpolarization (EDH). This mechanism requires a local increase in intracellular calcium in the endothelial cells. This leads to activation of the small and intermediate conductance calcium-activated potassium channels which causes hyperpolarization of the endothelial cells. This hyperpolarization is transmitted to the vascular smooth muscle cells via the myoendothelial gap junctions to produce dilation. Several transient receptor potential (TRP) channels are expressed in the cerebral endothelium and are thought to be crucial to the activation of this EDH-mediated dilation. At the level of the microvasculature, the endothelial cells can modulate blood flow by sensing blood-born agonists and changes in flow [16].

Hypertension causes significant impairments in endothelium-dependent dilation, and a variety of mechanisms are responsible for this. NO-mediated dilation is impaired in the cerebral vasculature of many rodent models of hypertension and is frequently associated with a marked increase in reactive oxygen species production [19]. Several NO-independent vasodilator mechanisms are also impaired in hypertensive models, including dilation mediated by the epoxyeicosatrienoic acids (EETs). Reduced EETs production from arachidonic acid may be responsible for this impaired NO-independent dilation. Preliminary studies from our lab suggest that PA dilation mediated by activation of the TRP vanilloid 4 (TRPV4) channel, a component of EDH-mediated dilation, is impaired in hypertension.

26.7 The Role of Neuroinflammation in the Pathogenesis of Cerebral Small Vessel Disease

There are several potential ways in which inflammation could affect the cerebral vasculature and cognitive function. Cerebral SVD can result in BBB breakdown which allows immune cells from the circulation to infiltrate into the brain parenchyma resulting in neuroinflammation. Astrocytes are important in providing support to the endothelial cells that form the BBB. However, the astrocyte cell can become activated in response to injury in a process called astrogliosis. Studies have shown an inflammatory response and astrogliosis in the white matter in regions where there is loss of myelin around the blood vessels. Both the reactive astrocytes and activated microglia release free radicals as well as a number of inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha. Reactive astrocytes and microglia also release matrix metalloproteinases (MMPs), including MMP-9, which can mediate the migration of T lymphocytes to white matter lesions in SHR [4, 5]. It is not clear at present if this increase in neuroinflammation is a cause or effect of cerebral SVD, although the recent studies described below point to a potential causative effect.

The brain contains two distinct macrophage populations: the perivascular macrophages (PVM) and the choroid plexus macrophages. The PVM are emerging as particularly important contributors to the genesis of cerebral SVD. The PVM are located in the intracerebral perivascular space and originate from hematopoietic precursors and are closely associated with the outer vessel wall in pial and parenchymal arteries/arterioles that are bigger than 20 μm in diameter. In pial arteries, about 80% of the wall is covered in PVM, and coverage in the PAs is slightly lower at about 60%. Neurovascular coupling is impaired in AngII-hypertensive mice, and removal of the PVM improved neurovascular coupling and endothelium-dependent dilation, returning it back to the levels observed in control mice. AngII was shown to cross the BBB and enter the perivascular space. The detrimental effects of AngII on the cerebral vasculature were linked to activation of the angiotensin receptor type 1 on the PVM, and this was associated with increased oxidative stress. Similar findings were also observed in genetically hypertensive mice (BPH/2J mice), in which macrophage depletion improved cognitive function [19].

High levels of dietary sodium contribute to cardiovascular disease development, and the association with cerebral SVD appears to be due, at least in part, to a gut-brain axis that

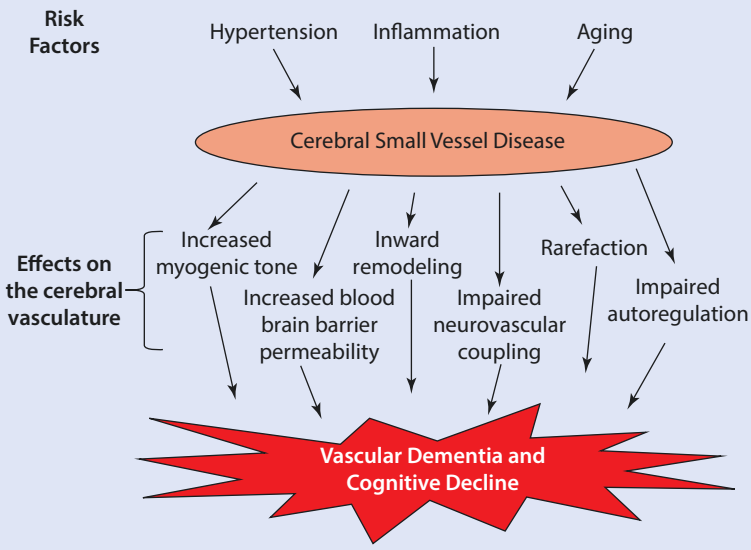

Fig. 26.2 Schematic of the risk factors associated with cerebral SVD and their effects on the cerebral vasculature that lead to cognitive decline

promotes inflammation and vascular injury. Although a high-salt diet alone did not increase blood pressure in mice, it did markedly reduce cerebral blood flow and impair both neurovascular coupling and endothelium-dependent dilation in the pial arteries. These changes in vascular function were associated with cognitive decline. This impairment was associated with an increase in the proinflammatory cytokine IL-17 which is produced by T-helper (TH17) cells within the gut. When T-cell deficient mice were treated with a high-salt diet, the cerebral vasculature and cognitive function remained normal. The link between vascular injury and IL-17 was confirmed by neutralizing IL-17 with specific antibodies in high-salt treated mice and by administering exogenous IL-17 to mice fed a normal diet [22].

Conclusions and Clinical Perspectives

There is no singularly perfect animal model of cerebral SVD. In many ways this is in keeping with the multifactorial development of the disease in humans. Hypertension is a major risk factor for the disease, and appropriate treatment of hypertension may prevent many of the vascular changes that contribute to the condition. Although inflammation is clearly an important contributing factor, it is likely that some of the inflammation involved in cerebral SVD is secondary to hypertension. Both hypertension and inflammation have numerous effects on cerebral artery structure and function that leads to vascular insufficiency in the brain and cognitive decline (Fig. 26.2). In the future, it will be important to identify treatments that can impact both the structural and functional impairments caused by hypertension and inflammation that mediate the development of cerebral SVD.

Gaps in Knowledge

There is a clear clinical need for an improved understanding of the pathogenesis of cerebral SVD, and several knowledge gaps remain. Despite the recent interest in sex as a biological variable, very few studies have compared the small cerebral arteries from males and females, and the information regarding the impact of cerebral SVD risk factors on the sexes is even more scant. Similarly, most studies focus on disease prevention. To be clinically relevant, future studies must focus on slowing the progression of the disease once it begins and ideally disease reversal. Clinically, this is particularly difficult as the

first symptoms of any neurological condition are often not noted until significant neurovascular injury has occurred. Thus, identification of easily measurable biomarkers for cerebral SVD would be a significant gain both to the research endeavor and the clinical realm.

As mentioned previously, very few studies utilize large animal models of cerebral SVD, yet this would clearly enhance the clinical relevance of any study. We also must consider the behavioral tests used to assess cognitive decline. It is important that these tests are extremely well controlled and that investigators are blinded to treatment groups. As the field moves forward, it will be important to identify tests and models that better reflect the cognitive changes observed in humans with cerebral SVD which include apathy and executive dysfunction which includes impaired impulse control, attention, focus, and task initiation.

The role that the venous system plays in cerebral SVD and in the associated neurological conditions remains to be investigated. The cerebral glymphatic system is a macroscopic waste clearance system that utilizes the perivascular space between the cerebral blood vessels and the astroglial cells to remove soluble proteins and metabolites from the brain [23]. The developing literature suggests the glymphatic system plays an important role in the development of Alzheimer's disease and perhaps other dementias. Recent improvements in methods to image the cerebral vasculature, such as multiphoton super-resolution microscopy, should allow for rapid progress in this field over the next few years.

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Ethnicity and Cardiovascular Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_27

Key Concepts

- Globally numerous ethnic groups reside in different regions, with unique exposures to specific environments, health behaviours, social norms and cultural traditions.
- These groups, including black Africans, white Europeans, Hispanic, South Asians, Asians, Aboriginal people, Pacific Islanders, and many others, also exhibit specific heritable traits which may be important in determining cardiovascular risk.
- Health disparities have been reported consistently indicating increased cardiovascular and metabolic risk in specific ethnicities, with many existing risk prediction models mainly developed for European populations not being effective in these populations.
- With large-scale population migration and also within country demographic transitions taking place, it is important to obtain a better understanding of ethnic-specific cardiovascular disease development in order to employ effective disease prevention strategies.

27.1 Introduction

Globally there is considerable heterogeneity in terms of life expectancy when comparing countries and regions [1], with cardiovascular disease remaining the primary contributor to mortality. When reviewing worldwide trends in blood pressure over the past decades, it becomes evident that the highest worldwide blood pressure levels have shifted from high- to low-income countries [2]. An array of explanations can be provided for the differences in blood pressure and cardiovascular disease prevalence, whether it is due to regional, environmental exposures or whether it is due to genetic origins.

Within this chapter there will be a specific focus on the concept of *ethnicity* and how it is defined. It is also important to realise what ethnicity means in terms of cardiovascular disease risk, its development, treatment and outcomes.

The word 'ethnicity' originates from the Greek word 'ethnos', referring to a nation. It is a complex, multidimensional social construct reflecting self-identification with cultural traditions, religion, customs and social identity [3]. It is also a term generally used for people sharing a specific physical appearance, such as skin colour, hence similar genetic origins. Other terms that are often used include race or ancestry, with each having a specific meaning. Race is primarily associated with physical features of a person, whereas ethnicity rather reflects culture. Notwithstanding the different terminology used, it is well known that diverse ethnic groups have ancestral origins in Europe, Asia, Africa or the Caribbean [3], and with ethnicity also comes distinct health features presenting specific cardiovascular risk profiles.

Disparities in cardiovascular risk was demonstrated in a study that evaluated different risk scores, including the Framingham cardiovascular disease risk score, over 10 years in a tri-ethnic population in the United Kingdom [4]. When including white Europeans, South Asians and African-Caribbeans, it was found that neither risk score performed consistently well in all ethnic groups, thereby indicating the inherent cardiovascular risk within specific population groups. Similar findings were reported in the United States, where persistent racial and ethnic disparities are reported in cardiovascular disease morbidity and mortality from 1999 to 2012, with black and Mexican-American populations indicating increased cardiovascular burden [5]. Ethnic comparison studies within Africa have also confirmed reports that black populations exhibit significantly greater blood pressures and overall cardiovascular disease risk when compared to white counterparts [6]. As cardiovascular risk prediction or stratification is a cornerstone of preventive strategies, it is essential that existing scores be improved and tailor-made according to the risk presented by different ethnic groups. With many ethnicities residing within single countries due to large-scale global population migration, a better understanding on ethnic differences in terms of cardiovascular disease prevention programmes and healthcare is needed.

27.2 Factors Contributing to Ethnic Differences in Cardiovascular Disease

27.2.1 Genetics of Ethnicity

The contribution of genetics to hypertension and cardiovascular disease risk has been questioned in the past, due to the multifactorial origins of cardiovascular disease. Importantly it was shown in early ethnic-specific studies conducted in rural populations, such as American Indians, Alaska Natives or rural Africans, that cardiovascular disease was extremely rare [7]. This was clear from cardiovascular disease outcome data and confirmed when evaluating cardiovascular risk factors, reporting very low blood pressures and serum lipids. Recent papers reviewing health marker trajectories over time report an alarming change in cardiovascular disease burden in these populations with many residing within low- and middle-income countries, which are known to carry a substantial burden of hypertension and cardiovascular disease [2]. Environmental factors that have contributed to this marked shift in cardiovascular disease burden will be discussed in subsequent sections, yet the contribution of genetics still requires further clarification. Although ethnicity in itself is important in explaining cardiovascular risk, so is the interaction of ethnicity with sex. It thus remains important to take sex into account in genetic studies along with ethnicity [8].

The International Consortium for Blood Pressure Genome-Wide Association Studies has confirmed recently that blood pressure is indeed a heritable trait [9], and their specific genetic risk score (based on 29 genome-wide variants) was associated with hypertension, left ventricular wall thickness, stroke and coronary artery disease. Although their analyses included 200,000 individuals of European descent, they also observed associations with blood pressure specifically in East Asian, South Asian and African ancestry individuals. Most importantly it was recognised that blood pressure is also influenced by multiple biological pathways and is highly responsive to environmental stimuli [9] – with different responses in ethnic groups [10].

Making sense of recent reports indicating a shift in the regional burden of hypertension [2] and mortality [1], it is plausible that these shifts would be largely explained by changes in demographics, health behaviours and environmental exposures.

27.2.2 Environmental Factors: Socio-economic Status and Demography

An important factor determining overall health status is a person's socio-economic status. Socio-economic status is a combination of the individual's or family's economic and social position when compared to others and is usually based on three main factors, namely, income, education and occupation. The actual health effect of this social standing or class of an individual or population is often neglected but is an essential determinant of health.

A meta-analysis reviewing the relationship between socio-economic status and hypertension including 51 studies found clear results indicating that a low socio-economic status is associated with higher blood pressure. A prominent finding was that those with lower education were twice as likely to have hypertension compared to those with higher levels of education [11]. In sub-analyses the authors combined studies from Africa and found a contrasting result, namely that higher income and occupation levels in Africa associated with higher rates of hypertension [11]. These findings clearly demonstrate that there is not necessarily a linear association between socio-economic status or its components and cardiovascular health and that certain demographic factors, such as the developmental status of a country or ethnic group, need to be considered.

The Global Burden of Disease Collaborators regularly report high-level global health data. To move beyond binary description of developed and developing countries and assessments of development status (only based on income), they developed the Socio-demographic Index. This was calculated by incorporating income per capita, average years of education and total fertility rate [1]. By including this measure in all analyses,

a better comparison between regions and ethnic groups can be performed.

To better understand ethnic differences while taking socio-economic status into account, a study was performed in South Africa comparing cardiovascular health between black and white school teachers followed over 3 years [12]. It was found that black teachers demonstrated greater increases in both systolic and diastolic blood pressure, abdominal obesity, total cholesterol, fasting glucose and other biomarkers such as fibrinogen and D-dimer, when compared to white teachers, suggesting that notwithstanding their similar socio-economic status, overall cardiovascular risk and the trajectory over time were greater in the black group. Further work is needed to determine the potential role of stress management and coping, environmental, behavioural and genetic factors to explain this increased cardiovascular risk.

27.2.3 Environmental Factors: Health Behaviours and Lifestyle

Lifestyle and health behaviours are the most important preventable cardiovascular risk factors and are relevant across populations. With recent dramatic demographic transitions from rural to urban areas, or migration and transitions across continents, adverse health behaviours are expected to escalate, with some strong indications already evident [13, 14].

It is furthermore important to understand the concept of lifetime risk applicable to the entire population from conception [15]. On a global scale, evidence indicates that the development of subclinical and clinical cardiovascular disease results from early life programming and lifetime exposure to cardiovascular risk factors. During pregnancy the adverse health behaviours of a mother could affect foetal health, and similarly health behaviours during childhood promote the development of cardiovascular disease in later life – promoting the trajectory of the so-called early vascular ageing already visible in young adulthood [6, 16].

Numerous health behaviours are known to directly contribute to increased cardiovascular risk, such as diet, physical activity, obesity, alcohol and tobacco use, but it will not be possible to discuss all in detail in this chapter. However, in the following sections, some behaviours will be highlighted that are known to have ethnic-specific implications.

27.2.3.1 The Nutrition Transition, Including Salt Intake

Certain nutrients and dietary intake of specific foods are known to be cardioprotective, including intake of fruits, vegetables, legumes, grains and dairy [17]. Yet, advances in food technology allowed the production of highly palatable, processed foods and beverages that are energy dense and in most instances more affordable than healthy options such as fruit and vegetables. Populations residing in low- and middle-income countries are greatly affected by these developments and in countries where specific ethnic groups have migrated, such groups often have lower socio-economic status and opt for cheaper processed food options, including sugary, fatty and salty foods.

Diets high in salt are also a well-known contributor to hypertension and cardiovascular disease. Specific ethnic groups, such as black populations, are known to include greater proportions of individuals with salt sensitivity [18]. This has important cardiovascular consequences, since a person with salt sensitivity exhibits greater increases in blood pressure when consuming similar amounts of salt than someone who is salt-resistant. A complex interaction between neuroendocrine factors and the kidney may underlie the propensity for such patients to retain salt and develop salt-dependent hypertension [18]. Hence, recent campaigns to reduce the sodium intake by reducing the salt content in processed foods, or to remove salt shakers from tables, are important.

27.2.3.2 Body Composition and Obesity

Obesity is a significant global threat to overall human health, with significant increases in obesity prevalence reported for most countries over the past decades [19]. Apart from efforts to turn this tide, it is important to identify patients at early phases of obesity development to reduce their risk for the subsequent development of non-communicable diseases such as cardiovascular disease and cancer. To determine whether a patient is obese, estimates such as body mass index (BMI) and waist circumference are widely used – with accepted cut-offs being 30 kg/m^2 for BMI and ≥94 cm for men and ≥80 cm for women in terms of

waist circumference. However, these cut-offs were defined based on data from European populations, and therefore it is important to use ethnic-specific cut-off values when evaluating patients from other ethnic groups, since body composition, such as height, muscle mass and fat distribution, is known to differ when comparing ethnic groups. Ethnic-specific BMI cut-offs were identified and validated for, e.g. Maori, Pacific Islanders, Asian Indians, Tongans, Japanese and Africans. Similarly specific cut-offs were also identified for waist circumference. It is particularly important that ethnic-specific cut-offs be used where appropriate, since many of the changes in cut-offs have proposed a lowering of, e.g. overweight cut-offs from 25 to, e.g. 22 or 23 kg/m^2. As an example, recently ethnic-specific waist circumference cut-offs for Africans were published [20], suggesting a similar cut-off of 80 cm for both African men and women. Taking this cut-off as example, the difference in the cut-off for European men of 94 cm signifies the dramatic gap of 14 cm when compared to the 80 cm advised for African men. This was further highlighted in a multi-ethnic population comparing white Europeans, African-Caribbeans and South Asians, indicating an ethnic-specific association of BMI at the diagnosis of type 2 diabetes with cardiovascular disease and all-cause mortality risk [21].

27.2.3.3 Tobacco and Alcohol Use

Tobacco use has remained one of the primary targets to reduce cardiovascular disease development, due to it being an established cardiovascular risk factor [17]. According to the Global Burden of Disease Study, there is a strong positive association between smoking and Socio-demographic Index, indicating that tobacco use remains popular among more affluent populations [17]. Yet many reports indicate that tobacco companies are targeting low- and middle-income countries where governments are less stringent in applying measures such as taxes to reduce population tobacco use. Yet smoking prevalence varies significantly among subgroups owing to religion, customs, traditions, social acceptance of smoking, attitudes and beliefs. In addition, 'chewing' tobacco is also common in certain communities [3].

On an ethnic level, research has shown that nicotine, which is the major addictive agent in tobacco smoke, is metabolised differently in African Americans when compared to Europeans [22]. This resulted in non-smoking African Americans excreting less nicotine and cotinine compared to Europeans when using similar nicotine patches. The cardiovascular implications of this finding are not yet clear but may suggest that longer exposure to circulating nicotine and cotinine may partly explain the increased risk for cardiovascular disease and cancer in Africans [22].

Excessive alcohol use also has significant cardiovascular consequences and is the seventh leading risk factor contributing to disability-adjusted life years – a measure of overall disease burden [17]. It is the leading risk factor between the ages of 15 and 49 years in 2016, and usage differs markedly between regions due to religion, with very low use in the Middle East. Regions such as South Asia, Southeast Asia and Central Asia have increased use over the past 25 years by 25%, in both men and women [17].

Previous studies have reported ethnic differences in the strength of association between habitual alcohol intake and risk of cardiovascular events – which may involve ethnic variations in genetic polymorphisms related to alcohol metabolism, cultural differences in drinking behaviour and patterns or other health risk factors [23]. Future studies are required to determine whether there is indeed ethnic variation in the acute and long-term effects of alcohol on cardiovascular risk and whether genetic differences are involved [23].

27.2.4 Environmental Factors: Metabolic Aspects

Several metabolic factors contribute to different disease outcomes observed in different populations. With ischaemic heart disease as the main cause of death for populations from European descent and South Asians, cerebrovascular disease including stroke seems more common in black populations [24]. Furthermore, the prevalence of type 2 diabetes is uniformly higher in South Asians than in many other populations – whether within India or in those residing in countries such as the United States or the United Kingdom [25]. This seems to be attributed to demographic and nutritional transitions as populations migrate rapidly from rural to urban settings.

These differences in disease outcome – to mention a few – can be attributed to a variety of factors, including metabolic factors associated with visceral obesity, glucose handling and

dyslipidaemia. South Asians, as an example, store a disproportionate amount of visceral fat and have higher levels of low density lipoprotein cholesterol [3, 25]. Due to higher amounts of visceral fat, South Asians present with greater insulin resistance when compared to other ethnicities with the same BMI [25]. This has a direct effect on the development of atherosclerosis and ischaemic heart disease. African populations, on the other hand, have a significantly greater risk for stroke than European populations, with higher blood pressures being an important contributor, and are less likely to have hypercholesterolaemia and ischaemic heart disease [6, 24].

27.3 Ethnic-Specific Pathophysiological Mechanisms Relating to Cardiovascular Risk

Globally there are indeed a range of different ethnic and race groupings, and where the majority share common risk factors, some disparities are also clear. Some of this is highlighted in a study that compared temporal trends in cardiovascular disease risk factors among white, South Asian, Chinese and black groups in Canada from 2001 to 2012 [26]. They found that the prevalence of diabetes increased more than twofold among South Asian men. The largest increases in obesity were observed in Chinese men. Overall, South Asian men and black men and women showed the greater declines in cardiovascular health over the study period – where the prevalence of hypertension increased most in black women [26].

In previous sections there were already alluded to potential mechanisms at play contributing to ethnic disparities in cardiovascular disease profiles. Embedded in many instances in genetic, environmental, behavioural and metabolic origins, some particular pathophysiological mechanisms observed in ethnic groups warrant further discussion.

Salt Sensitivity and Volume-Loading Hypertension A large proportion of black individuals exhibit salt sensitivity and a suppression of the renin-angiotensin-aldosterone system due to volume-loading hypertension [6]. Low plasma renin activity and aldosterone, as well as suppressed angiotensin I and II have been reported in black individuals compared to white groups, whether normotensive or hypertensive. This phenotype is characterised by a higher aldosterone-to-renin ratio, or both low aldosterone and renin, with elevated blood pressure. It further transpires that the aldosterone-to-renin ratio modifies the relationship between salt intake and blood pressure, reflecting a role in salt-sensitive low renin hypertension. Genetic polymorphisms in African populations confirm altered renal sodium handling, where sodium retention and volume expansion remain key role players in the development of hypertension in black populations [6]. This phenotype also guided Clinical Practice Guidelines for antihypertensive treatment indicating that medication directed towards the renin-angiotensin system should be avoided as first-line treatment.

Early Vascular Ageing The measurement of arterial stiffness (arteriosclerosis), which is a strong independent predictor of cardiovascular outcome, has proven to give more insight into accelerated vascular ageing [15]. When reviewing lifetime cardiovascular risk, it has become clear that especially black populations exhibit increased aortic stiffness when compared to others. This was confirmed in the Dallas Heart study where proximal aortic stiffness were compared between black, Hispanic and white populations, demonstrating that the black group had higher stiffness than Hispanics, which again had higher stiffness than whites – all independent of arterial pressure and other relevant risk factors [27]. This was also confirmed in 6–8-year-old black and white boys, with increased arterial stiffness in three segments of the arterial tree in black boys [6]. Different pathophysiological mechanisms may be involved in the process of arterial stiffening, where it has been suggested that chronic low-grade inflammation, which is more common in ethnic groups such as Africans, may be at play [6]. Related to arterial stiffening, an elevation in inflammatory biomarkers combined with an adverse metabolic profile is again involved in the early development of atherosclerosis in South Asians [25].

Autonomic Nervous System Regulation Altered activity of the autonomic nervous system has been put forward as a contributor to ethnic diversity in cardiovascular disease risk. This has been most studied in black and white populations, indicating sympathetic overactivity in black populations [6]. Yet, little is known about ethnic differences in other populations. In the Amsterdam Born Children and

their Development (ABCD) study, ethnic differences in autonomic regulation were studied in children [28]. It was found that at the age of 5–6 years, children from diverse ethnic backgrounds show large variation in sympathetic and parasympathetic drive of heart rate. Compared to European children, those from Ghana and African-Surinamese children showed more favourable autonomic regulation, while children from Turkey and Morocco had more unfavourable regulation. The authors suggested that a large genetic contribution may be at play due to the very young age of the children [28] – although early life programming may also play an important role.

27.4 Summary (Fig. 27.1)

Although this review is not exhaustive, collectively the evidence clearly point to ethnic disparities in cardiovascular disease risk and development. Although the role of genetic factors seems minor, environmental contributors such as nutrition, tobacco use and alcohol are involved in cardiovascular disease development, which may also take place by inducing epigenetic modifications. A better understanding of ethnic-specific inducers is therefore required. It has also been questioned whether ethnic disparities in health could not be merely explained by differences in socio-economic status, since in many instances ethnic groups (or minorities in developed countries) reside in low- and middle-income countries and have a lower socio-economic status. To some extent the question remains unanswered, yet studies on pathophysiological mechanisms point to certain aspects that cannot be explained by socio-economic status, such as ethnic-specific visceral fat distribution, renal sodium handling and salt sensitivity. Future research is therefore encouraged to involve larger populations including diverse ethnicities, in order to produce validated risk prediction models for all ethnic groups.

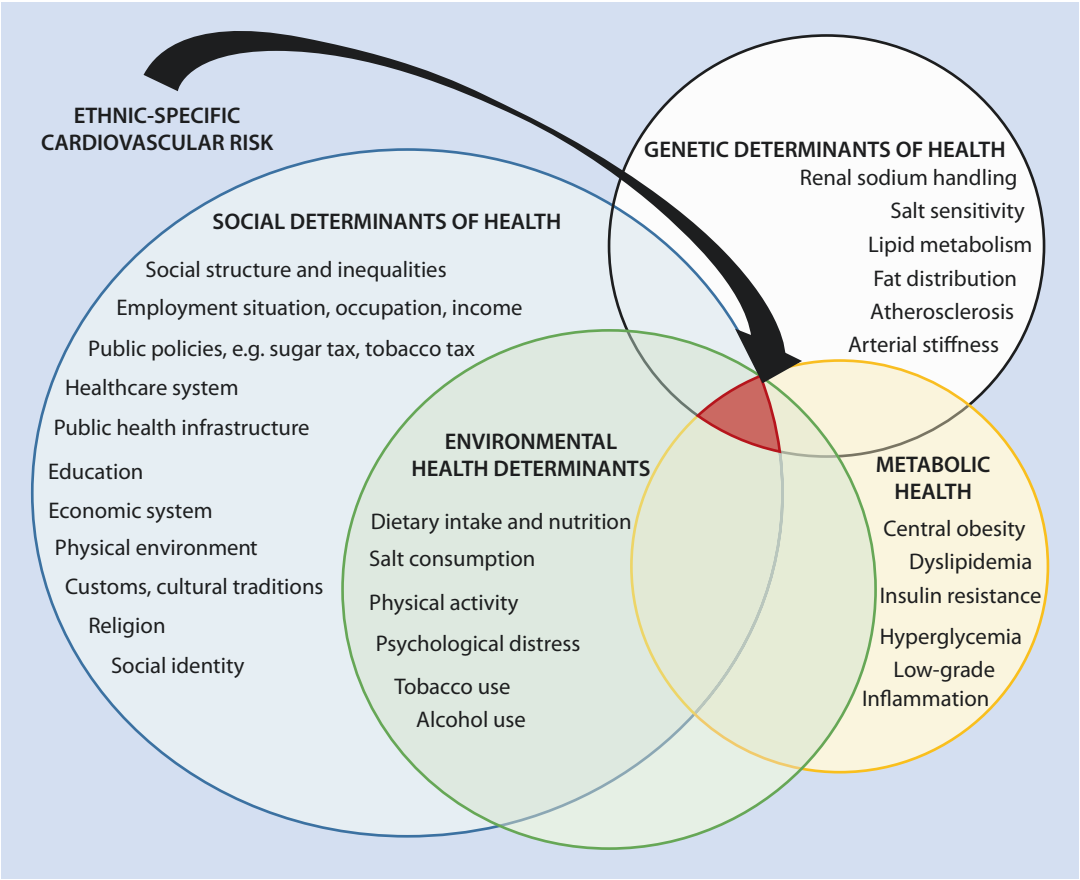

Fig. 27.1 Ethnic disparities in cardiovascular disease are largely explained by the convergence of social, environmental, metabolic and genetic determinants of health

Conclusion and Clinical Perspectives

- Persistent racial and ethnic disparities have been reported in terms of cardiovascular disease morbidity and mortality.
- An array of factors may explain these differences, with evidence supporting contributory roles of several factors, including genetics and environmental aspects.
- Social health determinants, such as education, income and occupation as well as access to healthcare are important in understanding ethnic health disparities.
- There are specific environmental, behavioural and metabolic aspects that are unique to specific ethnicities in terms of their contributions to cardiovascular health, such as salt intake and salt sensitivity.
- A better collective understanding of ethnic-specific disease development is needed to develop and validate risk prediction models, which form the cornerstone of cardiovascular disease prevention.

Gaps in Knowledge

- The evidence base by ethnic group on health status, health outcomes and cost-effectiveness of interventions is weak, and more large-scale population studies are needed.
- It is unknown whether the treatment regimes, e.g. the treatment algorithm for hypertension, are as effective in populations of African and South Asian descent.
- The ethnic-specific contributions of early life programming (exposures during pregnancy and the first 1000 days of life) towards cardiovascular disease development are largely unknown.

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Clinical Aspects

Contents

Epidemiology of Vascular Diseases

Paul Welsh and Stamatina Iliodromiti

R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_28

Key Concepts

- Epidemiology has played an important role in developing our understanding of vascular diseases and has underpinned development of successful pharmaceutical and public health interventions.
- There have been long-term declines in the incidence and prevalence of many cardiovascular diseases in the UK.
- Despite this, the burden of specific diseases associated with older age (such as dementia and heart failure) and adiposity (such as diabetes) is increasing.
- Precision medicine approaches may help further improve prevention of cardiovascular disease morbidity and mortality; for instance, sex-specific targeted interventions might help remedy gender inequalities.

28.1 Introduction

Amid another outbreak of cholera in Soho, London, during 1854, in the absence of knowledge of the method of transmission of the disease, the authorities could do little to limit the spread. Famously, a physician named John Snow used door-to-door surveys, and based on a pre-existing idea, he demonstrated that cholera suffers frequently used the Broad Street water pump. The handle was removed from the Broad Street pump, the outbreak resolved, and further outbreaks were prevented. All this achieved, before the germ theory of disease had even gained credibility, using an approach supported by simple but meticulously gathered observations. Epidemiology (from Greek *epi* meaning 'upon' and *demos* meaning 'people') was born.

For the next 100 years, early epidemiological research was largely restricted to the study of communicable diseases. That changed when in the 1920s, a long-term epidemiological study of cardiovascular diseases was proposed in St. Andrews, Scotland, but was unfortunately never completed. In 1945, US President Roosevelt died from haemorrhagic stroke, a complication of long-standing hypertension (a systolic blood pressure of >200 mmHg). His successor, President Truman, signed the 'National Heart Act' a few years later, with the aim of identifying and treating the causes of cardiovascular diseases in the population. The use of epidemiological approaches in the context of non-communicable diseases was a novel approach but gave rise to the hugely influential 'Framingham Study'. Recruitment of 5209 residents of the town of Framingham, Massachusetts, between 1948 and 1952 and collating their data (without modern digital capabilites) was a herculean task. The effort was rewarded by identification and definition of key risk factors for cardiovascular disease, for instance, defining hypertension as ≥160/95 mmHg based on a strong association with future cardiovascular events.

Since those early days, epidemiology has come a long way, as has our understanding of the causes of vascular diseases, and consequently our ability to prevent and treat them. However, underpinning it all remain the most important modifiable risk factors identified by those simple observations: blood pressure, cholesterol, smoking, obesity, and diabetes.

In this chapter we take a holistic view of cardiovascular diseases, including notable epidemiological trends and their causes (many of which are described in more detail in other chapters) in the UK population and more widely.

28.2 Temporal Trends in Classical Cardiovascular Diseases

The impact of Framingham and other epidemiological studies has allowed us to target the important root causes of cardiovascular diseases in an informed way. Since the 1950s, blood pressure can be targeted with thiazide diuretics, beta-blockers, calcium channel blockers, and more recently ACE inhibitors or ARBs. Moreover, with the advent of randomised controlled trials using precision medicine approaches, such as Systolic Blood Pressure Intervention Trial (SPRINT), we know that targeting low blood pressures (SBP of 120 mmHg) with intensive therapies is effective at reducing cardiovascular disease risk compared to traditional approaches [1]. Effective lipid lowering can be achieved with statins; this approach also safely reduces cardiovascular disease risk in secondary and primary prevention of cardiovascular disease. In fact, so far we have not identified a lower limit beyond which lipid lowering is not an effective means to reduce CVD. Recently, the

monoclonal antibody proprotein convertase subtilisin–kexin type 9 (PCSK9) inhibitors have been shown to further reduce lipid levels, a further 60% beyond that achieved by statins, and to also reduce cardiovascular disease in high-risk and secondary prevention groups [2]. In addition to pharmacological interventions, surgical interventions such as coronary artery bypass grafting (CABG) and stenting or balloon angioplasty can mitigate the ischaemic effects of atherosclerosis.

Pharmacological and surgical interventions to reduce cardiovascular disease risk have the apparent benefit of being able to target them to individual patients at risk of vascular diseases. Next to these, important public health interventions are sometimes overlooked. However, to do so is a critical error. Pharmacological and surgical interventions can only reduce risk in the high-risk patients allocated to them. Those at intermediate risk (the bulk of the population) are therefore untreated and experience a large number of cardiovascular events at the population level. The famous epidemiologist Geoffrey Rose argued that shifting the risk distribution curve by a small amount in the whole population has a greater beneficial effect than more aggressively treating only patients with high risk. Thus, public health interventions (such as removing the Broad Street pump handle) are an important tool. Since the 1970s public health interventions have included dietary recommendations and campaigns, salt reduction programmes, physical activity promotion, tobacco taxation, and public smoking bans. Over time, all of these interventions combined, together with pharmacological interventions, have had an important impact on public health in the UK and in other countries.

High rates of death from coronary heart disease were becoming a concern in the UK post-World War II. During the 1960s and 1970s, cardiopulmonary resuscitation (CPR) practices were established, and coronary care units were opened in NHS hospitals. In the UK in 1971, there were ~645,000 deaths from all causes, 52% of which were attributable to cardiovascular causes. By 2001 40% of deaths were attributed to cardiovascular disease. By 2015, it was 26% [3]. This impressive trend (Fig. 28.1) is against the

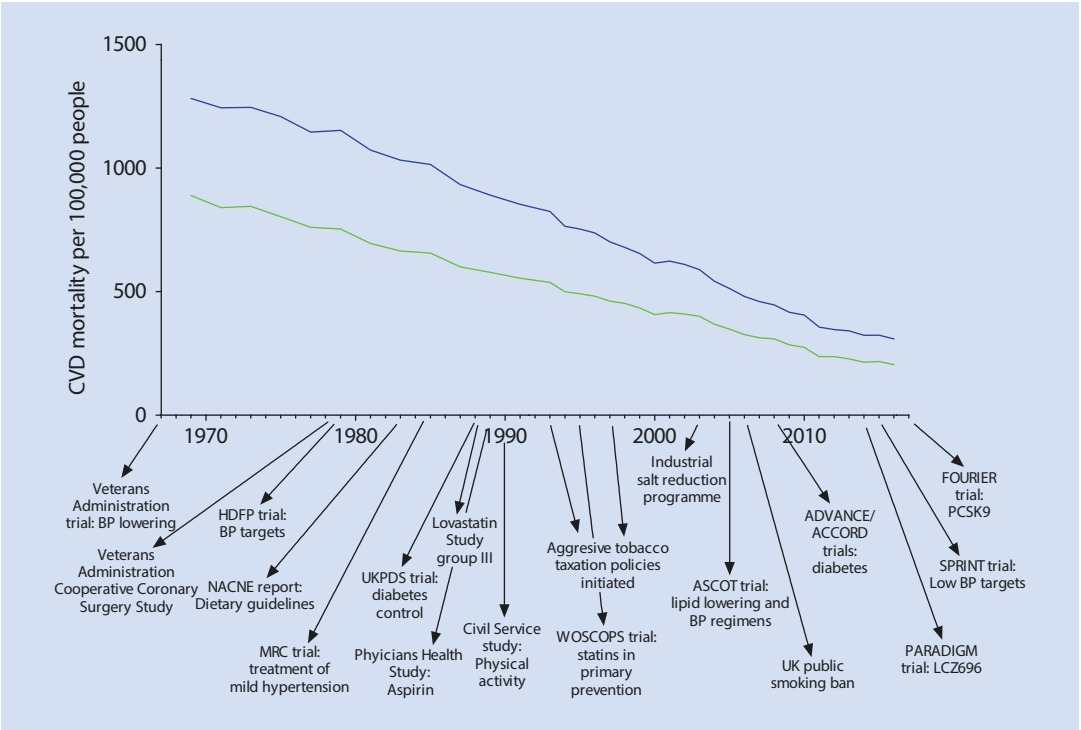

Fig. 28.1 Rates of cardiovascular disease mortality among men (blue line) and women (green line) since 1967. (From published British Heart Foundation statistics [3], with illustrations of key intervention trials and public health initiatives across the timeline)

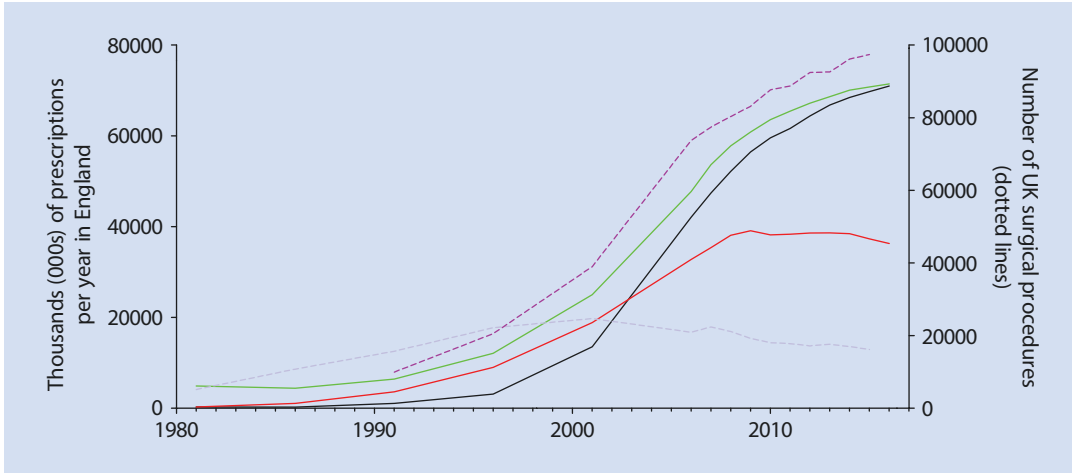

Fig. 28.2 Number of prescriptions of medication for the prevention of vascular diseases in England, and surgical procedures in the UK, since 1981. (From published British Heart Foundation statistics [3]). Medication groups are antihypertensives (solid green line), lipid-lowering drugs (solid black line), and antiplatelet drugs (solid red line). Procedures are coronary artery bypass graft (dotted light blue line) and percutaneous coronary intervention (dotted purple line)

background of an ageing population who are at greater risk of cardiovascular disease and coincides with the development of multifactorial interventions designed to reduce cardiovascular disease in the population. Over time, many more people are being prescribed drugs to improve their vascular health, and there are more surgical procedures being performed (Fig. 28.2). As a result of these, fewer people develop cardiovascular disease, and people who do develop cardiovascular disease are living longer [4]. Given these improvements, it is perhaps tempting to think that the vascular public health crisis is therefore over. This is simply not the case. The World Health Organisation still lists cardiovascular disease as the number 1 cause of death worldwide with 17.1 million deaths (31% of all deaths) every year.

28.3 Ageing and Vascular Diseases

The obesity crisis (▶ Chap. 39) and an ageing population are leading to increased prevalence of type 2 diabetes (▶ Chap. 40) in the UK population (increasing from 3.2% in 2004 to 5.3% in 2014) [5]. This burden has a high associated cost of the treatments that attempt to mitigate the increased risk of vascular diseases in people with type 2 diabetes. Because atherosclerosis is systemic, these include cardiovascular disease, retinopathy, nephropathy, neuropathy, and peripheral vascular diseases. As such, the NHS spends approximately 10% of its budget treating diabetes and its complications. Glycaemic control is particularly important in the prevention of small vessel disease. A recent study using the UK Clinical Practice Research Datalink (CPRD) showed that the prevalence of retinopathy was 48% in people with type 1 diabetes and 28% in people with type 2; the vascular complications of diabetes are therefore a public health concern [5].

As well as diabetes, the impact of vascular and degenerative cognitive impairment associated with ageing is also a concern. Specifically, dementia is a major public health concern in the UK and more widely (▶ Chap. 42). At age 60–64, the prevalence of dementia is <1% in both men and women in the UK, but by age 80–84, it is 11.7% in women and 10.3% in men and 33.0% and 22.6% at age 90–94, respectively [6]. The fact that people are generally living longer (in part due to reduction in cardiovascular disease deaths) is clearly a good thing. However, the impact of this is that diseases like dementia become more prevalent in the population. In 2012 there were over 800,000 people in the UK living with dementia, over 900,000 in 2017, and there will be over 1,000,000 by 2021, and 2,000,000 by 2051 [6]. This is despite a falling

age-adjusted incidence of dementia, due to improving risk factor control [7]. Similar patterns are also evident for heart failure, some of the causes of which are vascular [8]. Therefore in the coming years, dealing with the vascular diseases associated with ageing is going to be an important area of research.

28.4 Geographical and Ethnic Trends in Risk Factors for Vascular Diseases

These broad UK temporal trends should not be allowed to mask important geographical trends as well. There is a well-known North/South divide, such that those in the North of the UK are at greater risk of vascular disease [3]. This problem is particularly pronounced in more socioeconomically deprived post-industrial cities. In Scotland, the city of Glasgow has such profound health inequalities that the 'Glasgow effect' has become the topic of academic enquiry. The causes of such differences are in part socioeconomic, and in part cultural, but are difficult to explain fully. For instance, in 2011, deaths from cardiovascular diseases were around 20% higher in Scotland compared with England and Wales after adjustment for age, sex, and socioeconomic deprivation [9]. Other potential risk factors, with a range of plausibility, have been invoked to explain these differences including mineral content of drinking water, sunlight exposure (vitamin D), pollution, obesity, as well as legal and illegal drug use. Attempts to understand and intervene in these differences are ongoing. The influence of patterns of ethnic diversity on cardiovascular disease also requires greater understanding, both within the UK and more widely. A very recent cohort study of over 1 million patients registered with a GP in England, followed for over 5 years, studied the effect on ethnicity on the incidence of cardiovascular diseases (CALIBER study) [10]. Compared to patients of White ethnic background, South Asians were at increased risk of a first coronary heart disease event, whereas Black patients were at increased risk of stroke but were at reduced risk of coronary heart disease. In addition, the study reports a higher prevalence of diabetes and hypertension in South Asian and Black patients, which might help in targeting of prevention those at greatest risk.

While the Western world faces vascular problems associated with increased longevity, clearly there are wider global issues to consider too. The theory of the 'epidemiological transition' refers to a period when the causes of death within a region rapidly shift from infectious disease and undernutrition to chronic diseases associated with older age, due to shifts in complex social structures and health determinants. This can often occur before health-care systems are able to cope. Areas of sub-Saharan Africa are experiencing these changes now. For instance, a recent survey in Malawi suggests that among urban- and rural-dwelling men and women, the prevalence of hypertension is 13–16% and diabetes 2–3% [11]. Many of these conditions are undiagnosed, and therefore untreated, in the community. It is also widely understood that global trends in blood pressure are changing. There are long-term declines in blood pressure in affluent Western countries and increasing trends in south Asia and sub-Saharan Africa; for instance, in selected countries in sub-Saharan Africa, the mean systolic blood pressure among women was 132 mmHg [12]. Smoking is another important vascular risk factor, and adverse trends in tobacco usage have been noted in low-income countries (such as Africa) and have been reported. These trends in important risk factors are broadly mirrored by global trends in cardiovascular disease [13]. In Western Europe the age-standardised rate of death from cardiovascular disease per 100,000 people is 157, and in high-income North America, the rate is 171, whereas in Southern sub-Saharan Africa, the rate is 338, and in South Asia it is 369 per 100,000 people [14]. Therefore targeting effective public health and pharmacological therapies to those that need it most in the global context will be a key priority in the coming years.

28.5 Sex Differences in Cardiovascular Disease

Despite considerable gains in cardiovascular disease prediction and progression over the last two decades, cardiovascular disease accounts for one in four deaths for both sexes in the UK and remains the leading cause of mortality for women as well for men [3]. However, there is increasing evidence of sex-specific disparities in the onset, treatment, progression, and outcomes

of cardiovascular disease. The INTERHEART study, a large case control multi-ethnic study looking at the risk factors contributing to the first episode of myocardial infarction (MI), one of the most common manifestations of vascular disease, showed that women experience their primary MI on average 9 years later than men. Interestingly, the same risk factors contributed to the development of MI in both sexes; however, the earlier onset of MI in men was largely explained by higher prevalence of risk factors, including impaired lipid profile, smoking, and alcohol consumption at a younger age in men. In addition, it was postulated that endogenous oestrogens play a role on cardiovascular disease protection, which is possibly mediated through their beneficial effect on cholesterol carrying apolipoproteins and endothelial function; this may be lost with the onset of menopause [15]. The average age of primary MI corresponds closely with the mean age of menopause, and heart disease rises exponentially after menopause. This provides some temporal evidence for the vascular protective effect of endogenous oestrogens.

Over the last two decades, multiple cardiovascular disease risk prediction models, which mathematically combine risk factors to estimate the chance of an individual developing cardiovascular disease, have been incorporated into clinical guidelines (such as the ASSIGN score in Scotland and QRISK3 in the rest of the UK). These models are increasingly used in clinical practice to stratify individuals to risk categories and personalise preventive strategies. Women present distinct cardiovascular disease risk factors during their reproductive life-course that are associated with the exposure to endogenous hormones and pregnancy. For example, early menopause, before the age of 47 years, is associated with 33% greater risk of cardiovascular disease compared with average age of menopause (Fig. 28.3), and each miscarriage increases the risk of cardiovascular disease by 4% and each stillbirth by 14% beyond conventional cardiovascular disease risk factors [16].

Pre-eclampsia, a condition that complicates 2–8% of pregnancies, is associated with twofold greater risk of major coronary events and up to three- to fivefold when it is accompanied with reduced fetal growth or preterm delivery, respectively [17]. Inclusion of female-specific risk factors in the traditional prediction model may stratify women to more accurate risk categories and guide efficient therapeutic management.

More middle-aged men than women are diagnosed with heart attacks. However, within a year of a first MI, age-standardised survival rates are lower in women than in men; 47% of the women will die, develop heart failure, or suffer from a stroke, compared with 36% of the men within 5 years following a primary heart attack [18]. Delay in recognising acute signs of heart disease and receiving timely guideline-driven treatment along with limited access to efficient secondary preventative strategies for women have been proposed as potential causes for this striking disparity. Clinicians and the general public might be generally slower to recognise the potential seriousness of symptoms consistent with acute heart disease in women. For instance, women diagnosed with ST segment myocardial infarction have a 59% higher chance of an initial misdiagnosis compared with men, a delay that can lead to higher risk of mortality and long-term manifestations including heart failure [19]. Women are five times more likely than men to present with ischaemic chest pain, abnormal stress test results, and normal angiography, a condition known as microvascular angina, which is now established to be associated with increased mortality, reduced quality of life, hospital readmissions, and repeat coronary angiography [20]. Diagnosis of the condition remains a challenge, and these patients are often reassured that their pain is 'noncardiac' related and left untreated, leading to severe long-term implications.

Despite women representing 40–50% of cardiovascular disease sufferers in registries and cohort studies, they are massively underrepresented in trials, making up ~20% of the enrolled patients. Sex has been shown to modify the effect of treatment on outcomes, i.e. in trials of glycoprotein IIb/IIIa inhibitors, men in the treatment arm had 9% lower risk of mortality and subsequent MI within 30 days of an acute coronary event, whereas women in the treatment arm did not confer a similar risk reduction [21]. Attention to sex-specific characteristics and disparities will facilitate a step closer to personalised medicine by improving prevention, recognition, and treatment of vascular disease in women.

Fig. 28.3 Spline plots showing hazard ratios (95% CI in grey) for cardiovascular disease associated with women's age at menarche and age at natural menopause (after adjusting for age, Townsend deprivation index, smoking status, systolic blood pressure, history of diabetes, and body mass index). (Data from UK Biobank study. Figure reproduced from [16] with permission)

Conclusion and Clinical Perspectives

- Mortality from cardiovascular disease has decreased substantially over the last two decades in the UK but still remains the number one killer in the UK and worldwide.
- Application of evidence-based therapies in the high-risk populations, along with promotion of public awareness and lifestyle modifications in the low- and moderate-risk groups, may have led to the dramatic decline in mortality rates, which is encouraging.
- However, there are still an excess of deaths and reduced quality of life in the cardiovascular disease survivors, especially in women, socioeconomically disadvantaged groups, and many non-White ethnic groups.

Gaps in Knowledge

- Interventions to reduce, and effectively care for, the predicted tsunami of diabetes, heart failure, and dementia patients in the UK require urgent research.
- There is a need for targeted therapeutic options and culturally sensitive public health interventions to reduce cardiovascular disease risk in specific risk groups, including women, deprived communities, and ethnic minorities, in the UK.
- On the world stage, there is an increased understanding of the epidemiological transition and its potential implications. Research into the best practice to manage this transition is in its infancy.

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Healthy Vascular Ageing and Early Vascular Ageing

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_29

Key Concepts

- Age is an important determinant of cardiovascular risk.
- With advancing age, changes can be seen in the function and phenotype of various cell lines, as well as in the structure and physiology of large and small vessels.
- These changes result in arteriosclerosis, or vascular stiffness, and clinically result in hypertension, vascular remodelling and often clinical cardiovascular disease.
- However, vascular ageing may occur at different rates depending on individual characteristics and risk factor profiles. In certain chronic inflammatory and metabolic diseases, vascular stiffness can be observed at an earlier age, termed 'early vascular ageing'.
- The converse is also possible. 'Healthy vascular ageing' refers to individuals within a population who maintain a vascular phenotype that is measurably healthier than the mean for others of similar age and ethnicity.
- A number of tools are available for assessment of vascular age, and these may help to guide cardiovascular risk stratification in the future.

29.1 Introduction

Cardiovascular disease (CVD) remains a leading cause of death worldwide. A variety of scoring systems are available to assist clinicians in estimating an individual's overall cardiovascular risk, and with this information we are able to make decisions about prescribing evidence-based primary and secondary preventative therapies. Chronological age is a major non-modifiable cardiovascular risk marker, and consequently risk prediction models rely heavily on this parameter [1].

Ageing is associated with a number of structural and functional changes in the vasculature resulting in atherosclerosis and vascular stiffness, or *arteriosclerosis.* Whilst the presence of vascular disease increases across all territories with advancing age, this relationship cannot simply be ascribed to time-related accumulation of risk factor exposure [2]. It is now well-established that algorithms using traditional risk factors fail to accurately predict CVD in many cases, selected clinical measures of vascular structure and function have been shown to predict CVD independent of age and conditions such as diabetes, chronic kidney disease (CKD), obesity and hypertension which are increasingly prevalent in younger individuals also induce changes in the vasculature. It is clear therefore that chronological age does not always accurately reflect the biological age of the vessels and it has been suggested that the concept of 'vascular age' may in fact be a more effective means of communicating risk assessment to patients in the clinical setting. Against this background recent years have seen the emergence of early vascular ageing (EVA) as a concept for cardiovascular research and development of targeted preventative therapies [3]. This chapter will review the vascular physiology of normal ageing, biomarkers for measurement of vascular age, the concept of EVA and the impact of chronic diseases such as hypertension, diabetes and chronic kidney disease (CKD) on the vasculature.

29.2 The Cellular Ageing Process

At a cellular level the ageing process is characterised by loss of proliferative capacity, termed 'cell senescence'. Senescence can be further characterised into replicative and stress-induced premature senescence. Replicative senescence is a characteristic of normal ageing as a result of telomere shortening and subsequent DNA damage as described below. Conversely, premature senescence which may underlie EVA and associated conditions is induced by external factors such as radiation and oxidising agents.

29.2.1 Telomeres

Cell senescence is controlled by telomeres and the enzyme telomerase. Telomeres are small segments of DNA found at the end of chromosomes which act as a protective 'cap' to preserve chromosome stability. With each cell division, they are shortened and below a critical length lose their capacity to maintain cell integrity, at which point senescence ensues. This process is regulated by telomerase which acts to preserve telomere length. Mean leukocyte telomere length decreases by 6–9% with each decade and can therefore be a useful in vivo

biomarker of ageing, especially when measured repeatedly to calculate telomere attrition, a more relevant indicator of ageing that single cross-sectional measurement of telomere length. Whilst senescence may play a protective role in some conditions such as tumorigenesis, telomere attrition shortening has been associated with premature mortality, atherosclerosis and CVD [4, 5]. In vitro and in vivo work has shown inverse correlations between telomere length and age of human endothelial cells; inhibition of telomere function induces a senescent phenotype in human aortic endothelial cells which can be halted with telomerase activators; [6] and studies have linked telomere length to key mechanisms known to underpin development of CVD such as cholesterol, oxidative stress, nitric oxide availability and exercise [4]. Whilst collectively this evidence points to a link between telomeres, atherosclerosis and mortality, findings from prospective studies remain inconsistent [7].

29.2.2 Cell Senescence

Senescent cells display a number of phenotypic changes in addition to altered telomere length and telomerase activity. Aged endothelial cells become enlarged and have altered cytoskeletal integrity and proliferative capacity, as well as impaired nitric oxide production and increased endothelin-1 release. Endothelial cell ageing is therefore associated with impaired function and a pro-inflammatory phenotype. Vascular smooth muscle cells (VSMCs) derived from plaques are characterised by similar features including reduced proliferation, increased secretion of inflammatory cytokines and upregulation of adhesion molecules, again generating a pro-inflammatory environment promoting progression of vascular disease. Similarly inflammatory cells can also develop an 'ageing' phenotype as monocytes from patients with atherosclerosis exhibit increased secretion of inflammatory cytokines which can also be observed in aged compared to young monocytes [8].

29.2.3 Genes Implicated in Vascular Ageing

A number of candidate genes have been associated with longevity and mechanisms underlying vascular ageing. Epigenetic modifications such as histone acetylation and methylation play an important role in regulating gene activity under the control of mediators including the *sirtuin* family *(SIRTs)* of which *SIRT1* has been most extensively studied. *SIRT1* is thought to play a protective role against the development of CVD through promoting endothelium-dependent vasodilation and nitric oxide bioavailability, controlling cellular reactive oxygen species (ROS) levels and suppressing NF-κB signalling and inflammation [9]. Experimental studies in human endothelial cells have shown that inhibition of *SIRT1* results in a premature senescence phenotype, and murine *SIRT1* knockout models exhibit increased atherosclerotic plaque formation [9]. *SIRT1* expression declines with age, and reduced levels have been reported in patients with metabolic syndrome and coronary artery disease [7, 10], whilst particular *SIRT1* SNPs have been associated with increased longevity in the elderly [11]. Although less well studied, other members of the SIRT family have been shown to mediate the development of CVD and may represent interesting therapeutic targets in the future.

Adaptor protein $p66^{Shc}$ acts as a signalling molecule in mitochondria-mediated oxidative stress and apoptosis and is thought to be another key determinant of ageing. Knockout mice display reduced intracellular ROS levels, attenuated endothelial dysfunction, preserved left ventricular function and prolonged lifespan [7]. In human studies increased $p66^{Shc}$ expression has been demonstrated in peripheral blood mononuclear cells from subjects with coronary artery disease and correlates with systemic oxidative stress markers [12]. However, the data are limited and its role requires further clarification.

The *KL* gene, expressed primarily in the kidney and to a lesser extent in the vasculature, encodes the enzyme *Klotho* and has been highlighted as an ageing suppressor. Inactivation in mouse models has been shown to result in an ageing phenotype characterised by shortened lifespan and atherosclerosis [9]. Klotho is thought to protect against endothelial dysfunction by upregulating nitric oxide (NO), and vessels from knockout mice show impaired endothelium-dependent vasodilation with acetylcholine and has been shown to have anti-apoptotic and anti-senescent effects in human endothelial cells. Studies in humans suggest that *KL* gene polymorphisms are associated with longevity and cardiovascular disease [9].

Markers of vascular stiffness such as carotid-femoral pulse wave velocity (PWV) have demonstrated significant heritability in twin and family studies. Genome-wide association studies (GWAS) have identified a number of genetic variants associated with PWV including calcium- and integrin-binding protein-2 (CIB2) and collagen type 4 (COL-4A) gene polymorphisms; and a meta-analysis of GWAS in multiple cohorts identified an association between PWV and a locus on chromosome 14 in the BCL11B gene desert [13].

29.2.4 Oxidative Stress and 'Inflammageing'

The free radical theory of ageing describes the accumulation of ROS with age that eventually results in damage to DNA, proteins and structural components of the vasculature. This is supported by depletion of antioxidants such as superoxide dismutase [14]. As a result reduced nitric oxide bioavailability promotes endothelial dysfunction, the common denominator of CVD and atherosclerosis. Activation of NF-κB by ROS further stimulates release of other pro-inflammatory cytokines which further contribute to endothelial dysfunction and vascular disease. The renin-angiotensin-aldosterone system (RAAS) is also implicated in vascular ageing as angiotensin II enhances ROS production via activation of its receptor subtype 1 and has been shown to accelerate telomere shortening [15]. As such, vascular ageing can be characterised as a chronic low-grade inflammatory state also termed 'inflammageing', a principle thought to underlie cardiovascular conditions such as ischaemic heart disease, type 2 diabetes and obesity and associated with mortality in older people.

29.3 Effects of Ageing on Vascular Structure and Function

Many traditional cardiovascular risk factors perpetuate and accelerate inflammation, endothelial dysfunction and cell senescence; therefore recognising and treating these is a key factor in preventing or at least delaying the onset of overt CVD. The processes described above culminate in arterial stiffening and calcification, both are characteristic of vascular ageing and independently predict cardiovascular morbidity and mortality [16].

Vascular stiffness results from alterations of the collagen and elastin components of the vasculature, where elastin fibres become fragmented and subsequently depleted in number, whilst collagen fibres simultaneously accumulate in the vessel media. This process essentially begins in infancy although can be accelerated by exposure to cardiovascular risk factors.

The aorta and elastic arteries expand as much as 10% with each cardiac cycle, and this eventually results in fracture and fraying of the elastic lamellae of the vessel. Peripheral muscular arteries are not exposed to the same degree of pulsatile expansion and so are relatively protected from degeneration. However vascular stiffness is not solely the result of such 'wear and tear'. It is now well-established that there is crosstalk between the endothelium and vascular smooth muscle such that endothelial cells influence VSMC proliferation, migration and fibrogenic phenotype [17]. In addition, oxidative stress results in the generation of oxidised LDL which is taken up by the subintimal layer further contributing to arteriosclerosis; and of relevance in diabetes is the impact of hyperglycaemia and generation of advanced glycation end (AGE) products which are known to alter collagen cross-linking and thereby influence vascular stiffness. As a result of the mechanisms described above, the reservoir function of elastic arteries is diminished; systolic and pulse pressures are increased; and many elastic vessels show increased lumen diameter. Whilst remodelling and vascular stiffness affect the medial layer of the vessel wall and are often present in the absence of atherosclerotic disease, it is important to note that endothelial dysfunction, changes in VSMC phenotype and the increased systolic pressure seen as a result of stiffening vessels can also induce changes in the intima and thereby facilitate progression of atherosclerosis.

As the heart continues to pump into a stiffened aorta, there is a corresponding increase in left ventricular (LV) load which is clinically described as 'isolated systolic hypertension' and is thought to affect up to 80% of the population aged 80 and over. This increase in pulsatile LV load is further perpetuated by earlier wave reflection in a stiff aorta and can itself inflict a damaging effect on the vessel wall. As the aorta becomes less elastic, it is no longer able to cushion the effects of

increased LV load, and pulsatile pressure is eventually transmitted into the microvasculature of the heart, brain and kidneys [18]. Damage to the endothelial cell layer creates a pro-inflammatory and prothrombotic milieu and perpetuates this vicious cycle.

It is important to note that recent years have seen the recognition of what has been termed 'crosstalk' between the macro- and microcirculation. Remodelling and vascular stiffness are not exclusive to the large arteries, and the mechanisms described above can equally affect the microcirculation [19]. For example, inflammation, fibrosis and vasoconstriction have been shown to lead to changes in distensibility, remodelling and capillary rarefaction in small resistance vessels from human subjects with hypertension [19]. Vasoconstriction and altered reactivity in the microcirculation is a major determinant of peripheral resistance and therefore ultimately can exert influence on blood pressure and large artery stiffness. Small and large vessel changes are therefore closely interrelated, and rather than a clear-cut temporal relationship, it is likely that a crosstalk exists between the two leading to a vicious cycle of inflammation, endothelial dysfunction, vascular damage and remodelling and ultimately a synergistic contribution to target organ damage.

Although the changes described above are characteristic of ageing, there are a number of 'common soil' mechanisms involved which are dysregulated in cardiovascular conditions such as hypertension, obesity, renal disease and diabetes, all of which are becoming more prevalent in younger individuals. Vascular ageing therefore cannot be viewed solely as a phenomenon limited to the very elderly population (Fig. 29.1).

29.4 Measuring Vascular Age

As described above, ageing of the arterial wall is characterised by build-up of collagen and loss of elastin resulting in reduced compliance, or

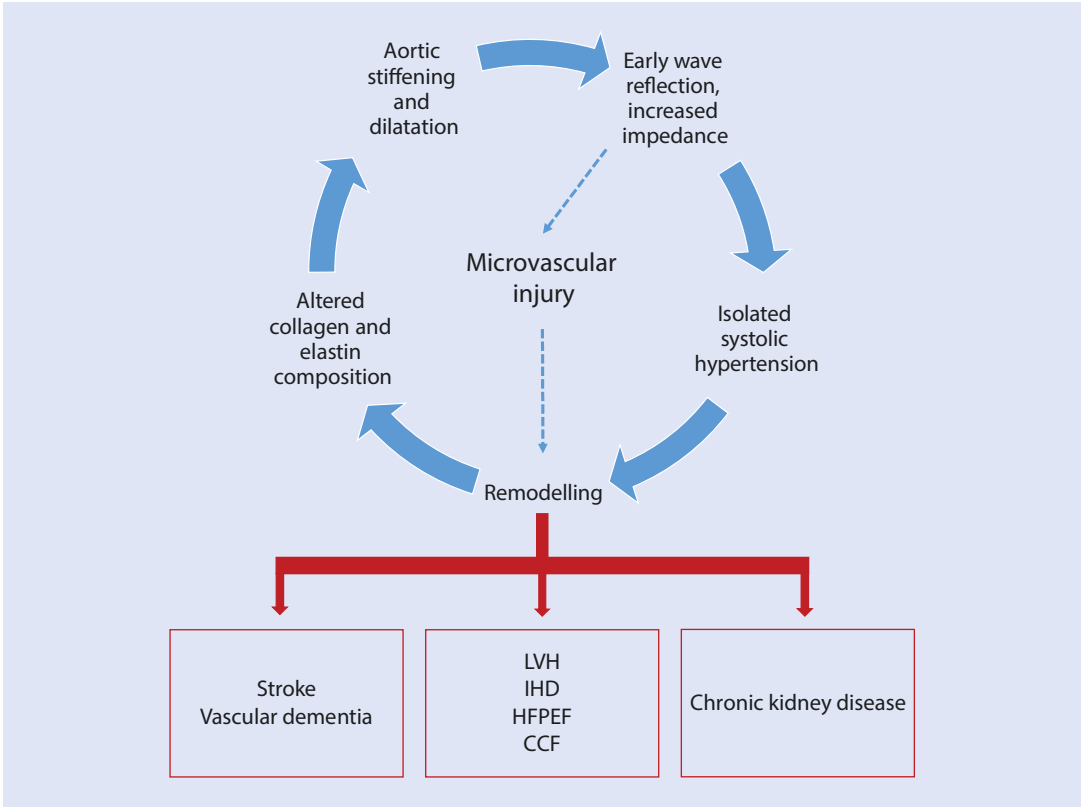

Fig. 29.1 The vascular ageing cycle. Illustration of vascular ageing cycle from changes in vessel composition to eventual remodelling and clinical vascular disease. LHV, left ventricular hypertrophy; IHD, ischaemic heart disease; HFpEF, heart failure with preserved ejection fraction; CCF, congestive cardiac failure

stiffness. A number of noninvasive techniques are available for assessment of stiffness, and some of these have now been included in clinical guidelines for management of cardiovascular conditions such as hypertension [20]. The aorta is a key vessel of interest for determination of arterial stiffness as the site of much of the circulation's buffering capability, but other vascular territories also provide information on vascular health beyond traditional clinical risk factors. This section will provide a brief overview of available tools for assessment of vascular stiffness.

29.4.1 Blood Pressure (BP)

Blood pressure measurement is an excellent metric for vascular ageing. As described above, progressive stiffening of the large arteries eventually leads to loss of their elastic reservoir function and increases in systolic blood pressure with widening of pulse pressure. The association between vascular stiffness, increased pulse pressure and ageing has been described in multiple populations, and therefore pulse pressure can be used as a surrogate measure of central elastic arterial stiffening.

29.4.2 Carotid-Femoral Pulse Wave Velocity (PWV)

This technique measures the speed of the pulse as it travels between the heart, carotid and femoral vessels based on the principle that reduced vascular compliance results in shorter pulse wave transit time. By measuring the flow velocity between the common carotid and femoral vessels, and thereby assessing the aorta's thoracic, abdominal and iliac branches, PWV is thought to accurately represent much of the load that the left ventricle is exposed to. PWV is universally accepted as a simple, robust and repeatable means by which to measure vascular stiffness, and reference values adjusted for age and gender are available [21]. Furthermore, this technique has been shown to be a robust predictor of all-cause and cardiovascular mortality as well as cardiovascular events independent of traditional cardiovascular risk factors across a variety of populations and disease states [22, 23]. It is therefore considered the 'gold standard' tool for evaluation of arterial stiffness, endorsed for cardiovascular risk reclassification in certain clinical circumstances.

29.4.3 Augmentation Index (AIx)

Other aspects of central haemodynamics can also be measured noninvasively. Augmentation index (AIx) denotes amplification of central pressure due to reflection of the pressure wave from peripheral vessels and provides an indirect estimation of aortic stiffness. The stiffer the peripheral vasculature, the more rapidly the reflected wave returns towards the heart, merges with the forward wave and hence 'augments' it. AIx is more relevant to determining vascular stiffness in younger populations in comparison to the elderly. Measured by applanation tonometry, AIx has been shown to independently predict mortality and cardiovascular events in populations including those with CKD, hypertension and ischaemic heart disease [24]. However, AIx is more susceptible to confounding by factors including age, gender and heart rate, and at the time of writing normal values adjusted for age and gender are yet to be determined. AIx has not yet been endorsed for risk stratification in clinical guidelines.

29.4.4 Ascending Aortic Distensibility (AAD)

Using MRI imaging techniques allows simultaneous measurements of local arterial stiffness as well as vascular structure and cardiac function. Through scanning the aorta during the cardiac cycle, it is feasible to capture maximal and minimal aortic lumen diameter and thereby determine change in aortic area, or aortic 'strain', which can be used to calculate AAD. AAD is thought to be an early marker of subclinical vascular disease, and reduction in AAD has been shown to predict cardiovascular events in individuals without overt CVD independent of traditional cardiovascular risk factors [25]. Limitations associated with this technique include the time and costs of MRI.

29.4.5 Carotid Intima-Media Thickness (CIMT)

Early in the development of atherosclerotic CVD, the subintimal layer of the vessel becomes infiltrated with lipids and inflammatory cells in advance of the build-up of overt atheroma. These early structural changes can be detected as a thickened intima-media complex (lumen-media interface to media-adventitia interface) by B-mode ultrasonography of the carotid vessels. CIMT can be measured at several carotid segments including the common carotid artery, carotid bulb and internal carotid artery though in terms of practicality and reproducibility, imaging of the common carotid is preferred. CIMT correlates with traditional risk factors such as age, sex, cholesterol and systolic blood pressure, and many studies have shown an increase in CIMT in individuals at higher cardiovascular risk. Although the capacity of CIMT for prediction of future cardiovascular events is well-established, there remains little evidence of association between CIMT progression or regression and manipulation of risk [26]. Local ultrasound measurement of carotid stiffness using high-resolution echo-tracking techniques offer the advantage of simultaneously measuring CIMT, pulsatile alterations in arterial diameter and local PWV, allowing assessment of stiffness, structure and remodelling patterns [27].

29.4.6 Retinal Vasculature

The retinal vessels can be imaged via retinal camera technology or optical coherence tomography angiography (OCTA) which offers an accessible window to the microvasculature which can be assessed noninvasively. Though the retina is routinely imaged in individuals with diabetes in order to determine the presence and extent of retinopathy, accumulating evidence points to a role for retinal microvascular measures as predictors of CVD. Retinal vascular measures such as arteriole and venule diameter and tortuosity correlate with other vascular stiffness markers such as CMT, AIx and PWV; and at a population level, studies have shown an association between retinal vessel calibre and risk of hypertension, metabolic syndrome, cerebrovascular disease and ischaemic heart disease [28].

29.4.7 Cerebral Vasculature

The prevalence of cognitive impairment and dementia increases with advancing age, and it has been consistently shown that increased vascular stiffness is associated with cognitive decline independently of traditional cardiovascular risk factors [29]. Structural changes within the brain such as white matter hyperintensities and cerebral microbleeds are predictive of dementia and cognitive decline; and subtle cerebral changes can often also be seen in younger individuals with hypertension [30]. Several studies have shown that WMH are associated with structural and functional markers of vascular ageing such as PWV and carotid stiffness (◘ Fig. 29.2).

29.5 Early Vascular Ageing

Chronological age is one of the strongest predictors of cardiovascular risk, and the changes in vascular structure and function described earlier in this chapter are to some degree an inevitable component of the ageing process. Many biomarkers of vascular structure and function as described above are independently associated with cardiovascular events, and some have demonstrated risk prediction benefit beyond traditional risk factors. Recently the Cardiovascular Risk Factors Affecting Vascular Age (CRAVE) study demonstrated that when cardiovascular risk factors are present, more rapid progression of vascular ageing biomarkers such as PWV and AIx is evident, even in younger individuals [31]. It has also been shown that early-life exposures play a role in the development of arterial structure and function; adverse risk trajectories can be identified in childhood/adolescence and predict increased vascular stiffness in early adulthood. It is evident therefore that chronological age and biological age are not necessarily analogous and many available cardiovascular risk calculators which focus heavily on chronological age may over- or underestimate risk at individual patient level. Within the last decade, the concept of EVA has evolved to denote more rapid progression of normal age-related arterial changes affecting both the micro- and macrocirculation, in some cases leading to early onset of target organ damage and CVD [3, 32]. At the time of writing, no standardised measurable definition of EVA has been agreed, although studies have

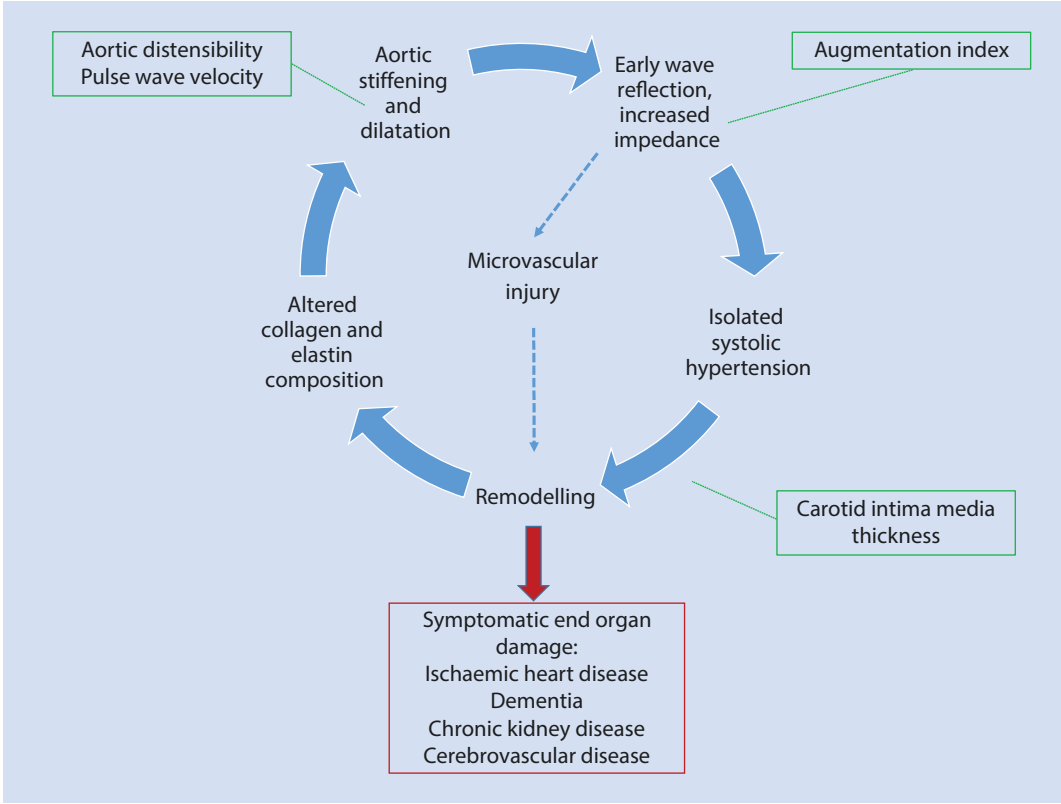

Fig. 29.2 Relation of clinical vascular phenotyping techniques to vascular ageing cycle. Highlighting points in the vascular ageing cycle where clinical techniques can be used to identify changes in advance of clinically symptomatic disease

employed thresholds for cardiovascular biomarkers of two standard deviations above the normal age- and sex-specific range from a relevant reference population to define this cohort [33].

29.6 EVA in a Clinical Context

As descried above a number of chronic conditions are associated with increased vascular stiffness and EVA. Although diverse, each of these conditions is characterised by mechanical vascular stress, inflammation and oxidative stress. To describe each of these in detail is outside the scope of this chapter, but some key examples will be highlighted below.

29.6.1 Hypertension

Systemic hypertension is well recognised as a risk factor for cardiovascular disease, and in the context of vascular ageing in hypertension, there could certainly be debate over whether vascular stiffness is a cause or consequence of elevated blood pressure. Hypertension and resultant vessel wall stress are known to lead to remodelling and loss of compliance within the central vasculature, changes which can of course be confirmed by demonstrating higher PWV, i.e. hypertension is a cause of vascular stiffness. This has now been shown to be a simplistic interpretation of complex vascular physiology. Firstly, large-scale meta-analyses have confirmed that PWV predicts cardiovascular morbidity and mortality independent of traditional risk factors including blood pressure [22]. Second, we now have strong evidence to suggest that the converse is true, i.e. higher PWV predicts onset of hypertension. Recently in a cohort of healthy, normotensive individuals aged between 30 and 45 years, elevated PWV was shown to predict onset of both systolic and diastolic hypertension 4 years later. Conversely, baseline blood pressure levels did not correlate with PWV at follow-up, and age was in

fact the weakest predictor of later hypertension; this is further evidence that vascular rather than chronological age more accurately reflects cardiovascular risk [34].

29.6.2 Chronic Kidney Disease

CKD is associated with increased incidence of cardiovascular morbidity and mortality; in fact many patients with CKD die from CVD before reaching end-stage renal disease. The impact of CKD on the vascular is complex and evolves as disease progresses. When individuals approach end-stage renal disease requiring dialysis or transplantation, arterial calcification is the predominant feature. However in the earlier stages of disease, increased vascular stiffness and remodelling are well-described. Altered collagen and elastin content of the vasculature evolves as CKD progresses, thereby impacting on vascular stiffness. At the same time remodelling of large arteries has been demonstrated, evidenced by thinning and luminal dilatation of the carotid vessels. Clinical studies have confirmed increased PWV, CIMT and pulse pressure in this patient group. Furthermore, PWV and carotid diameter have been shown to predict cardiovascular morbidity and mortality in patients with CKD independent of age and other traditional risk factors, and in end-stage disease, these parameters improve following transplantation. Tools such as PWV could thereby be useful for risk stratification of patients with CKD [35].

29.6.3 Diabetes

It is well established that diabetes is associated with high cardiovascular risk such that traditional risk calculators do not provide accurate estimations on an individual basis. Glycation of proteins such as collagen and elastin alters vascular wall structure and thereby influences compliance. Even at a young age, increased BMI in children affected by diabetes is associated with higher ambulatory blood pressure and increased CIMT into adulthood. In adults with diabetes, CIMT is higher than healthy control subjects, and using age-adjusted general population CIMT reference ranges, vascular age in diabetes is up to 9 years higher than chronological age in patients with no evidence of active CVD. Similarly, PWV is higher in patients with diabetes than the general population, with 10-year duration of diabetes affecting vascular stiffness to the same degree as a 10 mmHg rise in systolic blood pressure. PWV increases in parallel with urine albumin reflecting the tight link between diabetic kidney disease and vascular risk. Moreover it has been shown to predict mortality and cardiovascular events in diabetes independent of traditional risk parameters [36], and more recently PWV has been identified as a predictor of later type 2 diabetes in the general population [37].

29.6.4 Dementia

Cognitive impairment is very common in the elderly population, and its prevalence increases with advancing age. Hypertension, increasingly prevalent with age, is a risk factor for dementia, and arterial ageing has been shown to be an important predictor of accelerated brain ageing or cognitive decline. Studies have shown an inverse association between PWV and measures of cognition including global cognitive function, visuospatial organisation and memory suggesting that arterial stiffness influences multiple brain areas. This association increases in magnitude with advancing age [38]. Clearly cardiovascular disease burden also increases with age; however similar associations have also been shown in populations free from cardiovascular disease. For example, in the Baltimore Longitudinal Study of Ageing participants free from cardiovascular disease, based on repeated imaging over an 11-year period, individuals with increased CIMT were found to display accelerated decline in cognitive performance [39].

29.7 Healthy Vascular Ageing

Although huge advances in our understanding of the vascular pathophysiology of ageing have allowed us to identify individuals with higher vascular age, a key question remains: how do we achieve healthy vascular ageing (HVA)?

There are many cases in the published literature of cohorts who appear relatively protected from the effects of vascular ageing [40]. The

'Golden Years Cohort' is one such example. This group of individuals were diagnosed with Type 1 diabetes over 50 years ago, yet remarkably the majority remain free from hypertension, obesity and adverse lipid profiles. The lack of specific evidence-based interventions throughout the first 30 years of their disease suggests that a significant contribution to these individuals good vascular health is inherited, and indeed many had a family history of longevity [41]. Family history is however a non-modifiable risk factor. Framingham investigators have also evaluated HVA in the general population using the absence of hypertension and PWV of <7.6 m/s (which was the mean ± 2 SD of a reference population aged under 30 years) as defining criteria in a group of participants aged 50–70 years. One in six individuals fell into this category. This group were at significantly lower risk of cardiovascular events and had lower circulating levels of inflammatory biomarkers. Factors such as low BMI and the absence of diabetes or hyperlipidaemia were associated with HVA [42]. Further studies have shown that aerobic exercise, weight loss and dietary sodium restriction have all been shown to reduce PWV [43]. Additional evidence for the role of lifestyle and environmental factors in vascular ageing comes from research in specific populations. For example, work in indigenous populations has suggested that those who migrate to urban areas are at higher cardiovascular risk than those who maintain a traditional hunter-gatherer lifestyle [44] and similar findings have been described in closed quarter nuns in comparison to lay women from the same region [45]. These results suggest that vascular ageing is not necessarily 'inevitable' and HVA is achievable with lifestyle modification; although it should be noted that the phenotype was much less common in participants aged over 70 years, suggesting that chronological age becomes the key determinant of vascular stiffness in old age.

29.8 Pharmacological Interventions

There are a number of pharmacological strategies which have the potential to improve vascular stiffness. Numerous trials have evaluated the effect of antihypertensive treatments on PWV in populations with hypertension as well as a few in healthy volunteers. In general renin-angiotensin-aldosterone system inhibitors, beta-receptor blockers, calcium channel blockers, diuretics and vasodilators have all been shown to have some positive effect on vascular stiffness [43]; however outcomes may of course be due to achieving blood pressure lowering rather than a direct effect on stiffness per se, and several ongoing studies evaluating specific PWV rather than blood pressure treatment targets will add to this story in the coming years. Findings for trials of statin therapy have generally produced similar results in terms of improving vascular stiffness in patients with hypertension, hyperlipidaemia or obesity without any measurable effect on blood pressure. With regard to vascular stiffness, the effects of a number of novel pharmacological approaches including mTOR inhibitors, AMPK activators, anti-pro-inflammatory cytokine therapies, anti-fibrotic agents and specific angiotensin II type 2 receptor (AT2R) agonists such as compound 21 have been evaluated in animal models or small human studies [43]. Some of these agents may have a role in the management of accelerated or early-onset CVD in the future, but larger randomised studies evaluating their impact on hard endpoints are lacking.

Conclusions and Clinical Perspectives

- Ageing has direct effects on vascular structure and physiology. The cumulative effects of endothelial dysfunction, inflammation, cellular senescence and vascular remodelling are stiffening of large and small vessels, hypertension and cardiovascular disease.
- Vascular dysfunction is also evident in a number of chronic medical conditions characterised by inflammation, oxidative stress and metabolic abnormalities and can be seen at an earlier age in this context. This phenomenon is termed 'early vascular ageing'.
- Many of these structural and functional changes can be measured noninvasively and are independent predictors of cardiovascular morbidity and mortality. They may therefore have a future role in cardiovascular risk stratification. Monitoring alterations in vascular structure and function could help to determine effects of lifestyle and drug therapies, guiding therapeutic decisions.

Gaps in Knowledge

- The role of vascular age in risk stratifying individuals with chronic diseases such as hypertension, diabetes and CKD in a clinical context is not yet clear; as a result these tools have not been incorporated universally in clinical guidelines.
- A more exact definition of early vascular ageing is presently lacking, but at the core is arterial stiffness as measured by carotid-femoral PWV.
- Few treatments directed specifically at vascular stiffness have been evaluated in a clinical context.

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Biomarkers of Cardiovascular Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_30

Key Concepts

- A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."
- Biomarkers can be classified as predictive, diagnostic, prognostic, and representing pharmacodynamics or response to treatment; different types of biomarkers include genetic, imaging, and circulating biomarkers, with specific relevance at different points in the cardiovascular continuum. In this chapter we will focus on circulating biomarkers that represent biological processes.
- A clinically useful biomarker must show robust association with cardiovascular disease or risk, provide meaningful information about prognosis, and/or guide patient management beyond traditional risk factors or other measures that are already available in the clinical setting.

30.1 Introduction

Cardiovascular diseases (CVD) are the main cause of morbidity and mortality in the general population, a fact that underscores the importance of primary prevention [1]. However, the success of preventative measures depends in part on the accurate identification of individuals at risk of future cardiovascular events (risk prediction). In this regard hypertension, diabetes mellitus, obesity, smoking, and hypercholesterolemia, among others, are accepted as conventional risk factors for CVD and traditionally used as the main components of prediction models with clinical utility in the general population. Nonetheless, extensive research has revealed important limitations of such basic models. For instance, up to 20% of patients with coronary artery disease have no traditional risk factors, and 40% have only one. These data, as well as available results from other epidemiological studies [2], indicate that traditional risk factors do not fully explain the predisposition to CVD or how it evolves in different population groups and responds to treatment. Therefore, the incentive to improve upon current models with traditional risk factors has led to an increasing interest in discovering, validating, and translating to clinical practice novel biomarkers to better identify those individuals who will most likely experience cardiovascular events so that preventive measures can be applied.

Biomarkers generally represent a change at tissue or organ level that is associated with a physiological or pathological process. The National Institute of Health defines a biomarker as any "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Fig. 30.1). In addition, the World Health Organization proposes the following definition of a biomarker: "any substance, structure, or process that can be measured in the body or its products and influences or predicts the incidence of outcome or disease." On the whole, a biomarker should provide useful information to assist clinical decision-making and care, meeting at least one of the following criteria [3]:

1. Predict risk of developing clinically overt disease.
2. Diagnose and stage extent of disease.
3. Indicate disease prognosis.
4. Predict and/or monitor response to therapeutic intervention.

In addition, clinical usefulness of a biomarker requires that measurements render accurate and reproducible results in a standardized manner with high specificity and sensitivity. In biomarker development this often involves validation in independent populations and demonstration that the information provided adds meaningfully to already established clinical risk prediction tool or diagnostic tests. Biomarker data should not only be easily interpretable by clinicians but also cost-effective and thereby support implementation of affordable disease management strategies in the population [4]. It is important to note that biomarkers do not necessarily have to be more sensitive or specific than existing tools; it is the combination of performance parameters, ease of use, and costs that will ultimately inform clinical implementation.

Where these criteria are fulfilled, biomarkers can also be applied in clinical research and serve as endpoints in clinical trials. Substituting established "hard" clinical endpoints with surrogate markers is

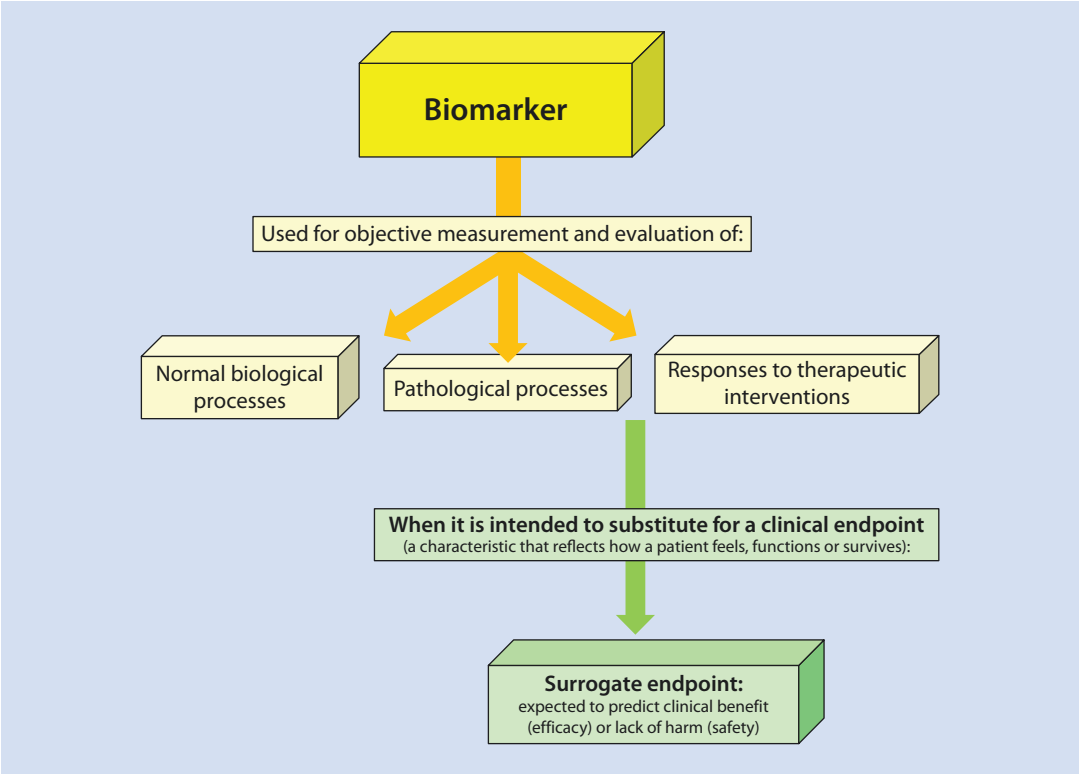

Fig. 30.1 Clinical applications of biomarkers

often more cost-effective and easier than assessing "true" endpoints (Fig. 30.1). These considerations play an important role in clinical trials where the use of surrogate endpoints that are closely linked to morbidity and mortality can result in smaller sample sizes, shorter duration of follow-up, and thereby more cost-effective trial designs. Biomarkers that are commonly used in the clinical trial setting include blood pressure, blood glucose levels, circulating markers of hemodynamic stress and cardiomyocyte injury, and echocardiographic parameters, all of which can help to evaluate the effects of drugs or other therapeutic regimens. However, it is important to note that surrogate endpoints are only useful in the context of specific disease mechanisms and depend on good understanding of disease pathophysiology.

30.2 Biomarker Types

Biomarkers are relevant to precision medicine, which according to the Precision Medicine Initiative is defined as "an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person" [5]. In this context, biomarkers are investigated as a source of information about a person's genes and proteins in order to prevent, diagnose, and treat disease. Thereby, biomarkers can be classified as predictive, diagnostic, prognostic, and reflecting pharmacodynamics.

A predictive biomarker can be used to estimate the risk of developing overt disease. Traditional risk factors such as blood pressure or body mass index are predictive biomarkers that provide reasonably exact information at population level. However, for individual risk, prediction markers that reflect specific disease processes have the potential to refine risk estimates and provide more precise information.

A *diagnostic biomarker* aids the diagnosis of a disease providing discrimination limits that allow separation of abnormal levels from normal levels for detecting the disease condition of interest. Ideally diagnostic markers should have both high sensitivity (low number of false-negative results) and high specificity (low number of false-positive results), but depending on the clinical context,

different performance characteristics can be accepted. This particularly applies to screening biomarkers that provide a first diagnostic step subsequently followed by further more specific confirmatory tests.

A *prognostic biomarker* provides information on the likely course of a disease or condition in an untreated or treated individual. Such markers may also identify individuals who are most likely to respond to a given therapy or help tailoring specific therapies to individuals depending on their biomarker profile. Changes in circulating levels of biomarkers that are intended to be used as prognostic tools in clinical practice should adequately reflect changes in mechanisms underpinning the disease of interest.

Biomarkers representing therapeutic response measure the effect of a drug or other interventions on the disease process itself. For instance, low-density lipoprotein (LDL) cholesterol is used as a pharmacodynamic biomarker where changes in its concentration are used to guide therapy with the ultimate aim of reducing the risk of future cardiovascular events.

For all types of biomarkers, derivation and validation of their use in a clinical context should be carried out in independent populations and different subsets of populations [6]. In an ideal scenario, a single biomarker can represent all of the above domains, i.e., predict risk, diagnose disease, and provide information about prognosis and response to treatment. However, complex diseases such as CVD develop over long periods of time, are multifactorial in origin, and involve different pathophysiological processes at different stages of disease. Therefore, biomarkers should be seen in the context of the development of disease, and while they may provide information during certain stages, they may not be universally useful.

30.3 Biomarkers and CVD

Extensive research within the cardiovascular context has evaluated new biomarker strategies in the "apparently healthy" general population and in patients with overt CVD [6]. These biomarker approaches include, among others, demographic features, imaging biomarkers, and proteomic, metabolomic, and genetic biomarkers, although in the context of CVD, the term biomarker is most often applied to circulating serum or plasma analytes beyond those used in routine hematology and biochemistry tests. As mentioned above, which of these biomarkers are most informative depends on the stage of the disease process. For instance, subclinical CVD can be present for decades before clinical symptoms are evident. In this regard, *imaging biomarkers* may detect the presence of subclinical CVD but are of limited utility for characterizing the very early stages at which not even subclinical changes in organ structure or function are present. In contrast, *genetic biomarkers* provide information about disease susceptibility, although without indication of whether or not subclinical disease has developed. *Circulating biomarkers* (and other biomarkers present in body fluids such as urine or saliva) may provide information at early or late stage of the disease process, with some reflecting activation of biological pathways that precede disease and others being influenced by the presence of subclinical CVD. Each of these biomarker categories should exhibit certain characteristics that determine their clinical usefulness (Fig. 30.2).

DNA transcription into RNA and translation into proteins that then regulate the metabolism often exert complementary actions to perform certain biological functions. Such synergistic interactions between omic layers can be captured by integrating genomics and transcriptomics as well as proteomics and metabolomics (Fig. 30.3).

Following this model, we will describe genetic, proteomic, and metabolomic biomarkers before we move on to imaging biomarkers and finally provide examples of biomarkers representing specific aspects of cardiovascular pathophysiology.

30.3.1 Genomic Biomarkers

Genetic factors play an important role in the development of CVD. Identification of new genetic susceptibility variants has contributed to the understanding of the pathophysiological processes underlying CVD. Although genetic factors are not influenced by environmental factors, gene-environment interactions will often determine transcription and translation of genes into RNA and proteins and thereby the development of disease. A key difference between genetic biomarkers and other circulating or imaging biomarkers is that the systemic genome itself remains largely unchanged throughout the lifespan, thereby allowing for risk

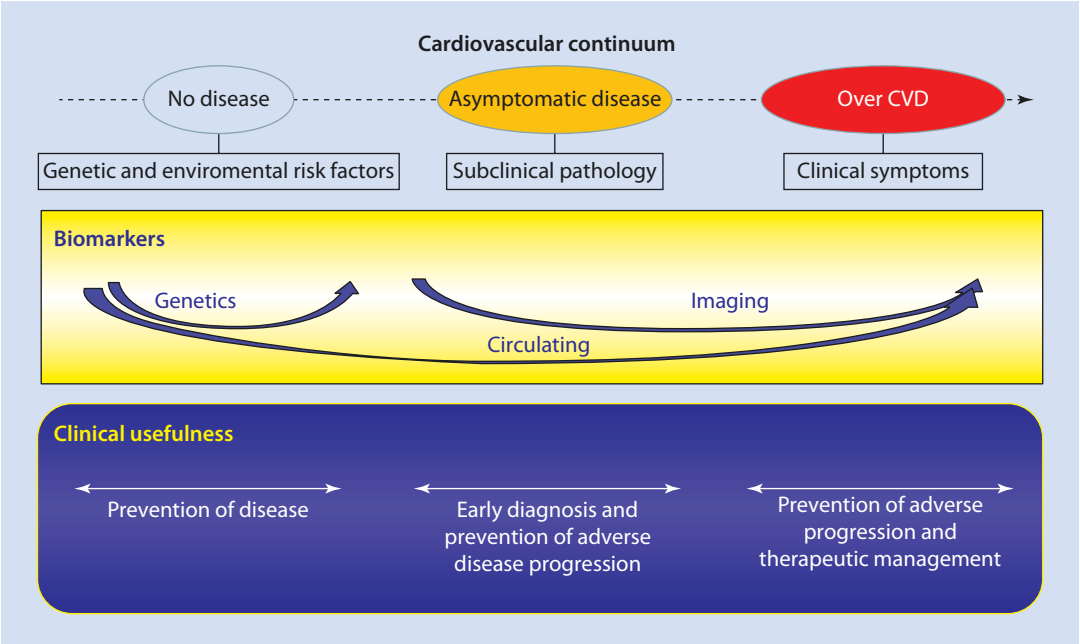

Fig. 30.2 Clinical usefulness of different types of biomarkers along the stages of the CVD continuum

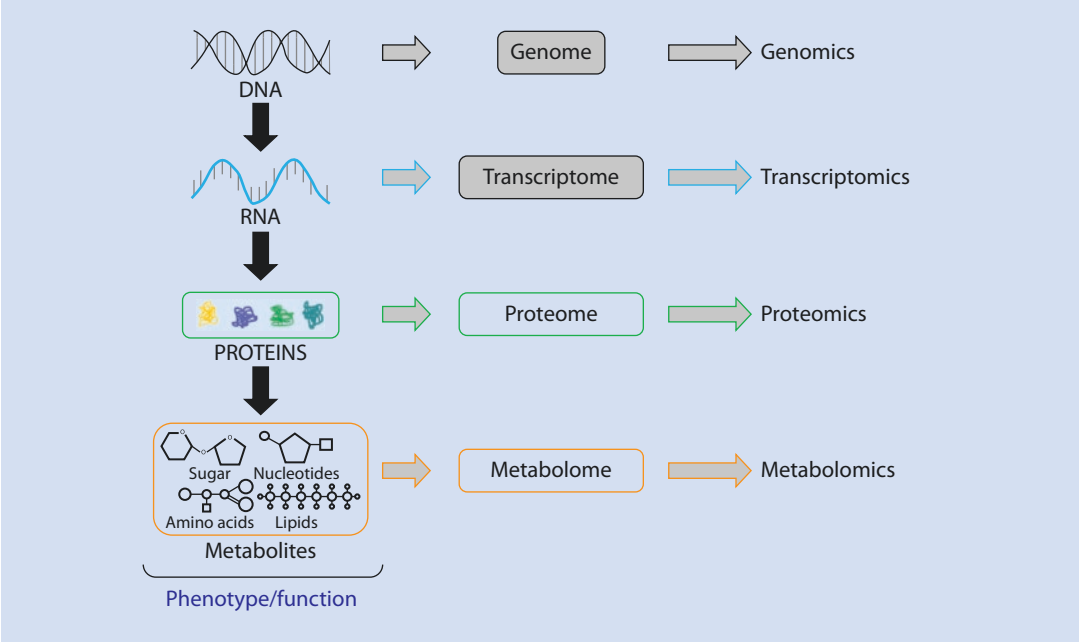

Fig. 30.3 Diagram depicting the flow of information from genes to metabolites and the "omic" sciences

prediction and primary prevention at early stages. In fact, recent genetic studies have shown some consistent loci or genes independently associated with CVD risk factors and with a higher risk of developing CVD. For instance, certain changes in the DNA sequence and epigenetic modifications resulting in altered gene expression and phenotypes have been associated with adverse cardiovascular phenotypes [7]. These properties allow genetic information to be evaluated to guide drug

therapy based on the presence or absence of markers associated with cardiovascular outcomes. Several pharmacogenomic assays are now approved by the FDA for clinical use to assess risk of adverse events and mode of drug action and to predict the response to treatment. Importantly, the sequencing of the human genome in the past decade has enabled genome-wide association studies, a popular experimental design that surveys the whole genome to create single-nucleotide polymorphism (SNP) maps and databases [8]. More details about the genetic makeup of CVDs are provided in ▶ Chap. 16 of this book. The field of proteomics in human pathology is still young, and defining the proteome in different cardiovascular diseases still awaits extensive research.

30.3.2 Proteomic Biomarkers

Proteins play an important role in almost every physiological process of cellular life; therefore it is not surprising that dysregulation in protein expression and activity can result in pathology. Mass spectrometry (MS) has become one of the most powerful technologies in recent years to examine peptide and protein expression in a variety of biological samples such as blood, urine, or tissues. This methodology has been utilized in the past two decades to evaluate the association between a wide range of proteins and CVDs [9, 10]. In particular, MS has been used to create large-scale databases of proteins that inform experimental studies to characterize changes in protein expression associated with adverse cardiovascular phenotypes. By studying large numbers of proteins in an unbiased approach, proteomic techniques can support the characterization of pathophysiological pathways and ultimately the assessment of the CVD risk. In addition, the large amount of information provided by proteomic techniques facilitates further progress in drug discovery and therapeutic approaches in different CVDs [10].

30.3.3 Metabolomic Biomarkers

Metabolomic techniques allow identification and quantification of small molecules that provide information about the state of the organisms at a certain time. Recently developed high-throughput metabolomic profiling technologies allow the quantification of hundreds of circulating metabolites that may help identify metabolic changes preceding irreversible organ damage and symptomatic disease. Characterization of the interrelation between identified metabolites can contribute to the identification of individuals at high CVD risk and provide a "fingerprint" of disease and preclinical disease states and a better understanding of the pathophysiological mechanisms involved in development of CVD. In particular, metabolites such as acylcarnitines, dicarboxylacylcarnitines, and TMAO, several amino acids such as phenylalanine and glutamate, and several lipid classes have been associated with CVD risk. Of interest, some of these metabolites (e.g., branched-chain amino acids) have been found to be associated with obesity, insulin resistance, and diabetes mellitus through underlying processes such as inflammation and oxidative stress. Although comprehensive metabolomics profiling applied to CVD is still in its infancy, metabolomics is currently considered as a tool that holds considerable promise for the search of novel biomarkers in the CVD context [11].

30.3.4 Imaging Biomarkers

Currently, several imaging-based techniques have been developed to study CVD progression. For instance, the assessment of carotid intima-media thickness (cIMT) by ultrasonography is a simple and noninvasive technique that allows characterization of early atherosclerotic changes and thereby visualizes more advanced consequences of the atherosclerotic disease process. cIMT has been found to be correlated with clinical outcomes, making it an attractive biomarker of atherosclerosis and CVD risk. However, although data support the use of cIMT as a valuable tool in clinical atherosclerosis research, it remains unclear how exactly assessment of cIMT can inform clinical decision-making and if changes in cIMT over time that result from a particular therapy correlate with future clinical events [12].

Cardiac magnetic resonance (CMR) is another imaging tool which is increasingly being used to differentiate the etiology of cardiomyopathies but also to assess the structure and function of blood vessels. CMR allows accurate measurement of

cardiac morphology and function due to its three-dimensional nature with excellent spatial resolution and high tissue contrast. In particular, late gadolinium enhancement is the reference imaging procedure for noninvasive assessment of the myocardial scar and focal fibrosis, facilitating differentiation between ischemic versus non-ischemic cardiomyopathy. However, this technique does not allow detection of diffuse fibrosis. In this regard, parametric mapping methods, such as native and post-contrast T1 mapping, have shown potential to detect and quantify both focal and diffuse alterations in myocardial structure, with promising results as novel biomarkers to support diagnostic, therapeutic, and prognostic decision-making in cardiac patients [13].

30.3.5 Circulating Biomarkers

Several systems exist to classify circulating biomarkers of CVD. Most commonly, biomarkers have been grouped based on disease specificity such as biomarkers of heart failure (HF) or cardiomyocyte injury. They have been also classified according to their use in acute versus chronic disease stages or as prognostic biomarkers. In addition, they may be categorized according to the pathophysiological process they represent, such as inflammation, oxidative stress, or myocardial fibrosis (an overview of these categorizations is shown in ◘ Table 30.1). In this section, examples of traditional and novel biomarkers that are currently being investigated for different pathophysiological processes involved in CVD are presented.

30.3.5.1 Biomarkers of Myocardial Stress

Natriuretic peptides are the most commonly used biomarkers to support the diagnosis of heart failure in patients with dyspnea. They are a closely related family of ring-shaped peptides involved in sodium and water balance and regulation of vascular tone, with several structurally similar natriuretic peptides identified: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and dendroaspis natriuretic peptide (DNP). Of these, ANP and BNP are produced in the myocytes of the atria and ventricles, respectively. In conditions of myocardial strain, induction of the BNP gene results in the production and secretion of the prohormone, which is cleaved into the biologically more stable N-terminal pro-B-type natriuretic peptide (NT-proBNP). Natriuretic peptides are also produced in other organs, especially the kidney.

The diagnostic strength of natriuretic peptides, and in particular of NT-proBNP, is their high sensitivity for heart failure, which is more likely as the value of this biomarker increases [14]. Natriuretic peptides have powerful negative predictive value at low levels. However, as with any other biomarker, there are caveats to the interpretation of circulating levels of natriuretic peptides. For instance, natriuretic peptide levels may be elevated in non-heart failure cardiac diseases such as tachycardia and myocarditis where they reflect ventricular stress rather than a clinical diagnosis of heart failure, as well as in non-cardiac diseases such as advanced chronic kidney disease where circulating levels are increased due to reduced renal clearance.

30.3.5.2 Biomarkers of Myocardial Injury

Cardiac troponin I and T, as proteins unique to the heart, are specific and sensitive biomarkers of myocardial damage. Troponin is a complex of three globular contractile regulatory proteins (troponin T, I, and C) that reside in the thin filament of striated muscle and inhibit contraction by blocking the interaction of actin and myosin. In contrast to troponin C, which is identical in type 2 fibers of the skeletal muscle and the cardiac muscle, troponins T and I are different between skeletal and cardiac muscle and are therefore preferred as cardiac-specific biomarkers.

The detection of cardiac troponins in peripheral blood is used as an estimate of cardiomyocyte damage. Technological advances have led to a refinement in troponin assays, improving its sensitivity to detect cardiomyocyte injury. In addition, these high-sensitivity troponin assays have expanded the role of cardiac troponins from biomarkers only used in the diagnosis of acute cardiac damage (e.g., myocardial infarction) to indicators of myocardial injury in chronic cardiac conditions (e.g., those evolving with HF). Interestingly, detectable levels of cardiac troponins have been observed in apparently healthy subjects from the general population as well as in asymptomatic individuals with stable CVD and predict future cardiovascular events [15]. Moreover, cardiac troponin levels have been found to be associated with

Table 30.1 Circulating biomarkers of pathophysiological processes involved in cardiovascular diseases

Pathophysiological processes associated with cardiovascular disease								
Vascular damage				Cardiac damage				
Inflammation/endothelial dysfunction	Plaque instability	Platelet activation	Oxidative stress	Neurohormonal activation	Myocardial injury	Myocardial stress	Myocardial fibrosis	
							Collagen characteristics	Other
ICAM-1	PAPP-A	sPLA2	Ox LDL	ADM	Troponin T	BNP	PICP	OPN
VCAM-1	MPO	Lp-PLA2	MPO	MR-proADM	Troponin I	NT-proBNP	PIIINP	MMP-2
E-selectin	MMPs	sCD40L	BOM	Copeptin	H-FABP	sST2	CITP:MMP-1	MMP-3
CRP	IL-6	PF4	F2-IsoPs		MLCK	Gal-3		MMP-8
sCD40L	Lp-PLA2	CXCL5	8-OHdG		CK-MB	GDF-15		MMP-9
IL-18	IL-18	CXCL7	pMDA		sFAS	ET-1		TIMP-1
MCP-1	sPLA2	CXCL8 (IL-8)			HSP 60	NRG-1		TIMP-4
Fibrinogen	OPN	CXCL12			sTRAIL			Myo-statin
MPO	OxApo A-I	MIP-1α						Syn-decan-4
FFA	PGF	CCL5						
	MCP-1	IL1β						
	OxLDL							
	TMAO							

8-OHdG 8-hydroxydeoxyguanosine, *ADM* adrenomedullin, *BNP* B-type natriuretic peptide, *BOM* bilirubin oxidative metabolites, *CCL* chemokine ligand, *CITP:MMP-1* carboxy-terminal telopeptide of collagen type I to matrix metalloproteinase-1 ratio, *CK-MB* creatine kinase MB isoenzyme, *CXCL* CXC chemokine ligand, *CRP* C-reactive protein, *ET1* endothelin-1, *F2-IsoPs* F2-isoprostanes, *FFA* free fatty acids, *Gal-3* galectin-3, *GDF-15* growth-differentiation factor-15, *H-FABP* heart-type fatty acid-binding protein, *HSP* heat shock protein, *ICAM-1* intercellular adhesion molecule-1, *IL* interleukin, *MCP-1* monocyte chemoattractant protein-1, *MIP* macrophage inflammatory protein, *MLCK* myosin light-chain kinase, *MMP* metalloproteinase, *MPO* myeloperoxidase, *MR-proADM* adrenomedullin precursor, *NT-proBNP* N-terminal pro-B-type natriuretic peptide, *NRG-1* neuregulin-1, *OPN* osteopontin, *PAPP-A* pregnancy-associated plasma protein-A, *PGF* placental growth factor, *PICP* carboxy-terminal propeptide of procollagen type I, *PIIINP* amino-terminal propeptide of procollagen type III, *pMDA* plasma malondialdehyde, *sCD40L* soluble CD40 ligand, *sFAS* soluble FAS ligand, *sST2* soluble form of suppressor of tumorgenicity, *sTRAIL* soluble tumor necrosis factor-related apoptosis induced ligand, *TIMP-1* tissue inhibitor of metalloproteinase-1, *TMAO* trimethylamine-N-oxide, *VCAM-1* vascular cell adhesion molecule-1

established heart failure risk factors, including diabetes mellitus, left ventricular hypertrophy, chronic kidney disease, and elevated natriuretic peptide levels, independently of prior myocardial infarction. In fact, troponins evaluated with high-sensitivity assays exhibit prognostic value in patients with established heart failure [16].

30.3.5.3 Biomarkers of Myocardial Fibrosis

The myocardial extracellular matrix consists of an intricate weave of (predominantly) collagen fibrils that play a crucial role in maintaining the structural and functional integrity of the heart, among other organs. Imbalance between synthesis and degradation of collagen types I and III results in myocardial fibrosis, a lesion characteristic of more advanced stages of cardiac diseases. Importantly, the functional impact of myocardial fibrosis is not just a matter of the quantity (i.e., severity of deposition) but also of the quality (i.e., degree of cross-linking among collagen fibrils) of the collagen fibers. Therefore, it is proposed that the assessment of these characteristics of collagen fibers may help to identify cardiac patients vulnerable to adverse clinical outcome [17].

Among the many circulating molecules proposed as biomarkers of myocardial fibrosis in humans, only two collagen-derived serum peptides have been shown to be associated with the quantity of collagen fibers in the myocardium: the carboxy-terminal propeptide of procollagen type I (PICP), formed during the extracellular conversion of procollagen type I into mature fibril-forming collagen type I by the enzyme procollagen type I carboxy-terminal proteinase, and the amino-terminal propeptide of procollagen type III (PIIINP), formed during the extracellular conversion of procollagen type III into mature fibril-forming collagen type III by the enzyme procollagen type III amino-terminal proteinase. Serum PICP levels have been found to be highly correlated with the abundance of collagen fibers in the myocardium of patients with hypertensive heart disease. In addition, serum PIIINP has been found to be highly correlated with extent of collagen type III deposition in the myocardium of HF patients with ischemic heart disease or idiopathic dilated cardiomyopathy [17].

On the other hand, a more rigid and stiffer collagen fiber due to excessive cross-linking may be more resistant to degradation by matrix metalloproteinase-1 (MMP-1), resulting in diminished cleavage of a small carboxy-terminal telopeptide of the collagen type I fiber (CITP). In accordance with this, low serum levels of the CITP:MMP-1 ratio have been found to be independently associated with high myocardial cross-linking [17]. Recent findings suggest that the biochemical phenotyping of myocardial collagen cross-linking may be useful to guide anti-fibrotic therapies in patients with HF [18].

In addition, the biomarkers galectin-3 (Gal-3) and soluble suppression of tumorigenicity (sST2) are markers of myocardial fibrosis which have been endorsed by the American College of Cardiology (ACC)/AHA HF guidelines, with potential interest for additional stratification of HF patients [19]. Gal-3 is a beta-galactosidase-binding protein implicated in diverse biological processes and expressed in multiple tissues and in different types of cells, including macrophages, eosinophils, neutrophils, and mast cells. Plasma levels of Gal-3 are increased in patients with heart failure showing additional prognostic value to NT-proBNP levels [20]. ST2 is a member of the interleukin-1 family and exists in two forms, a transmembrane receptor (ST2L) as well as a soluble receptor (sST2). Several clinical studies have shown elevated sST2 levels in plasma from patients with both acute and chronic heart failure, with predictive value for heart failure outcomes. sST2 is produced by cardiomyocytes and cardiac fibroblasts although elevated plasma levels have been also observed in diseases other than those cardiac-related such as gastric and breast cancer, nephropathy, and liver disease [20]. The inclusion of these novel biomarkers in guidelines supports their potential value over and beyond established risk factors, although their exact potential to inform clinical decisions remains vague. In general, circulating biomarkers can derive from multiple and also non-cardiovascular sources and should be interpreted with caution; they may be influenced by systems other than those directly involved in CVD.

30.3.5.4 Biomarkers of Inflammation

Among all circulating inflammatory markers of the atherosclerotic process, C-reactive protein (CRP) has been most extensively studied. CRP is a member of the pentraxin family of innate immune response proteins and its secretion is stimulated in the liver by cytokines such as IL-1

and IL-6. Among other properties, the fact that the largest set of data in terms of cardiovascular biomarkers have been obtained from studies evaluating CRP may be in part explained by ease of measurement using reliable and affordable technology. In recent years, the role of CRP as a proatherogenic factor has emerged. In particular, it has been proposed that CRP has a role in modulating the network between the endothelium and both inflammatory and smooth muscle cells of the arterial wall, a mechanism that probably favors the atherosclerotic process [21]. In the general population, CRP is associated with cardiovascular events independent of other CVD risk factors.

However, despite the robust statistical association, several studies indicate that CRP measurements provide only modest improvements in predictive accuracy, raising the issue of whether CRP is merely a marker of itself or a causal factor for CVD [22]. In this regard, Mendelian randomization analyses evaluating the association between CRP and coronary heart disease indicate that CRP concentration itself is not a causal factor in this condition [23]. Nonetheless, results from the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) demonstrate that targeting the interleukin-1β to interleukin-6 to CRP signaling pathway in patients with a history of myocardial infarction and high levels of circulating hs-CRP (≥2 mg/L) is beneficial for the secondary prevention of CVD, being this benefit independent of the cholesterol levels [24].

Other inflammatory biomarkers that hold promise in the context of atherosclerosis include advanced glycation endpoints, oxidized LDL, heat shock proteins, lipoproteins, tumor necrosis factors, interleukins 1 and 6, platelet-derived activation products, and myeloperoxidase [25]. These biomarkers have been comprehensively reviewed in ► Chap. 21 of this book.

Conclusion and Clinical Perspectives

Numerous cardiovascular biomarkers have been evaluated for their use in the clinical setting as predictive, diagnostic, prognostic, and therapy guidance tools. Importantly, a biomarker must reflect a pathophysiological mechanism and help making decisions on patient management, providing information beyond the clinical tools already available. More specifically, the prognostic value of a given biomarker should include improved discrimination, calibration, and reclassification with respect to standard variables already implemented in the clinical setting. In addition, biomarkers of CVDs have to be robustly validated in independent cohorts prior to approval for clinical practice. They should exhibit adequate precision and optimal intraindividual reproducibility, be easy to measure preferably at a point of care over a short time period, and demonstrate cost-effectiveness. These evaluation processes are needed to establish noninvasive tools as surrogate measures to be used for predictive and prognostic purposes in clinical trials, contributing to improve future precision medicine strategies in CVD treatment.

Gaps in Knowledge

- More reliable methods for diagnosis and guided clinical management of patients with CVD are needed.
- Limited reproducibility of proteomic data has been reported repeatedly. This may originate directly from the biology of protein expression: gene expression is highly variable even in healthy people, and disease changes expression of and diversity within protein families.
- Even if a protein is correctly identified as a potentially useful biomarker, it may be technically impossible to quantify it by affordable techniques (e.g., ELISA), precluding its use in the clinical setting. Current research based on methodological adjustments and multidimensional approaches is addressing these limitations so that the heterogeneity and diversity of biomarkers within proteomic approaches can be taken into account [26].
- A novel biomarker should add incremental information about a condition of interest, above and beyond traditional risk or disease factors. However, several studies suggest that many of the biomarkers currently used in the clinical setting, including multimarker models, may not consistently and substantially improve risk prediction or diagnosis of CVD compared to established criteria [6, 27].

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Hypertension

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_31

Key Concepts

- Hypertension is a common condition and growing public health burden.
- The majority of patients have primary hypertension although clinical assessment should include focussed evaluation for secondary causes, especially in young patients.
- Successful treatment to BP targets as per national or regional guidelines is a key priority for the management of all patients.
- Clinical evaluation should include an assessment of overall cardiovascular risk and target organ damage.
- Treatment involves lifestyle modifications with a relatively low threshold for pharmacological therapy.
- Single-pill combination therapy may achieve efficacious BP control with improved compliance and reduced side effects, although the availability and use of fixed drug combinations is limited in the United Kingdom.

31.1 Introduction

Hypertension is a common condition characterised by persistently elevated blood pressure (BP) in the systemic arteries, which affects 30–45% of the adult population [1–3]. Worldwide, more than 1 billion people have elevated BP, and the figure is expected to increase to 1.5 billion by 2025 [4]. Hypertension is the most important modifiable risk factor and is the largest contributor to the global burden of cardiovascular disease [2, 5, 6]. Indeed, hypertension is the most common preventable risk factor for cardiovascular diseases that include ischaemic heart disease, heart failure and stroke, chronic kidney disease and cognitive impairment [2]. More than half of patients with hypertension are unaware of their condition, and many patients with elevated blood pressure are inadequately or not treated [2]. Prevention of hypertension and effective blood pressure control is an urgent public health priority to prevent a global burden of cardiovascular morbidity and mortality [6].

31.2 Aetiology and Definitions

Blood pressure is referred to as the ratio of systolic BP (the pressure exerted on artery walls when the heart contracts) and diastolic BP (the pressure when the heart relaxes) [2]. The BP thresholds that define hypertension vary depending upon the measurement method and international guideline (Table 31.1) [4, 7–9]. The European Society of Cardiology (ESC)/European Society of Hypertension (ESH) published updated guidance on the management of hypertension in 2018 [4], whilst the most recent guidance from the American College of Cardiology (ACC)/American Heart Association (AHA) was published in 2017 [8], and the National Institute for Health and Care Excellence (NICE) guidance was published in 2011, reviewed in 2016, and is currently being updated [9]. Herein we focus on the 2018 ESC/ESH guideline, although reference is made to the 2017 ACC/AHA and 2011/16 NICE guidelines.

Many aetiologies can underlie hypertension although the vast majority of patients (90–95%) have primary or essential hypertension, which is a condition with a multifactorial gene-environment aetiology [2]. Secondary causes of hypertension include primary aldosteronism (Conn's syndrome), phaeochromocytoma and renal artery stenosis, as well as many other causes as described in [2]. Rare, monogenic causes of hypertension have also been described including Liddle syndrome and glucocorticoid-remediable aldosteronism, which is a state of mineralocorticoid excess [2]. Other common causes of secondary hypertension include drugs, e.g. non-steroidal anti-inflammatory drugs, anti-angiogenic drugs (VEGF inhibitors) and dietary factors (salt, liquorice).

31.3 Pathophysiology

31.3.1 Blood Pressure Regulation

Cardiovascular parameters that contribute to the determination of BP include circulating blood volume, cardiac output and the balance of arterial tone which can be affected by both intravascular volume and neurohumoral activation. There is complex interplay from a variety of integrated neurohumoral systems including the renin-angiotensin-aldosterone system, natri-

Table 31.1 Classification of blood pressure in adults

Definition	Systolic BP (mmHg)		Diastolic BP (mmHg)
2018 European Society of Cardiology/European Society of Hypertension guideline			
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Hypertension			
Grade 1	140–159	and/or	90–99
Grade 2	160–179	and/or	100–109
Grade 3	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90
2017 American College of Cardiology/American Heart Association guideline			
Normal	<120	and	<80
Elevated	120–129	and	<80
Hypertension			
Stage 1	130–139	and/or	80–89
Stage 2	≥140	and/or	≥90
2011 National Institute for Health and Care Excellence			
Normal	<140	and	<90
Hypertension			
Stage 1	≥140	and/or	≥90
Stage 2	≥160	and/or	≥100
Stage 3	≥180	and/or	≥110

uretic peptides, endothelium, sympathetic nervous system and immune system. Interruption or disruption of any component of these closely integrated systems can directly or indirectly upset the balance of mechanisms controlling BP and lead to hypertension [2]. Genetic predisposition interacts with environmental factors that include high salt intake, alcohol excess and obesity to contribute to the development of hypertension. Arterial stiffness increases with age which increases the probability of developing hypertension due to changes in vascular collagen and atherosclerosis [2]. This is especially important in isolated systolic hypertension in the elderly.

31.3.2 Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is a master component in the regulation of BP and plays a central role in the pathogenesis of hypertension due to effects on vasoconstriction, endothelial dysfunction, sodium retention and pressure natriuresis, where increased renal perfusion pressure leads to increased sodium excretion, as illustrated in ▶ Chap. 9, ◘ Fig. 9.1 [2, 10]. The most important physiological role of the RAAS is to maintain perfusion in states of intravascular volume depletion by regulating pressure-volume homeostasis in the kidney, although up-regulation of the RAAS con-

tributes to the development of elevated BP and hypertension. Renin is stored and secreted by juxtaglomerular cells of the kidney and cleaves angiotensinogen to form angiotensin I, which is then converted to angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II is central in the pathogenic role of the RAAS by acting on type 1 angiotensin II receptors (AT1) to stimulate smooth muscle contraction, vasoconstriction and increased vascular resistance, aldosterone release, sodium reabsorption, endothelial dysfunction, fibrosis, inflammation and oxidative stress. It is through these mechanisms that angiotensin II is closely linked to the development of target organ damage in hypertension [2, 10].

Angiotensin II also acts on type 2 angiotensin II receptors (AT2) to induce opposite effects including vasodilation and natriuresis. A second angiotensin-converting enzyme, ACE2, has emerged as a modulator of the harmful effects of angiotensin II by metabolising angiotensin II to angiotensin (1–7). Angiotensin (1–7), by binding to its G protein-coupled receptor Mas, has protective effects by inducing vasodilation, natriuresis and anti-proliferative effects [2, 11].

Aldosterone is the final component of the RAAS and also plays a key role in the development of hypertension by activating mineralocorticoid receptors to induce sodium reabsorption. Aldosterone contributes to endothelial dysfunction, vasoconstriction, vascular smooth muscle cell proliferation, oxidative stress, cardiovascular fibrosis and vascular remodelling [2, 12].

31.3.3 The Endothelium

The endothelium plays a major role in regulating vascular tone since endothelial cells release a variety of vasoactive substances including the vasodilators nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factors (EDHF), as well as vasoconstrictors such as endothelin-1 (ET1), locally derived angiotensin II and thromboxane A2. NO is the most important endothelial-derived vasoactive factor involved in BP regulation as it is continuously released by endothelial cells in response to shear stress, leading to vascular smooth muscle cell relaxation through guanylate cyclase activation and intracellular cyclic GMP generation. Inhibition of endothelial NO synthase (eNOS) can interrupt NO production and lead to the development of hypertension. ET-1 is a potent vasoconstrictor that induces effects by activating vascular smooth muscle cell ET1 receptors (ET_A). The fine balance between endothelium-derived vasodilators and vasoconstrictors is crucial in determining the effect of the endothelium on vascular tone. Endothelial dysfunction is key in the pathogenesis of hypertension through reduced NO bioavailability and oxidative stress via the NADPH oxidase, xanthine oxygenase and cyclooxygenase enzyme systems that lead to increased generation of reactive oxygen species [2].

31.3.4 Sodium Homeostasis

Sodium is a key regulator of intravascular volume, since elevated serum sodium concentration promotes fluid retention, which can increase intravascular volume and BP [2]. Reduction of dietary sodium intake can reduce BP, prevent hypertension, improve BP control and may reduce the intensity of antihypertensive medications required to achieve BP targets [2]. Salt sensitivity and high dietary salt intake can therefore contribute to the development of hypertension, whilst endothelial dysfunction is a risk factor for salt sensitivity and the subsequent development of hypertension [2].

31.3.5 Natriuretic Peptides

The natriuretic peptides atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) also play an important role in the development of hypertension, as they exert natriuretic and vasodilator properties that facilitate the maintenance of sodium balance and BP. Deficiency of the natriuretic peptides can promote the development of hypertension, as well as contribute to insulin resistance and type 2 diabetes mellitus [2].

31.3.6 Sympathetic Nervous System

The sympathetic nervous system has baroreceptors throughout the arterial tree that detect arterial stretch in the context of elevated blood pressure, particularly at the carotid sinus which is situated at the base of the internal carotid artery,

and act to reduce sympathetic outflow and hence blood pressure. Patients with hypertension exhibit increased sympathetic nervous system activity which contributes to the generation and maintenance of hypertension [2].

31.3.7 Inflammation

Inflammation contributes to the pathogenesis of hypertension and target organ damage through associations with increased vascular permeability and release of important vascular mediators such as reactive oxygen species, cytokines and matrix metalloproteinases (MMPs). Pro-inflammatory cytokines contribute to neo-intima formation, reduced arterial diameter, arterial remodelling and vascular fibrosis, leading to increased vascular resistance. MMPs promote extracellular matrix degradation, immune cell infiltration, apoptosis and increased collagen synthesis that contribute to target organ damage in hypertension [2].

31.4 Diagnosis

31.4.1 Definitions and Confirming the Diagnosis of Hypertension

Hypertension is generally an asymptomatic condition, and therefore it is important that adults have regular BP measurement to improve global screening and detection of hypertension. Conventional office BP measurement using a validated device is common practice for the screening, diagnosis and management of hypertension. The diagnosis should be based on at least two measurements in the sitting position on at least two visits using a validated device [1, 4]. Out-of-office BP measurement with home or ambulatory BP monitoring (ABPM) may be used as an adjunct to diagnose hypertension, titrate BP-lowering medications and identify patients with distinct BP phenotypes, including white-coat or isolated clinic hypertension and masked or isolated ambulatory hypertension [1, 2, 4, 8]. White-coat hypertension is characterised by elevated clinic BP with normal home BP or ABPM, whilst masked hypertension is characterised by normal clinic BP with elevated home BP or ABPM [2].

Hypertension has traditionally been defined as office systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg, although the BP thresholds for diagnosis of hypertension are now generally lower following the Systolic Blood Pressure Intervention Trial (SPRINT), which demonstrated reduced mortality and cardiovascular events with lower BP targets [4, 7, 8, 13]. The 2017 American College of Cardiology/American Heart Association guideline defines hypertension as systolic BP ≥130 mmHg and/or diastolic BP ≥80 mmHg, whilst the 2018 European Society of Cardiology/European Society of Hypertension guideline defines systolic BP ≥130 mmHg and/or diastolic BP ≥85 mmHg as "high-normal" and systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg as hypertension [4, 8]. Clinic BP ≥140/90 mmHg is equivalent to mean daytime BP ≥135/85 mmHg, whilst clinic and mean daytime BP ≥130/80 mmHg are equivalent [4, 8]. In the United Kingdom, the 2011 NICE guideline defines hypertension as clinic BP ≥140/90 mmHg with average ABPM or home BP ≥135/85 mmHg [9], although NICE is in the process of updating this guidance.

Patients with elevated BP can be subcategorised into stages or grades of hypertension. The 2017 American guideline categorises patients into stage 1 hypertension (systolic BP ≥130–139 mmHg or diastolic BP ≥80–89 mmHg) or stage 2 hypertension (systolic BP ≥140 mmHg or diastolic BP ≥90 mmHg) [8]. In contrast, the 2018 European guideline categorises patients into grade 1 hypertension (systolic BP 140–159 mmHg or diastolic BP 90–99 mmHg), grade 2 hypertension (systolic BP 160–179 mmHg or diastolic BP 100–109 mmHg) or grade 3 hypertension (systolic BP ≥180 mmHg or diastolic BP ≥110 mmHg) [4].

Treatment-resistant hypertension is defined as clinic BP >140/90 mmHg despite treatment with ≥3 drugs at adequate doses, including a diuretic, and when secondary hypertension has been excluded [2]. Low treatment adherence is often a cause of apparently resistant hypertension and should be excluded, for example, with observed treatment in a hospital setting.

31.4.2 Investigations and Detecting Secondary Causes of Hypertension

A small proportion of patients with hypertension have a potentially reversible cause that, if identified, may lead to curative treatment or substantial

improvement in BP control and reduction in cardiovascular risk. Patients should therefore be screened for secondary causes of hypertension, based on history, clinical examination and routine laboratory investigations. Secondary hypertension should also be suspected in patients who present with sudden worsening of hypertension, poor response to treatment or severe target organ damage out of proportion to the extent and duration of hypertension. Clinical signs that may suggest secondary causes of hypertension include features of Cushing syndrome, abdominal bruit (renovascular hypertension), enlarged kidneys (polycystic kidney disease), neurofibromatosis (phaeochromocytoma) and precordial murmurs (aortic coarctation) [2].

Routine investigations that should be performed in all patients with a new diagnosis of hypertension include renal function, serum electrolytes, lipid profile, fasting glucose, urine albumin to creatinine ratio (ACR) and 12-lead electrocardiogram. Additional investigations that may be indicated could include out-of-office BP measurements using ABPM or home measurements, cardiac echocardiography, abdominal ultrasonography, renal magnetic resonance angiography (MRA), aldosterone and renin concentrations with aldosterone to renin ratio (ARR), and urinary catecholamine concentrations or plasma metanephrines as determined by local laboratory facilities [2]. It is also essential to assess patients' family history, diet and alcohol intake, as well as taking a comprehensive drug history that includes any recreational drug use.

31.4.3 Assessing Cardiovascular Risk and Target Organ Damage

Evaluation of a patient with elevated BP should include an assessment of overall cardiovascular risk, target organ damage and additional clinical conditions that may affect BP and/or target organ damage [2, 4, 8]. Patients who are at highest risk of cardiovascular disease will derive the greatest benefit from pharmacological therapy in addition to lifestyle measures [2, 14]. Cardiovascular disease risk should be established using a validated tool, and particular focus should be placed on current and previous smoking habits, dyslipidaemia and diabetes mellitus [2, 4, 8]. Physical signs of target organ damage may include hypertensive retinopathy, features of cardiac congestion, atrial fibrillation, cognitive impairment, carotid murmurs and diminished or absent peripheral pulses. Investigations to screen for target organ damage may include urine albumin to creatinine ratio, serum creatinine and eGFR, fundoscopy, echocardiography, carotid ultrasound, abdominal ultrasound, pulse wave velocity (an index of arterial stiffness), ankle-brachial pressure index (to screen for evidence of peripheral arterial disease), cognitive function testing and brain imaging [4]. The presence of target organ damage guides lower BP treatment thresholds and targets.

31.5 Management

31.5.1 Treatment Principles and Targets

The treatment of patients with elevated BP should include non-pharmacological and pharmacological approaches, with treatment decisions dependent upon risk of cardiovascular disease and the presence of pre-existing cardiovascular disease, diabetes mellitus or chronic kidney disease [7]. There has recently been debate regarding the BP thresholds that should be used to diagnose hypertension and for treatment targets [2]. This is largely due to the publication of results from the Systolic Blood Pressure Intervention Trial (SPRINT) which was a randomised, open-label, controlled trial that was stopped early after treating to an intensive BP target of <120 mmHg reduced cardiovascular outcomes and mortality compared to a standard target of <140 mmHg [13]. As a result, some newer guidelines have adopted more aggressive BP thresholds and targets, at least for patients at high overall cardiovascular risk [2].

The 2017 American guideline recommends patients with stage 2 hypertension or stage 1 hypertension with 10-year risk of cardiovascular disease ≥10% should be treated with a combination of lifestyle advice and mediation aiming for a target BP <130/80 mmHg. A period of 3–6 months of lifestyle modifications is recommended in the first instance for patients with stage 1 hypertension and 10-year risk of cardiovascular disease <10%, followed by pharmacological therapy if BP <130/80 mmHg is not achieved with lifestyle measures [8].

The 2018 European guideline recommends immediate drug treatment and lifestyle advice in all patients with grade 2 or 3 hypertension, life-

style advice and immediate drug treatment in high-risk patients with grade 1 hypertension or very high-risk patients with high-normal BP and a period of lifestyle modification followed by drug therapy if target BP is not achieved in low-moderate risk patients with grade 1 hypertension or high-normal BP. The first objective is to lower BP to <140/90 mmHg in all patients. If treatment is well tolerated, BP should be targeted to 130/80 mmHg or lower in most patients and especially in patients with diabetes mellitus, coronary artery disease or chronic kidney disease [4]. The 2011 NICE guidelines recommend a BP target <140/90 mmHg for most patients, although these are in the process of being updated [9]. In general, an individualised approach should be adopted for older (age ≥65 years) or very old (≥80 years) patients. BP targets should be similar to younger patients for those who are relatively independent with few comorbidities, whilst a more pragmatic approach should determine BP thresholds for patients who are frail, have a high comorbidity burden or have limited life expectancy, taking account of individual patient preferences.

31.5.2 Non-pharmacological Approaches

Lifestyle advice should be given to all patients for the prevention and treatment of hypertension since targeted dietary approaches can reduce BP in patients with hypertension [2]. The key lifestyle measures are salt restriction; moderation of alcohol consumption; increased consumption of fruit, vegetables and low-fat dairy products; weight loss; regular physical exercise; and smoking cessation [1]. Strict restriction of dietary salt intake below 6 g per day, weight loss if overweight or obese, regular physical exercise, moderation of alcohol intake and enhanced intake of potassium are each likely to reduce systolic BP by 3–8 mmHg and diastolic BP by 1–4 mmHg [7, 15].

31.5.3 Pharmacological Therapies

Many large clinical trials have demonstrated that reduction of BP using pharmacotherapy results in reduced cardiovascular morbidity and mortality [2, 7, 16]. The four main drug classes used as initial antihypertensive medications are ACE inhibitors (ACEi), angiotensin receptor blockers (ARBs), dihydropyridine calcium channel blockers (CCBs) and thiazide/thiazide-like diuretics [7, 17]. The choice of antihypertensive medication should be based on drug efficacy and tolerability, as well as patient clinical characteristics, comorbidities and lifestyle.

31.5.3.1 ACE Inhibitors and Angiotensin II Receptor Blockers

ACE inhibitors or ARBs are first-line antihypertensives for most patients as they have been extensively tested in large-scale clinical trials, are effective and have an acceptable side effect profile in most patients [2, 18]. A cough develops in up to 20% of patients treated with ACEi, whilst angioedema is a less common but potentially serious side effect. Cough and angioedema are more common in black individuals and can usually be managed by substituting the ACEi for an ARB [2, 7]. Deterioration in renal function and hyperkalaemia can occur with both ACEi and ARBs; renal function and serum electrolytes should therefore be monitored after starting or increasing the dose of an ACEi or ARB. Alterations in renal function after initiating an ACEi or ARB may indicate a potentially beneficial reduction in glomerular pressure, and treatment should not be modified if serum creatinine does not increase by ≥30% or estimated glomerular filtration rate (eGF) does not fall by ≥25% from baseline [19].

31.5.3.2 Dihydropyridine Calcium Channel Blockers

Dihydropyridine calcium channel blockers are effective antihypertensive drugs that can be combined with all other first-line agents and induce vasodilation by blocking vascular smooth muscle L-type calcium channels [2, 18]. Calcium channel blockers are generally the first-line antihypertensive agents for black individuals, and ankle oedema is a relatively common side effect [7].

31.5.3.3 Thiazide Diuretics

Thiazide diuretics and thiazide-like diuretics inhibit sodium and chloride cotransporters in renal tubules to promote natriuresis and reduce BP, although they can induce hyponatraemia, hypokalaemia or worsening renal function and should be used with caution in patients who are elderly or have a history of hyponatraemia [2].

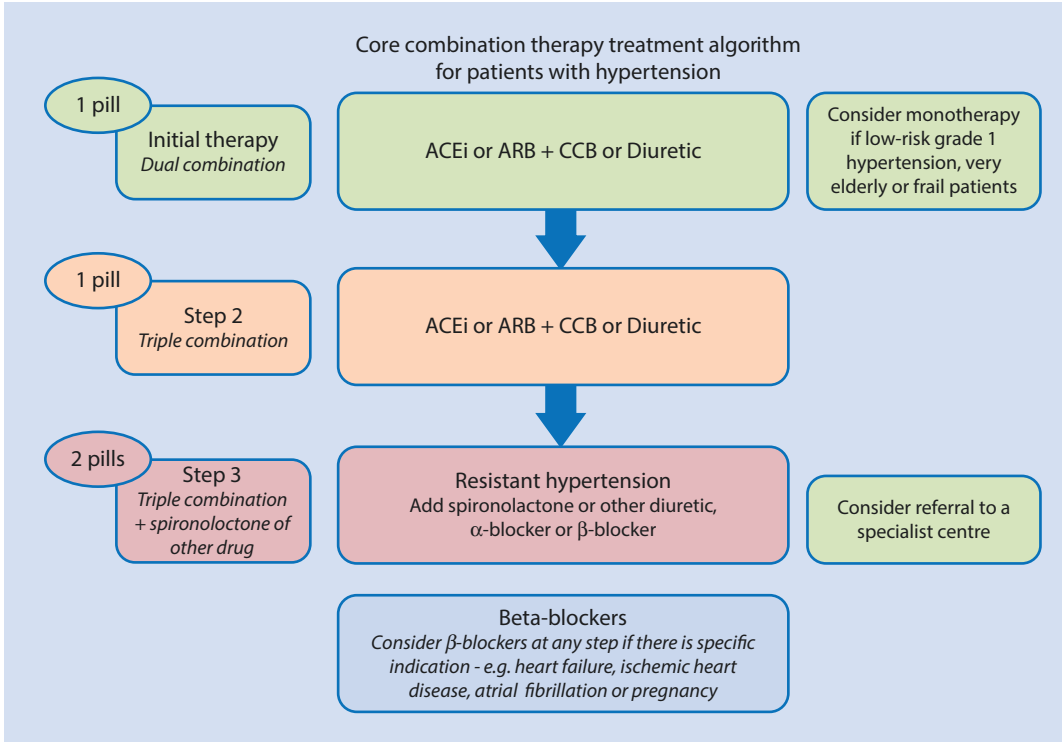

Fig. 31.1 Core combination therapy treatment algorithm for patients with hypertension. The algorithm is based on 2018 ESC/ESH guidelines and recommends initial dual therapy as single-pill combination (SPC) with an ACEi or ARB + CCB + Diuretic. Monotherapy may be considered for low-risk grade 1 hypertension or very elderly/frail patients. Step 2 is triple therapy as SPC with an ACEi or ARB plus a CCB and a diuretic. Step 3 is triple therapy as in Step 2 plus spironolactone or another antihypertensive agent. Referral to a specialist centre should be considered at this point. Beta-blockers should be considered at any step in the treatment algorithm if there is an indication, such as heart failure, ischaemic heart disease, atrial fibrillation or younger women with, or planning, pregnancy. *ACEi* angiotensin-converting-enzyme inhibitor, *ARB*, angiotensin receptor blocker; *CCB*, calcium channel blocker

31.5.3.4 Combination Therapies

The traditional approach to managing patients with elevated BP involved initial monotherapy with subsequent dose titration and stepped care. This approach may increase the propensity to develop side effects due to higher drug doses and poor compliance due to multiple-pill burden, which may contribute to less effective BP control. Indeed, fewer than 50% of treated patients currently achieve BP control, and this will become more challenging with lower treatment targets. Initial treatment with single-pill combination therapy (SPC) may provide quick, well-tolerated and effective BP control and could be considered a preferred treatment option for some patients. SPC drugs are available as dual and triple therapy combinations which may improve BP control through a simplified treatment approach, fewer side effects and reduced pill burden leading to improved compliance [4, 7, 20]. A core treatment strategy has been proposed by the 2018 European guidelines which suggests initial treatment using dual therapy in a single combination pill containing an ACEi or ARB plus a CCB or diuretic (Fig. 31.1). Monotherapy may be considered for patients with low-risk grade 1 hypertension or patients who are very elderly or frail. Step 2 suggests triple therapy in a single combination pill containing an ACEi or ARB plus a CCB and a diuretic. Step 3 suggests triple therapy as in Step 2 plus spironolactone or another antihypertensive agent, such as an alpha- or beta-blocker, and referral to a specialist centre should be considered. Beta-blockers may be initiated at any step in the treatment algorithm if there is a specific indication, such as heart failure, ischaemic heart disease, atrial fibrillation or younger women with, or planning, pregnancy. The main challenges associ-

ated with SPC therapy can include a higher relative cost compared to more traditional drugs and limited availability in some countries.

31.5.3.5 Resistant Hypertension

A mineralocorticoid receptor antagonist such as spironolactone is the best choice of fourth-line agent in patients with resistant hypertension and is more effective than an α-blocker or β-blocker, although serum potassium concentrations should be closely monitored due to risk of inducing hyperkalaemia [2]. Treatment non-adherence is a common cause of pseudo-resistant hypertension that may be detected by urinary drug screening or directly observed drug administration, and greater use of single-pill combination therapy may prevent pseudo-resistant hypertension by reducing side effects and pill burden.

Device-based treatments have also emerged for patients with severe resistant hypertension that cannot be controlled using medication. Device-based approaches include renal denervation, carotid body denervation, baroreceptor stimulation and arteriovenous fistula formation, although these approaches should currently only be used in a clinical trial until further evidence is available regarding their safety and efficacy [2, 4].

31.5.3.6 Agents for Specific Comorbidities

Patients with specific comorbidities may benefit from a particular agent, for example, a β-blocker can and should be considered for patients with heart failure and following myocardial infarction. However, β-blockers are less effective than the first-line agents at reducing BP and cardiovascular disease morbidity and mortality in patients without ischaemic heart disease or heart failure [2, 7, 21, 22]. ACE inhibitors or ARBs are recommended for patients with heart failure or diabetic nephropathy [2].

31.5.3.7 Newer Agents and Precision Medicine

The current range of antihypertensive drugs is generally inexpensive and relatively well tolerated which has resulted in limited development of new antihypertensive medications over recent years [2]. New drugs have been approved for other indications which may be useful in treating patients with hypertension, such as combined angiotensin II receptor and neprilysin inhibitors which improve prognosis for patients with heart failure or sodium-glucose cotransporter 2 (SGLT2) inhibitors which improve cardiovascular outcomes in patients with diabetes [2, 23–25]. These drugs have not been tested for treating hypertension, and it remains to be seen whether trials in this context may emerge. Other agents in development, often for indications other than hypertension, include newer mineralocorticoid receptor antagonists, aldosterone synthase inhibitors, ACE2-angiotensin (1–7) activators and natriuretic peptide receptor agonists [2, 26].

Precision medicine uses diagnostics to predict responses to treatment and identify treatments that are more likely to be efficacious for specific groups. For example, a genotype-guided algorithm has the potential to identify patients with a specific form of salt-sensitive hypertension who may benefit from greater BP response to a loop diuretic [27]. The era of precision medicine therefore presents exciting new opportunities for targeted treatment of patients with hypertension.

Conclusions and Clinical Perspectives

Hypertension and associated cardiovascular morbidity and mortality are a common and growing public health burden worldwide. Approximately half of patients with elevated BP are not aware that they have the condition, fewer than 50% of patients achieve BP control, and the global population affected by hypertension is expected to increase to 1.5 billion by 2025. Prevention strategies are essential and should include public health and lifestyle measures, whilst improved public awareness to facilitate increased detection and diagnosis of patients with elevated BP is crucial. Approaches to drug treatment of hypertension must also be improved, and the greatest benefit may be achieved through affordable and effective single-pill combination therapy containing two or three drugs that include an ACEi or ARB with a CCB and/or diuretic. Spironolactone can be used as a fourth-line agent in most patients with resistant hypertension, and BP control using one or two tablets should be achievable for the majority of patients. Improving the awareness of hypertension, routinely screening for elevated BP and using a combination of lifestyle modifications, public health strategies and affordable combination drug therapies will help to reduce the global public health burden.

Gaps in Knowledge

Key outstanding research questions and gaps in knowledge:

1. Achieving international consensus on the most appropriate BP thresholds to diagnose hypertension and target treatment
2. Assessing whether device-based strategies will prove an effective approach for patients with resistant hypertension
3. Determining if drug therapy with a single-pill combination approach will become established clinical practice
4. Unravelling the molecular causes of primary hypertension

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Diabetic Retinopathy

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_32

Key Concepts

- Multiple molecular pathways contribute to the pathogenesis of diabetic retinopathy including metabolic and hemodynamic pathways, advanced glycation end products, oxidative stress and inflammation.
- Increasing evidence indicates that the retinal neurovascular unit is damaged in diabetes and is comprised of vascular cells, neurons, glial cells and immune cells as well as the retinal pigment epithelium and choroid.
- Diabetic retinopathy is the sequela of progressive damage to the retinal microvasculature which can progress to vision-threating proliferative diabetic retinopathy and diabetic macula oedema.
- Current treatments target the retinal vasculature in subjects with proliferative diabetic retinopathy and diabetic macula oedema and include laser or the intravitreal injection of anti-vascular endothelial growth factor.
- Preventative treatments for diabetic retinopathy are limited with management of hyperglycaemia and high blood pressure of importance.

32.1 Introduction

The global prevalence of diabetes mellitus is considerable with 415 million people estimated to have the disease in 2015, a number expected to grow to 642 million by 2040 [1]. This increase in diabetes has occurred in all countries including in urban and rural areas and does not include the large proportion of people with undiagnosed diabetes who are living with high blood glucose and may be at risk of developing the complications of diabetes. Diabetic retinopathy (DR) is a major complication of diabetes and the leading cause of vision loss and blindness in people of working age. DR occurs in people with type I or type II diabetes, and its hallmark feature is slow and progressive damage to the retinal microvasculature. The major risk factors for DR are hyperglycaemia, hypertension and the duration of diabetes. Dyslipidaemia may also be a risk factor with elevations in circulating apolipoproteins A and B stronger risk factors for DR than total cholesterol and triglyceride levels. Other risk factors for DR include the severity of diabetic nephropathy, obesity, puberty and pregnancy [2].

Approximately one third of people with DR will progress onto developing vision-threatening forms of the disease which are proliferative DR (PDR) and diabetic macula oedema (DME). The global prevalence of DR is surging with the number of people with DR and vision-threatening DR (VTDR) estimated to be 191 million and 56.3 million people, respectively, by 2030 [3]. This rise in DR is alarming given that these forms of VTDR often have an insidious onset and subsequently can have a negative impact on schooling, employment and everyday life. This chapter will discuss the pathogenesis of DR, current treatment approaches and how there is an urgent need to develop new medical treatments that prevent the advancement of DR to VTDR.

32.2 The Structure of the Retina

32.2.1 The Layers of the Retina

To understand the effect of diabetes on the retina, it is important to appreciate the structure of this delicate tissue as well as its blood supply. More information on this topic can be found in the excellent review by Hildebrand and Fiedler [4].

Light penetrates through the vitreous body of the eye to reach the neurosensory retina, comprised of a complex mix of vascular cells, neurons, glial cells, immune cells (microglia) and retinal pigmented epithelium (RPE) arranged in ten layers (described below and Fig. 32.1a).

1. The *internal limiting membrane* (ILM) is adjacent to the vitreous body and is formed by the end-feet of macroglial Müller cells.
2. The *nerve fibre layer* (NFL) is formed by the axons of ganglion cells which travel towards the optic nerve head and are surrounded by glia (astrocytes and the cell processes of Müller cells).

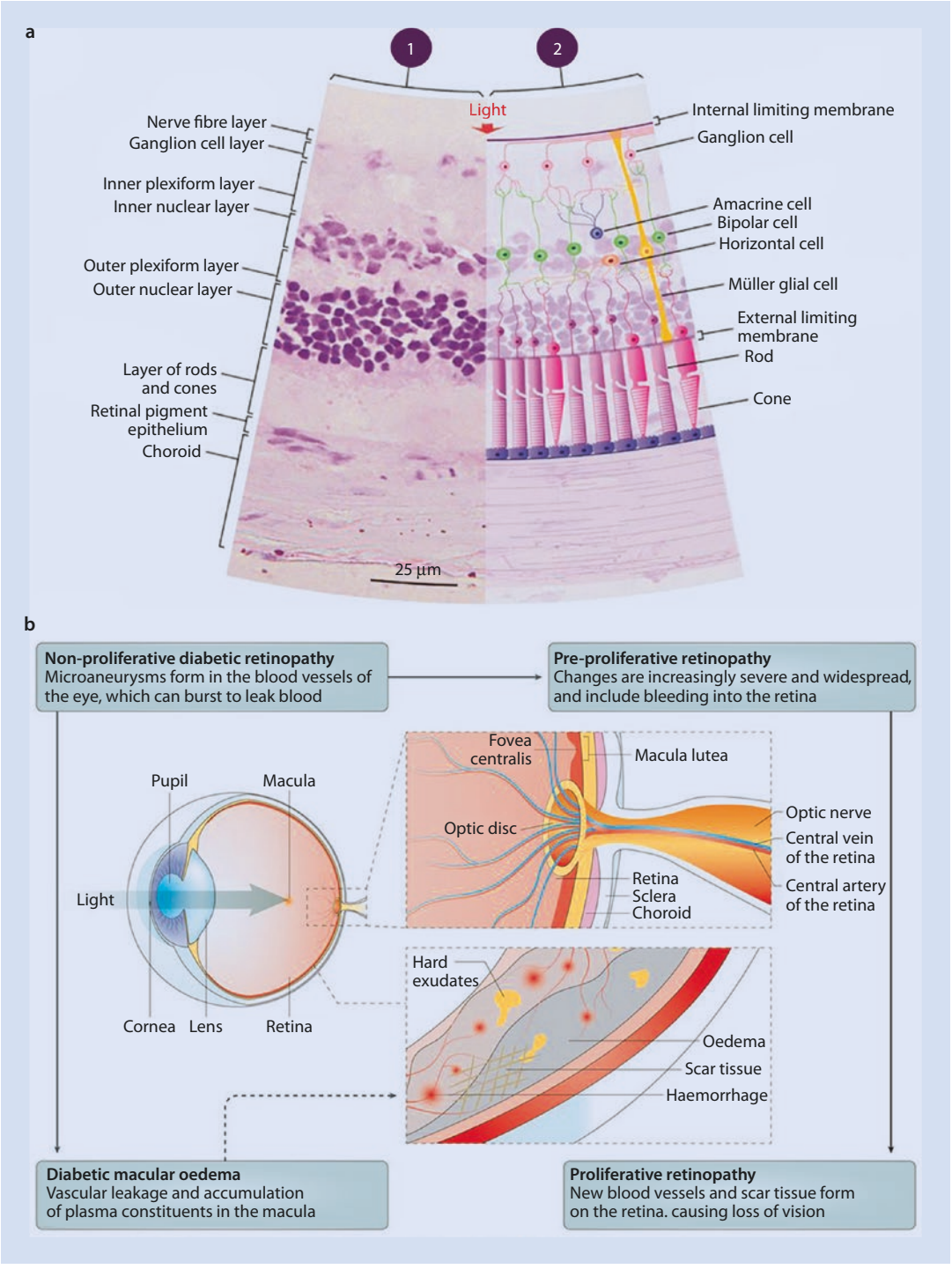

Fig. 32.1 **a** The basic structure of the retina. (1) A section of rat retina stained with cresyl violet and showing the layers of the retina. (2) A schematic diagram of the retina showing neuronal and glial cell populations. (Reproduced with permission from [26]). **b** Clinically, DR can progressively develop from NPDR, pre-proliferative DR to PDR. DME can occur at any stage of DR and has serious consequences on vision. (Reproduced with permission from [2])

3. The *ganglion cell layer* (GCL) contains ganglion cells as well as displaced endothelial cells, pericytes, astrocytes and neuronal amacrine cells.
4. The *inner plexiform layer* (IPL) consists of the synapses between ganglion, bipolar and amacrine cells.
5. The *inner nuclear layer* (INL) contains the nuclei of neuronal cells (bipolar cells, horizontal cells and amacrine cells) as well as Müller cells.
6. The *outer plexiform layer* (OPL) is comprised of the synapses between bipolar cells and photoreceptors and also contains horizontal interneuron cells.
7. The *outer nuclear layer* (ONL) is comprised of the nuclei of photoreceptors (rods and cones).
8. The *outer limiting membrane* (OLM) is created by the junctional complexes between adjacent Müller cells as well as between Müller cells and photoreceptor cells.
9. The *photoreceptor layer* is comprised of the inner and outer segments of rods and cones.
10. The *RPE* is a single layer of cuboidal cells that separates the neural retina from the choroidal circulation.

32.2.2 The Inner Retinal Blood Supply

The central retinal artery in the optic nerve head supplies the retinal circulation (Fig. 32.1b). The retinal arteries travel towards the peripheral retina within the nerve fibre layer, and the smaller arterioles give rise to two capillary systems: horizontal branches supplying the superficial nerve fibre layer and deeper branches supplying the inner retina. Therefore, all layers of the retina are nourished by these blood vessels, except for the avascular photoreceptor layer which receives nutrients via the choroidal circulation. All retinal capillary blood is returned via retinal venules into the central retinal vein located in the optic nerve head (Fig. 32.1b). The vascular pathology that is the hallmark feature of DR involves dysfunction of these blood supplies due to diabetes-mediated damage to the blood-retinal barrier (BRB). The BRB is divided into two regions: the inner BRB (iBRB) in the neural retina and near the vitreous body and the outer BRB (oBRB) near the choroid. The iBRB is the main site of vascular injury in diabetes, albeit increasing evidence indicates that the oBRB also becomes compromised (discussed in ▶ Sect. 32.2.4). The iBRB is comprised of capillaries (endothelial cells, pericytes and associated basement membrane) and the end-feet of glial cells (astrocytes and Müller cells) and functions to maintain retinal homeostasis and protect the neural retina from exposure to toxic substances.

32.2.3 The iBRB in Diabetes

The iBRB is a highly selective barrier, and in normal circumstances, the entrance of molecules from the circulation into the neural retina is regulated, with molecules entering via a transcellular route across endothelial cells or a paracellular route between endothelial cells [5]. The transcellular route may involve passive diffusion for small lipophilic substances or energy-dependent mechanisms reliant on receptor, carrier or ion transporters as well as efflux pumps. Paracellular transport occurs through tight junctional complexes including claudins, zona occludens (ZO) and the junctional adhesion molecule (JAM), as well as adherens junctions (vascular endothelium cadherin) and gap junctions. In diabetes, alterations in the functionality of some of these transport systems occur and may be mediated by inflammatory cytokines and vasoactive factors as well as growth factors including the potent vascular permeability and angiogenic factor, vascular endothelial growth factor (VEGF), leading to increased vascular permeability. Indeed, alterations in the integrity of the iBRB resulting from endothelial dysfunction are one of the early events in DR.

32.2.4 The oBRB and Choroidopathy in Diabetes

The oBRB is established by the RPE cells that form a single layer separating the neural retina from the underlying choroid (Fig. 32.1a). In normal retina, the RPE provides a number of important functions including immune privilege, secretion of factors to protect the neural retina, absorption of light and protection against photo-oxidation, phagocytosis of shed photoreceptor membranes and the supply of 11-*cis*-retinal for visual function [6]. Further, the RPE mediates the transport of

water and nutrients from the circulation into the retina by a variety of cellular transport mechanisms and tight junctional complexes [6]. Although the effect of diabetes on the integrity of the oBRB is less well studied than the iBRB, there is increasing evidence that diabetes-mediated damage to RPE cells compromises essential functions leading to increased vascular permeability in the neural retina and DME as well as damage to photoreceptors. The choroidal vasculature also becomes progressively damaged in diabetes and features thinning of the capillary bed but may also thicken if choroidal neovascularization develops. The causal factors involved in the development of this damage are yet to be fully determined, albeit inflammatory factors and the increased presence of leukocytes may contribute to choroidopathy in diabetes [7].

32.3 The Clinical Stages of DR

Classically, the clinical diagnosis and management of DR rely on the assessment of vascular pathology that is viewed with fundoscopic ophthalmoscopy. However, it is increasingly appreciated that damage to neurons, glial cells and resident immune cells, as part of the retinal neurovascular unit, contributes to the pathogenesis of DR (see ▶ Sect. 32.5). Clinically, DR is characterised into the early stage of non-proliferative DR (NPDR), formerly called background retinopathy, and may advance to pre-proliferative DR (◘ Fig. 32.1b). PDR is the advanced stage of DR with the formation of abnormally formed blood vessels and potentially fibrous scar tissue (◘ Fig. 32.1b). It is estimated that NPDR will develop in about 75% of people who have had diabetes for approximately 10 years [8]. An additional and important characterisation of both NPDR and PDR is central thickening at the macula (the central focal point of the retina). This thickening can be due to blood, lipid or serous fluid leakage from microaneurysms in the region of the macula. DME is a major cause of VTDR estimated to occur in 39% of patients with DR [3] (◘ Figs. 32.1b and 32.2). As patients with VTDR may not have any symptoms, lifelong evaluation of the retina is required. A key pathogenic factor in the development of the aforementioned vascular disease is the increased local production of VEGF. Indeed, current treatment strategies for PDR and DME focus on attenuating the effects of VEGF (see ▶ Sect. 32.4).

32.3.1 Non-proliferative DR and Pre-proliferative DR

Some of the early features of NPDR are clinically visible by ophthalmoscopy because they occur in the superficial retinal layers and include small microaneurysms (focal dilations), retinal haemorrhages either dot (small) and blot (larger) or flame-shaped haemorrhages.

Protein leakage from damaged blood vessels can result in hard exudates that appear as yellow deposits. As DR advances, the aforementioned vascular signs increase in frequency. Features of pre-proliferative DR are intraretinal microvascular abnormalities (IRMAs) which develop due to arterial abnormalities and remodeling of retinal capillary beds. The development of IRMA as well as areas of tissue non-perfusion due to the demise of existing capillary network (hypoxia) heralds an increase in the production of vascular permeability and angiogenic factors such as VEGF. Approximately 50% of retina exhibiting these features will progress to PDR within 1–2 years.

32.3.2 PDR

The hallmark feature of PDR is neovascularization in areas of tissue ischaemia, which may represent an attempt to restore blood flow to the under-perfused retina. However, the characteristics of these new retinal vessels are that they are excessive, fragile and have a tendency to bleed and exhibit a haphazard growth pattern.

They may therefore cause vision loss by recurrent bleeding in the form of intravitreal or preretinal haemorrhage and in the advanced stage of the disease regress and be replaced by a fibrous tissue scar predisposing to retinal detachment.

32.3.3 Diabetic Macula Oedema

The aforementioned vascular pathology that occurs in DR may be largely confined to the periphery of the retina. However, if there is vascular leakage and exudation of fluid and proteins in the central part of retina, this is known as DME. This accumulation of fluid into the neural retina leads to increased retinal thickening and

Fig. 32.2 Retinal images showing DME and PDR. **a** PDR with retinal laser treatment (green spots). **b** PDR showing vitreous haemorrhage. **c** Centre involving DME. **d** Fundus fluorescein angiography showing severe maculopathy characterised by multiple points of macula leak predisposing to macula ischaemia. Areas of capillary non-perfusion are seen in the superior retina. Both carry a poor visual prognosis. **e** Fundus fluorescein angiography showing an irregular and enlarged foveal avascular zone typical of macular ischaemia. **f** Fundus fluorescein angiography showing non-centre involving DME. **g** Corresponding image from OCT showing macula oedema (asterisk) illustrating that follow-up of all patients with DME is imperative

Table 32.1 International classification of clinical DME disease severity scale

Proposed severity scale	Findings observed on ophthalmoscopy
DME apparently present	Some apparent retinal thickening or hard exudates at posterior pole
Mild DME	Some retinal thickening or hard exudates but distant to the macula centre
Moderate DME	Retinal thickening or hard exudates approaching the macula centre
Severe DME	Retinal thickening or hard exudates involving the macula centre

often cystoid oedema of the macula. The severity of DME can be graded clinically which may be helpful in instituting treatment (Table 32.1).

32.4 Current Treatments for DR

The current treatments for DR (see below) are focussed on the assessment of damage to the retinal microvasculature and applied to patients with PDR and DME (see below). Fundus fluorescein angiography (FFA) is a test in which retinal photographs are taken after the systemic injection of a fluorescent dye and is used to locate the sources and extent of vascular leakage in the retina. Improvements in the evaluation and management of DR have occurred with the development of new ocular imaging platforms. Optical coherence tomography (OCT) is a non-invasive diagnostic test providing detailed cross-sectional anatomic images of the retina. OCT allows for early detection of anatomical changes in the macula particularly the presence of DME.

The Diabetic Retinopathy Study (DRS) and Early Treatment of Diabetic Retinopathy Study (ETDRS), conducted in the 1970s and 1980s, respectively, demonstrated the beneficial effects of laser treatment in eyes with PDR and DME [9, 10]. The DRS established that pan-retinal photocoagulation (PRP), which uses laser to produce multiple small burns scattered throughout the retina and spares the macula, is an efficacious treatment for PDR, reducing the risk of severe vision loss by 50% [9]. The ETDRS demonstrated that focal photocoagulation to seal leaky vessels was an effective treatment for DME reducing the risk of moderate vision loss by 50% [10]. As a result of these clinical trials, PRP and focal laser became standard of care for patients and may still be performed today. Although both procedures reduce the risk of vision loss and the progression of DR, they are essentially destructive to retinal tissue and may impair peripheral vision. Therefore, more effective treatments were sought.

The search for a secreted factor X that stimulated vascular permeability and angiogenesis eventually led to the identification of VEGF. Several clinical trials showed the efficacy of administering anti-VEGF agents (e.g. ranibizumab, aflibercept, bevacizumab) into the vitreous cavity as a treatment of DME [11]. Indeed, repeated monthly intravitreal injections lead to a new standard of care for DME. However, 35–65% of patients with DR may not respond to anti-VEGF agents and have persistent DME that can result in irreversible functional damage [12]. This suggests that there are factors in addition to VEGF that likely mediate the development of DR (see ▶ Sect. 32.6).

32.5 The Retinal Neurovascular Unit

It is now appreciated that DR is more than a disease of the retinal microvasculature. Retinal capillaries are closely associated with retinal neurons, glial cells and immune cells to form the retinal neurovascular unit which regulates retinal function including local blood flow and metabolic demands of the tissue. In diabetes, injury to the neurovascular unit will influence the functionality of the unit and integrity of the iBRB, and therefore each cellular component will be briefly discussed.

32.5.1 Endothelial Cells and Pericytes

Capillaries in the iBRB are comprised of endothelial cells and pericytes embedded in a common basement membrane composed of collagen types IV and V, laminin and heparin sulphate proteoglycan. Pericytes are specialised mural cells that have key roles in the retinal microvasculature including the regulation of blood flow, vessel stabilisation, angiogenesis and the integrity of the iBRB. The cytoplasmic processes of pericytes span several

endothelial cells, and communication between these two cell populations via signaling factors, such as platelet-derived growth factor-B, transforming growth factor-β and angiopoietin-1/Tie2, assists in maintaining the integrity of the blood vessel wall.

One of the earliest morphological features of DR cannot be readily discerned with ophthalmoscopy. The evaluation of post-mortem human eyes and studies in diabetic animals revealed that thickening of retinal capillary basement membranes develops early in DR and disrupts communication between endothelial cells and pericytes and promotes the apoptosis of pericytes (known classically as pericyte drop-out). Indeed, in DR the ratio of pericytes to endothelial cells drops from 4:1 to 1:1 [13]. This loss of pericytes is viewed to contribute to the development of microaneurysms, neovascularization and vascular leakage. The apoptosis of endothelial cells in conjunction with pericytes drop-out results in naked basement membrane tubes (acellular capillaries). The outcome are areas of tissue non-perfusion and ischaemia resulting in the upregulation of the transcription factor, hypoxia inducible factor-1 (HIF-1). Subsequently, the increased production of angiogenic and permeability factors, notably VEGF, induces breakdown of the iBRB and neovascularization.

32.5.2 Glial Cells

The cell processes of retinal glia cells, astrocytes and Müller cells surround retinal blood vessels providing structural support as well as influencing retinal homeostasis by regulating the uptake of nutrients from the blood stream into the neural retina [14]. Müller cells react to diabetes by undergoing a reactive gliosis that is apparent with microscopy as increased immunolabeling for the protein, glial fibrillary acidic protein (GFAP). In healthy retina, GFAP labeling is restricted to astrocytes, but in diabetes GFAP immunolabeling can be readily discerned in the long cytoplasmic processes of Müller cells. The reaction of Müller cells to the ischaemia and hyperglycaemia of diabetes boosts the secretion of VEGF from Müller cells as their increased production of inflammatory factors such as tumour necrosis-factor-α (TNFα) and intercellular adhesion molecule-1 (ICAM-1). This reaction of Müller cells overcomes their production of factors such as pigment epithelium-derived growth factor and thrombospondin-1 which act to oppose the actions of VEGF and maintain the tightness of the iBRB. The end result is diabetes-induced vascular pathology including breakdown of the iBRB.

Müller cells also have a critical role in regulating ion and water transport in the retina and regulating neuronal activity [14]. For instance, Müller cells remove external potassium ions generated from neuronal activity via the Kir4.1 channel, protect neurons via the release of antioxidants and neurotrophic factors and contribute to neuronal signaling by the uptake and recycling of neurotransmitters. In diabetes, the dysregulation of these Müller cell-dependent functions negatively influences the neurovascular unit. Contributing to these events is evidence that although Müller cells may proliferate in diabetes, they may also undergo apoptosis.

32.5.3 Neuronal Dysfunction

Neuronal dysfunction occurs in the retina of diabetic patients, as measured by the electroretinogram (ERG), and includes deficits in colour vision and contrast sensitivity [15, 16]. Previous studies of post-mortem tissue from diabetic subjects as well as retinas from diabetic animals have clearly shown the apoptosis of retinal ganglion cells and amacrine cells resulting in thinning of the inner retina [15]. These deficits in neurons occur before the appearance of damage to the retinal vasculature, suggesting that neuronal degeneration may be a valuable therapeutic target. Recently, a 4-year longitudinal study of subjects with type I and type II diabetes, who had no or minimal retinal vascular pathology, found thinning of the retinal nerve fibre layer occurred at 0.25 μm/year and the ganglion cell layer/inner plexiform layer at 0.29 μm/year [16].

32.5.4 Immune Cells

Microglia are resident immune cells of the retina with similar features to macrophages. In healthy retina, microglia have long ramified cell processes and constantly survey the tissue to perform various protective functions including synaptic pruning and the release of neurotrophic and

anti-inflammatory factors [17]. In response to the chronic hyperglycaemia and tissue ischaemia of DR, microglia exhibit an altered phenotype and state of activation. They proliferate, become amoeboid in shape and secrete a range of injurious factors including increased levels of reactive oxygen species (ROS), TNFα, interleukin (IL)-1β, IL-17, IL-18 and IL-6. In this chronic activation state, microglia are viewed to damage cells within the neurovascular unit.

32.6 Overview of the Mechanisms That Contribute to Diabetic Retinopathy

The pathogenesis of DR is complex and involves a range of molecular pathways that appear to be interlinked by hyperglycaemia-mediated increases in oxidative stress. The end-result is the excess production of ROS, angiogenic and vascular permeability factors such as VEGF and inflammatory mediators that damage the neurovascular unit. A brief overview of some of the main pathways involved in the development of DR will be presented.

32.6.1 Hyperglycaemia

The role of hyperglycaemia in diabetic complications including DR has been clearly established by large-scale prospective studies: the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) [Reviewed [18]]. Of interest is that DCCT and the Epidemiology of Diabetic Interventions and Complications (EDIC) study of type I diabetic patients demonstrated that early intensive glycaemic control resulted in a persistent benefit for diabetic complications even when followed by conventional control of hyperglycaemia. These findings implied that a mechanism of glycaemic or metabolic memory provided long-lasting protection against diabetic complications. Although yet to be fully understood, increasing evidence indicates that epigenetic modifications involving transcriptionally suppressive cytosine DNA methylation and histone post-translational modifications may influence oxidative stress and inflammatory pathways involved in diabetic complications including DR.

32.6.2 Blood Pressure and the Renin-Angiotensin System

Hypertension is an independent risk factor for the development of DR in patients with type I and type II diabetes. The DCCT and UKPDS demonstrated that controlling blood pressure reduced the risk of developing PDR and DME. The detrimental effects of hypertension and diabetes on the retina are strongly linked to upregulation of the renin-angiotensin-aldosterone system (RAAS) with its main effector, angiotensin II, a powerful vasoconstrictor. However, angiotensin II has other actions that are relevant to the pathogenesis of DR including the upregulation of oxidative stress, VEGF production and inflammatory factors. Furthermore, a local RAAS exists within the retina with studies in animal models of DR demonstrating that angiotensin type 1 receptor blockade (ARB) or angiotensin-converting enzyme (ACE) inhibition prevents damage to the neovascular unit including vascular leakage [19].

The largest clinical study to date to evaluate the effects of RAAS blockade with retinopathy as the primary endpoint is the DIabetic REtinopathy Candesartan Trial (DIRECT). This study involved over 5000 patients recruited from 309 centres worldwide and a follow-up of individuals for 5 years [20, 21]. DIRECT reported that the oral administration of the ARB, candesartan, elicited a modest prevention of the onset of retinopathy in normotensive people with type 1 diabetes of 18%, which on post hoc analysis was 35%. In type 1 diabetic individuals, there was no effect of candesartan on progression. On the other hand, in people with type II diabetes, candesartan treatment resulted in a 34% regression of retinopathy. A greater benefit for RAAS blockade has been reported in the renin-angiotensin-aldosterone system (RASS) study of 258 normotensive and normoalbuminuric individuals with type 1 diabetes, which after a 5-year follow-up showed a reduction in progression with ACE inhibition (65%) or ARB (70%). A further study generated from RASS indicated that these treatments reduced DR progression only in patients with HbA1c levels greater than or equal to 7.5%. Overall, these largely positive effects of ARB and ACE inhibition on the diabetic retina highlight the importance of the RAAS in DR.

32.6.3 Dyslipidemia

The contribution of lipids to the development of DR and DME is not entirely clear. There is evidence that total cholesterol and low-density lipoprotein cholesterol are associated with the presence of hard exudates in the retina of patients with DR. Furthermore, nontraditional lipid markers such as apolipoprotein A1 are associated with the severity of DR. Of interest is the drug fenofibrate, a peroxisome proliferator-activated receptor α (PPARα) agonist and cholesterol-lowering and lipid-modifying agent. The oral administration of fenofibrate has been demonstrated in two large clinical trials (FIELD, Fenofibrate Intervention and Event Lowering in Diabetes study; ACCORD, Action to Control Cardiovascular Risk in Diabetes eye trial) to reduce the progression of DR and the need for laser treatment [22]. Although fenofibrate's main mechanism of action was originally considered to be the lowering of lipids, fenofibrate can modulate a wide variety of genes and actions that are relevant to DR including VEGF, angiogenesis, cellular apoptosis and inflammation. Fenofibrate has been approved for use in Australia as an adjunct treatment to slow the progression of existing DR in patients with type II diabetes.

32.6.4 Advanced Glycation End Products

The non-enzymatic glycation of long-lived proteins leads to the formation of advanced glycation end products (AGEs) which progressively accumulate in various tissues through the normal process of ageing. The hyperglycaemia of diabetes is a major stimulus for the increased glycation of a variety of proteins including plasma proteins (e.g. fibrinogen, IgG, albumin) and essential structural proteins such as collagen. The AGEs that are formed in diabetes as well as their intermediate precursors such as methylglyoxal (MGO) have a number of deleterious effects and contribute to the development of diabetic complications including DR.

AGEs are elevated in the retina during diabetes and present in various cell types including the vasculature, neurons and glial cells [23]. The accumulation of AGEs in these components of the neurovascular unit is linked to endothelial cell and pericyte apoptosis and the closure of retinal capillaries as well as the dysfunction of Müller cells, resulting in the increased production of VEGF. Furthermore, AGEs through the receptor for AGEs (RAGE) activate intracellular signaling pathways such as protein kinase C, mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) to result in the increased expression of inflammatory mediators such as TNFα and IL-1β as well as ROS in the diabetic retina. The inhibition of AGEs and the antagonism of RAGE have been examined in animal models of DR and may be useful targets for the treatment of DR.

32.6.5 Oxidative Stress

ROS are essential for various normal cellular processes including intracellular signaling mechanisms and homeostasis. However, oxidative stress can occur when there is a disturbance in the balance between the production of ROS such as superoxide and the ability to neutralise their harmful effects through antioxidants. Excess levels of ROS can lead to the oxidation of proteins, lipids, carbohydrates, RNA and DNA and result in severe cellular and tissue damage. Oxidative stress has a central role in the development of diabetic complications including DR [24]. The hyperglycaemia of diabetes causes damage to tissues by (a) increased flux through the polyol pathway also known as the sorbitol-aldose reductase pathway, (b) activation of protein kinase C, (c) the increased intracellular production of AGEs and (d) overactivation of the hexosamine pathway. The elevated levels of ROS in diabetes are from various sources including the mitochondrial electron transport chain, upregulation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme family and myeloperoxidases. Further, vasoactive factors such as angiotensin II can increase the production of ROS from NADPH oxidase. The increase in ROS in diabetes is exacerbated by a decline in the functionality of antioxidant enzymes such as glutathione peroxidases, catalase enzymes, superoxide dismutases and peroxiredoxins. The imbalance in these mechanisms leads to ROS-mediated upregulation of growth factors, cytokines and pro-fibrotic factors that promote the development of DR. Various treatments that inhibit excess ROS production have been evaluated in

animal models of DR and include inhibitors of NADPH oxidase isoforms. Furthermore, treatments that boost the antioxidant capacity of the retina may have protective effects in the diabetic retina.

32.6.6 Inflammation

Inflammation is emerging as a key contributor to DR. Hyperglycaemia induces the increased expression of leukocyte adhesion molecules such as ICAM-1 in the vascular endothelium resulting in the adherence of leukocytes, a process known as leukostasis [25]. This is one of the earliest events in animal models of DR, with leukostasis also observed in patients with DR. Leukostasis is viewed to lead to the occlusion of retinal capillaries to result in areas of tissue non-perfusion. As mentioned in ▶ Sect. 32.5.4, retinal microglia become chronically activated in DR and produce pro-inflammatory cytokines which injure the neurovascular unit. The inflammatory environment of the diabetic retina is further exacerbated by the release of inflammatory factors from retinal Müller cells (▶ Sect. 32.5.2). Anti-inflammatory agents such as corticosteroids reduce DME in patients but have adverse effects leading to cataract formation and increased intraocular pressure in some patients. Other anti-inflammatory agents being evaluated as possible treatment strategies for DR include inhibition of TNFα and non-steroidal anti-inflammatory medications.

Conclusion and Clinical Perspectives

- The neurovascular unit plays a critical role in the development of VTDR resulting in vascular disease, a decline in retinal function and inflammation.
- Current treatments for DR target vision-threatening vascular disease and include laser photocoagulation and the intravitreal administration of anti-VEGF agents. However, laser treatment can be destructive to healthy retina, and anti-VEGF agents are ineffective in the treatment of all cases of DME.
- There is an urgent need to more fully understand the pathogenic factors that contribute to DR in order to develop preventative treatments as well as effective approaches for early intervention in the treatment of VTDR.

Gaps in Knowledge

- Limited treatments to reduce the progression of DR to PDR and development of DME
- Identification of biomarkers that predict DR and disease progression
- Increased knowledge about how metabolic and hemodynamic pathways interact to influence the health of the neurovascular unit early in disease
- New technologies to allow sustained release of medications (e.g. nanoparticles, topical drug delivery) to replace current invasive ocular treatments

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Ischaemic Heart Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_33

Key Concepts

- Ischaemic heart disease usually develops in the presence of a number of readily identifiable lifestyle and medical risk factors.
- The initial investigation of suspected angina typically requires a multimodality assessment including a combination of history taking, clinical examination and non-invasive testing.
- Treatment of ischaemic heart disease centres on medical therapy for relief of angina and the primary and secondary prevention of acute coronary syndromes.
- Invasive medical procedures such as percutaneous coronary intervention and coronary artery bypass graft surgery are most commonly employed in the treatment of angina which is refractory to medical therapy or in the acute setting of obstructive coronary disease which has caused a myocardial infarction.
- Medical advances and improved care pathways for patients with acute coronary syndromes have helped to reduce the burden of ischaemic heart disease in the United Kingdom over the past 15 years.

33.1 Introduction

Up until 2015, when it was replaced by Alzheimer's disease and other dementias, ischaemic heart disease (IHD) was the leading cause of death in the United Kingdom. An estimated 76,400 people died due to IHD in 2016, and it remains the leading cause of death among males [1]. The proportion of deaths in the United Kingdom attributed to IHD has, however, declined since the turn of the millennium, accounting for 11% of deaths registered in 2016, compared with 19.9% in 2001 [2]. IHD is primarily caused by a build-up of fibro-lipid deposits called atheroma in the walls of the coronary arteries (the blood vessels which supply the heart muscle). This is a process known as atherosclerosis and leads to progressive narrowing of the artery, restricting the flow of blood to the heart. In many people this will provoke symptoms of chest pain called angina.

33.1.1 Angina

Angina is commonly described as a heaviness or tightness in the chest. The sensation can also spread to the neck, jaw or arms. Angina can be broadly classified into two main categories, stable and unstable. Stable angina is typically triggered by physical exertion and is relieved by rest and/or the administration of nitrate medication. Unstable angina differs in that the attacks are less predictable and can occur at rest, without any apparent trigger. Symptoms of angina generally indicate that a person is suffering from ischaemic heart disease and is at increased risk of a heart attack.

33.1.2 Heart Attack

A heart attack, or myocardial infarction, occurs when a coronary artery becomes obstructed by a blood clot (thrombus), almost always, superimposed on a pre-existing narrowing in the blood vessel. If the interruption to blood flow is short-lived, then the damage to the heart muscle may be minimal. A complete occlusion persisting for more than 20–30 minutes, however, starts to cause irreversible myocardial necrosis (cell death). Depending on the extent of the final damage to the heart, patients may develop heart failure or abnormal heart rhythms (arrhythmias). Heart attacks can also cause sudden cardiac death. Unstable angina and myocardial infarction are classified as acute coronary syndromes and are medical emergencies that require urgent treatment.

33.2 Causes and Risk Factors

There are a number of risk factors associated with the development of IHD, many of which are modifiable and therefore targets for interventions to reduce the burden of disease.

33.2.1 Lifestyle

Smoking has been well established as a risk factor of IHD [3]. Obesity and a sedentary lifestyle also have strong associations with the condition [4–6]. However, the role of dietary fats (saturated fats in particular) remains controversial [7]. Links have also been made between certain psychosocial

stressors and heart attacks, with negative emotions [8–11], low socioeconomic status [12–16] and shift work [17] being identified as potential risk factors.

33.2.2 Family History/Genetics

IHD frequently affects successive generations of families, and a family history of a first-degree relative with IHD is recognised as strong risk factor for developing the condition. Genome-wide association studies have identified about 30 gene locations (loci) that are associated with increased risk of myocardial infarction [18]. This is likely only the tip of the iceberg however, and further genetic associations will doubtless continue to be discovered as our understanding of the human genome expands.

33.2.3 Associated Medical Conditions

High blood pressure (hypertension), abnormal cholesterol levels (dyslipidaemia/hypercholesterolaemia) and diabetes mellitus are all recognised as risk factors for heart disease. These conditions are frequently treated with medications for both the primary and secondary prevention of atherosclerotic vascular disease (including IHD) with a goal of reducing risk by normalising blood pressure and appropriately regulating cholesterol and blood sugar levels.

33.2.4 Age/Sex

Older age increases the risk for IHD. Men are more at risk than women, particularly before menopause, and tend to have heart attacks at younger ages.

33.3 Diagnosis

The exact pathway for the diagnosis of IHD is dependent upon the manner in which a person presents to medical services but will invariably involve progression through a sequence of tests of increasing invasiveness and complexity.

Screening for, and assessment of, the risk factors described above is also frequently performed at first presentation. This involves non-invasive blood pressure measurement and obtaining blood samples for analysis.

33.3.1 Electrocardiogram (ECG)

An ECG is a simple, non-invasive test that records the electrical activity of the heart over a period of time (usually 10–20 seconds) and gives valuable information regarding the heart rate and rhythm in addition to indications of the health of the electrical conduction system, the heart muscle and even the structure of the heart. The ECG is performed by attaching ten adhesive electrodes to the skin over the chest and limbs. It is the standard baseline test for all patients with suspected heart disease.

Patients presenting with symptoms of stable angina, and asymptomatic people with strong risk factor profiles, will usually progress to the next diagnostic tier of non-invasive functional assessment. This typically involves assessing the heart's response to stress, induced either by exercise or by using pharmacological agents which increase heart rate and blood pressure in a manner analogous to exercise. The baseline investigation at this level is the exercise ECG. The patient exercises, usually on a treadmill, with continuous ECG monitoring which is then scrutinised for changes indicative of myocardial ischaemia.

33.3.2 Cardiac Imaging and Functional Assessment

More complex modalities of assessment involve imaging of the function and/or perfusion of blood to the heart and include dobutamine stress echocardiography, radioisotope myocardial perfusion scans and stress perfusion cardiac magnetic resonance imaging (MRI).

Echocardiography obtains real-time images of the heart using an ultrasound probe applied externally to the chest wall. This provides a wealth of information about cardiac structure and function and can reveal evidence of compromised blood flow to the heart muscle. When this is coupled with a pharmacological stressor such as

dobutamine, it can unmask signs of ischaemia that are not evident at rest.

Isotope perfusion scans (at rest and under stress) can detect how well the heart muscle (myocardium) is being perfused by blood and identify areas at risk from or previously damaged by ischaemia.

Modern cardiac computed tomography (CT) and MRI scans can provide high-definition anatomical and functional assessment of the heart and have increasing utility in the assessment of patients with IHD.

CT coronary angiogram (CTCA) has established a role in the diagnostic algorithms for the assessment of patients thought to have a low to intermediate pretest probability of having IHD as it has a high negative predictive accuracy. A reassuring CTCA in an appropriately selected patient can often obviate the need to progress to further testing. Though largely non-invasive, there is an associated radiation exposure with CT which must be considered.

Cardiac MRI can provide both detailed structural and functional assessments of the heart without radiation exposure.

33.3.3 Invasive Coronary Angiography

The final common step in the diagnostic pathway is the invasive coronary angiogram. This remains the gold standard test for the diagnosis of IHD but, due to the potential procedural risks involved, is rarely the most appropriate first-line investigation for asymptomatic patients with high-risk profiles or those with symptoms of stable angina. These patients will generally only proceed to coronary angiography when non-invasive testing has either indicated or failed to exclude the presence of IHD and/or ischaemia.

Coronary angiography is performed in a dedicated catheterisation laboratory (cath lab) in a hospital setting. Small, flexible tubes (catheters) are advanced under x-ray screening guidance from the radial artery (at the wrist) or femoral artery (at the top of the leg) through the arterial system to the root of the aorta (the body's main artery which arises directly from the heart). These catheters are then manipulated under direct x-ray visualisation to engage, in turn, the openings of both the right and left coronary arteries as they branch off the aorta to supply the heart muscle. A radiopaque contrast dye is injected into the coronary arteries to opacify their inner lumen, and live digital recordings of each injection are made by an x-ray camera which is sequentially rotated around the torso.

A standard angiogram will usually contain anywhere from six to nine such images in order to optimally visualise the three-dimensional nature of the coronaries using a sequence of two-dimensional video recordings. In this manner, the angiogram can identify areas of narrowing or obstruction as the dye flows through the arteries.

However, the diagnostic angiogram essentially only provides detail on the dimensions of the inner lumen of the blood vessel. In order to obtain information about coronary physiology and the composition of the vessel walls (i.e. the burden of plaque and calcium), additional tools can be employed during an angiogram. These methods require a more invasive approach than simply injecting dye and require the operator to advance diagnostic equipment into the coronary arteries themselves.

Where there is doubt over the functional significance of a narrowing (stenosis) on angiographic appearances alone, additional information about its impact on coronary physiology can be very helpful in deciding how best it should be treated. A specialised device commonly referred to as a "pressure wire" can be introduced into the coronary artery to measure the lesion's effect on coronary blood flow using a method called fractional flow reserve (FFR). It has been established that the routine use of FFR in patients with multivessel IHD (and only intervening on those lesions causing greater than 20% reduction in maximal flow) improves patient outcomes compared to relying on the angiogram alone [19].

Using tiny guide wires, intravascular imaging equipment can also be introduced into the coronary arteries, allowing detailed radial and longitudinal visualisation of the artery. This provides information on vessel size, plaque burden and composition and can also be used to assess and optimise the results of percutaneous coronary interventions (PCI). The two most common intracoronary imaging modalities are intravascular ultrasound (IVUS) and optical coherence tomography (OCT).

When the data obtained at a diagnostic coronary angiogram is correlated with that from non-invasive and/or invasive functional assessments, it provides a wealth of information on the extent and effects of a patient's coronary disease and helps physicians decide on the optimal treatment.

33.4 Primary Versus Secondary Prevention

The term "primary prevention" refers to delaying or, ideally, preventing the onset of disease. Typically, this is approached through lifestyle and risk factor modification such as weight loss, exercise, dietary modification, smoking cessation and, where necessary, medication to treat associated medical conditions such as hypertension, dyslipidaemia and diabetes.

Secondary prevention is focused on preventing progression of established disease and includes all of the interventions above in addition to more invasive treatments such as PCI and coronary artery bypass graft (CABG) surgery.

33.4.1 Medications

Medications remain a cornerstone of treatment for IHD.

- Antiplatelet drugs such as aspirin which inhibit the formation of blood clots are a first-line treatment of IHD. Following myocardial infarction and coronary stent implantation, a second antiplatelet agent (clopidogrel, prasugrel or ticagrelor) is recommended for up to 12 months in addition to aspirin [20].
- Statins (simvastatin, atorvastatin, pravastatin, rosuvastatin) are a class of drug which reduce the body's production of cholesterol [21].
- Beta blockers (metoprolol, bisoprolol, carvedilol, atenolol) are used to regulate heart rate and blood pressure and have antianginal properties. They are also employed in the management of cardiac arrhythmias [22, 23].
- Angiotensin-converting enzyme (ACE) inhibitors (ramipril, lisinopril, perindopril, enalapril, captopril) and angiotensin II receptor blockers (ARBs) (valsartan, telmisartan, candesartan, losartan) treat high blood pressure and congestive cardiac failure. They have multiple effects, but among the most important is the dilation of systemic arteries which reduces blood pressure and oxygen demand from the heart.
- Glyceryl trinitrate (GTN) produces an antianginal effect by causing vasodilation of both systemic and coronary blood vessels. It can be administered under the tongue via a buccal spray, in tablet form or as a transdermal patch.
- Other antianginal drugs include ranolazine, nicorandil and ivabradine which each have varying individual mechanisms of action.

33.4.2 Percutaneous Coronary Intervention (PCI)

In patients with stable angina whose symptoms are not adequately controlled by optimal medical therapy, treatment of a haemodynamically significant coronary stenosis with PCI is recommended and can potentially improve both their symptoms and prognosis [24]. Diagnostic coronary angiography and assessment is performed as detailed above. If a lesion suitable for PCI is identified, the operator will pass a very fine guide wire down into the coronary artery and through the area of stenosis or obstruction to the distal vessel beyond. Using the wire as a guide rail, balloon catheters can be advanced into the coronary artery to stretch open the area of stenosis or occlusion. With flow restored or improved, an appropriately sized coronary stent can be deployed to the diseased portion of the artery to maintain its patency (Fig. 33.1).

Coronary stents are produced in an array of diameters and lengths and are fabricated using a number of different metals and drugs which confer variable characteristics allowing for tailoring of stent selection based on patient factors. However, the basic method of deployment is common to all. The stent is a tubular, mesh framework of cells (not unlike a roll of chicken wire) that is mounted on a cylindrical balloon. This is delivered to the area of the coronary artery requiring treatment, and, by inflating the balloon, the stent is expanded to its prespecified diameter inside the artery. After deployment, the stent is

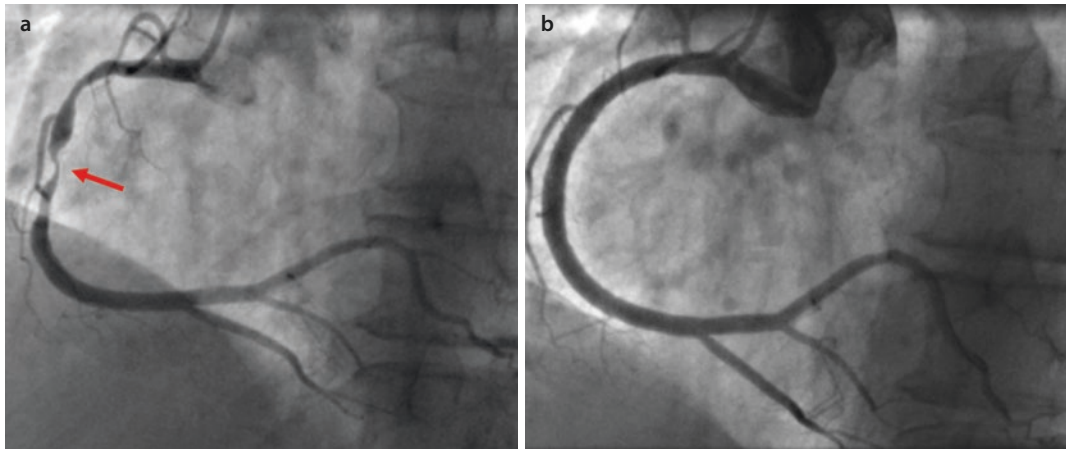

Fig. 33.1 Coronary angiograms. Panel (**a**) shows a severe stenosis in the mid portion of the right coronary artery (arrow), while panel (**b**) shows the final result following PCI and stenting of the vessel

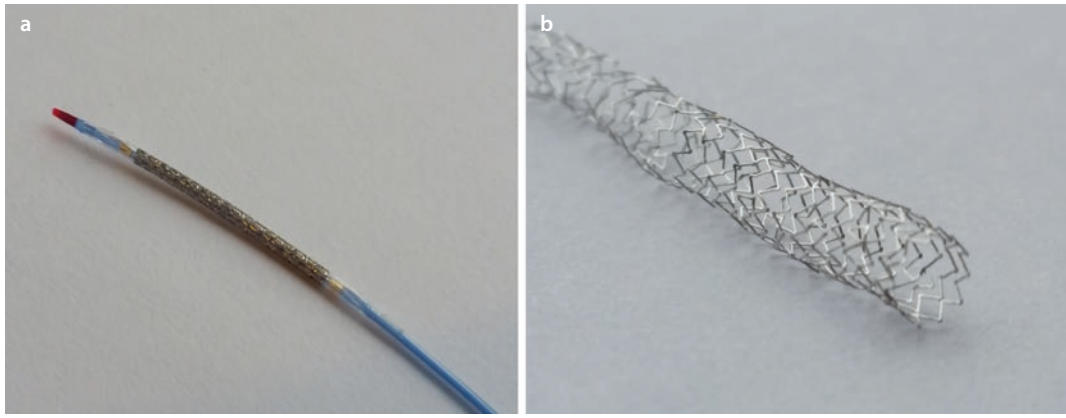

Fig. 33.2 Undeployed stent mounted on a balloon catheter (Panel **a**). Expanded stent post balloon inflation, illustrating the cell structure and tubular mesh framework (Panel **b**)

usually optimised by performing post-dilation to high pressure with a non-compliant balloon to ensure good apposition to the vessel wall (Fig. 33.2).

33.4.3 Surgical Revascularisation

Prior to the advent of PCI, coronary artery bypass grafting (CABG) surgery was the only revascularisation option available. It is a major surgical procedure during which the chest cavity is opened by cutting through the breastbone (sternum) and blood vessels (veins or arteries) harvested from other areas of the body are attached (grafted) to the coronary arteries, distal to the areas of stenosis, so as to provide an alternative conduit for blood to flow into the native vessel. While CABG surgery can obtain durable, long-term results (particularly in patients with diabetes and complex multi-vessel disease), there are significant morbidity and mortality risks associated with the procedure, and patient selection is important. Nowadays, it is recommended that decisions on whether to offer a patient with complex coronary artery disease revascularisation with surgery or multi-vessel PCI should be made by consensus at multidisciplinary heart team meetings [24]. Patients who are deemed too high risk for surgery may often be suitable for PCI, while patients who require concurrent heart valve surgery, have anatomy unfavourable for PCI or have developed

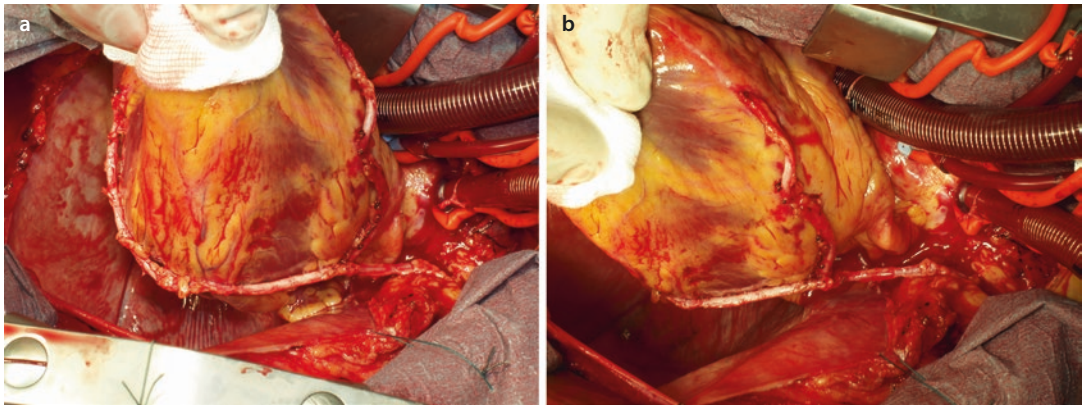

Fig. 33.3 Intraoperative photographs of coronary artery bypass grafting surgery demonstrating vein grafts attached to the exterior surface of the heart. (Images courtesy of Prof. Nawwar Al-Attar)

issues with recurrent in-stent restenosis may be better served by surgery. Occasionally, some patients will undergo staged hybrid procedures combining the two modalities (Fig. 33.3).

33.5 Acute Coronary Syndrome (ACS)

ACS usually arises as a result of a myocardial infarction. A patient is typically diagnosed with a myocardial infarction if two (probable) or three (definite) of the following criteria are satisfied: (1) history of ischaemic-type chest pain lasting more than 20 min, (2) changes in serial ECG tracings and (3) rise and fall of serum cardiac biomarkers on blood testing (most commonly a protein called troponin).

ACS is broadly classified into three types based on the patient's initial ECG appearances and subsequent troponin levels:

1. ST segment elevation myocardial infarction (STEMI, troponin positive).
2. Non-ST segment elevation myocardial infarction (NSTEMI, troponin positive).
3. Unstable angina (troponin negative) (Fig. 33.4).

STEMI requires emergency treatment, ideally with primary percutaneous coronary intervention (PPCI), or thrombolysis if PPCI is not available within an appropriate time frame (the current recommendation is within 120 min) [25]. PPCI involves an urgent coronary angiogram to establish the location of the coronary stenosis or occlusion followed by PCI and stenting of the culprit lesion.

Thrombolysis involves administration of a fibrinolytic ("clot-busting") medication with the aim of breaking down the blood clot obstructing flow in the coronary artery. It is a systemic treatment and can be complicated by severe bleeding, so, again, appropriate patient selection is vital, and there are a number of relative and absolute contraindications to its use. In approximately one third of patients, thrombolysis fails to result in reperfusion, and so-called rescue PCI is performed as an emergency procedure.

NSTEMI is usually managed medically in the first instance with antiplatelet, anticoagulant and antianginal therapies. Current guidelines recommend that diagnostic coronary angiography (with PCI or CABG as appropriate) should be performed within the first 24 h following presentation for high-risk patients and within 72 h for all others, assuming that co-morbidities do not preclude an invasive treatment strategy [20]. Very occasionally, patients with NSTEMI are so medically unstable that they need to follow an emergency care pathway.

Unstable angina with no biomarker evidence of myocardial infarction can usually be managed successfully with optimal medical therapy. Angiography is not mandatory but is used if patients have ongoing symptoms or non-invasive evidence of prognostically important ischaemia.

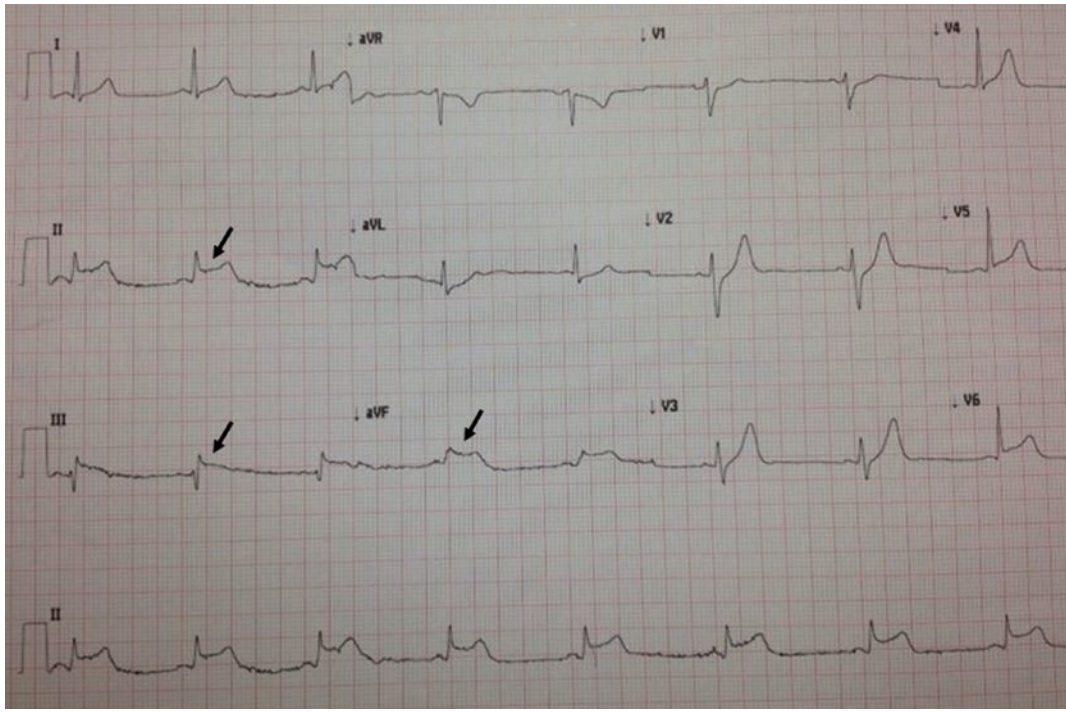

Fig. 33.4 Electrocardiogram (ECG) showing ST segment elevation (arrows) in leads II, III and aVF, consistent with an inferior ST segment elevation myocardial infarction (STEMI)

Conclusion and Clinical Perspective

IHD remains a leading cause of death in the United Kingdom, but encouragingly the actual number of deaths attributed to the condition reduced by approximately 45% between the years 2000 and 2016 [1]. While the explanation for this change is multifactorial, there can be little doubt that advances in medical therapies and devices, improved pathways of care for patients with acute coronary syndromes and dedicated public health initiatives and policies have all played important roles in reducing the burden of disease. Hopefully, with continued research and innovation, we will see this trend continue.

Gaps in Knowledge

- Will the identification of genetic predictors of IHD allow for implementation of targeted personalised prevention therapies earlier in life?
- What will be the role of lipid-lowering and anti-inflammatory therapies based on monoclonal antibodies or microRNA modification in primary and secondary prevention treatment strategies?
- Will highly reversible antithrombotic therapies (or agents with specific titratable antagonists) become the new standard of care?
- Can we develop personalised therapies in which the doses, combinations and durations of treatment are determined for each individual patient on the basis of clinical characteristics, genetic profiling and biomarkers?
- Can the benefits of these newer therapies be provided to low- and middle-income countries at affordable costs?

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Pre-eclampsia

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_34

Key Concepts

- Pre-eclampsia is a leading cause of maternal morbidity and mortality worldwide.
- Pre-eclampsia is characterised by hypertension and generalised endothelial dysfunction; multiple organs including kidneys, liver and brain can be affected.
- Anti-angiogenic placental factors including sFLT-1 and PlGF are promising diagnostic biomarkers and may be key to pathogenesis.
- A history of pre-eclampsia is an important risk factor for future cardiovascular disease.

34.1 Introduction

Traditional medical student teaching dictates that pre-eclampsia has a single cause, the pregnancy itself, and a single cure, delivery. Well over a century after its first description, it could perhaps be argued that our understanding of, and our ability to manage, pre-eclampsia has progressed little. To this day pre-eclampsia, a multi-system characterised by hypertension and proteinuria, continues to affect 2–5% of pregnant women in the Western world.

The Confidential Enquiry into Maternal Deaths and Morbidity (UK) showed that only 2 women died from pre-eclampsia and eclampsia in the UK between 2012 and 2014, a reduction from 19 and 10 deaths in 2006–2008 and 2009–2011, respectively. This reduction largely relates to improved obstetric care [1]. These improvements in mortality, however, have not been matched on a global level; pre-eclampsia continues to cause over 40,000 annual deaths, equating to 5 deaths per hour worldwide. In addition it is now known that the effects of pre-eclampsia are not confined to the pregnancy and that the disease can continue to affect mother and baby well after the pregnancy.

There is therefore a need, more than ever, to improve our ability to predict, to diagnose and to treat this major condition. In this chapter we will explore what is known about the origins of the disease, will review research that has been undertaken and will discuss the relationship with future cardiovascular diseases.

34.2 Definitions of Disease

To understand the impact of a disease, we must first be able to accurately diagnose it. Diagnosing pre-eclampsia can be difficult, particularly since definitions of pre-eclampsia have changed over the years and continue to be different throughout the world. The UK National Institute of Clinical Excellence (NICE) defines pre-eclampsia as new-onset hypertension occurring after 20 weeks of gestation, with significant proteinuria (urinary protein/creatinine ratio greater than 30 mg/mmol or 24-h urine protein greater than 300 mg). Oedema, often seen in affected women, is no longer part of the diagnostic criteria. Whether proteinuria itself even needs to be present for diagnosis is also for debate; the 2017 American College of Obstetricians and Gynecologists (ACOG) guidelines no longer necessitate proteinuria, permitting pre-eclampsia to be diagnosed if new-onset hypertension is accompanied by signs or symptoms of significant end-organ dysfunction such as neurological disturbance, renal dysfunction or pulmonary oedema.

Pre-eclampsia can also be defined by the time of its recognised onset. Early-onset pre-eclampsia is usually defined as being diagnosed before 34 weeks of gestation, or requiring delivery before 37 weeks of gestation, and is often considered to be a more severe variant of the disease. It should be noted, however, that although pre-eclampsia by definition is diagnosed after 20 weeks of gestation, the disease process begins earlier in pregnancy, and subclinical manifestations of the disease may be detectable before 20 weeks.

Pre-eclampsia is in many ways a condition of generalised endothelial dysfunction, with variable organ involvement. Disease severity can be defined by the presence of end-organ dysfunction, such as neurological or renal involvement, or by blood pressure readings above 160 mmHg systolic or 110 mmHg diastolic. “HELLP” syndrome (haemolysis, elevated liver enzymes, low platelets) and eclampsia, the development of grand mal seizures in a woman with pre-eclampsia, are also considered to represent severe forms of disease.

Gestational hypertension is part of the pre-eclampsia spectrum of disease and refers to hypertension occurring after 20 weeks of gestation in the absence of significant proteinuria or end-organ dysfunction, while chronic hypertension either precedes pregnancy or is diagnosed before 20 weeks of gestation.

34.3 Risk Factors

34.3.1 Pre-existing Disease

Pregnancy puts an enormous strain on a woman's physiology, and as pregnancy progresses, nearly every organ is affected by an increasing fetal and placental demand. Women in the Western world are now conceiving at an older age with both an increased prevalence of underlying medical diseases and predisposition to disease, through obesity, cigarette smoking and other factors. As a result they are often unable to keep up with the increased demand, leading to adverse outcomes of which pre-eclampsia is the most prominent. Many of the underlying diseases commonly encountered are themselves associated with an increased risk of pre-eclampsia. Pre-existing type 1 diabetes is associated with an up to fourfold increased risk of developing pre-eclampsia. Type 2 diabetes, now increasingly encountered in women of child-bearing age, is associated with a similar risk. Chronic kidney disease is associated with a twofold increased risk of pre-eclampsia, although in women with chronic proteinuria, it can be difficult to diagnose superimposed pre-eclampsia. A history of chronic hypertension or autoimmune diseases such as anti-phospholipid syndrome and systemic lupus erythematosus also put women at two- to threefold increased risk.

34.3.2 Other Factors

Nulliparity has long been known to be a major risk factor for the development of pre-eclampsia; women having their first baby are at almost three times the risk of developing pre-eclampsia compared to parous women. A long interval between pregnancies and a pregnancy with a new partner have also been traditionally reported to be associated with an increased risk. More recently, however, partner change has been shown to also have a potentially protective influence in women with a history of early-onset pre-eclampsia, suggesting a paternal role and that certain partners may be more or less favourable for successful placentation [2]. Other more established risk factors for pre-eclampsia are listed in the table below. A body mass index of >30 kg/m^2 increases risk of pre-eclampsia, and the risk doubles with each 5–7 kg/m^2 increase in BMI [3]. Increasing maternal age is also a risk factor; the risk begins to rise from the age of 34 years, while women with multiple pregnancy, or with a family history in their mother or sister are also at increased risk.

34.4 Prevention

Delivery is the only known cure for pregnancy, and management of women with early-onset disease requires a balance between the risks associated with early delivery and the risks of elevated blood pressure. An intervention that could help to prevent pre-eclampsia would therefore be of enormous clinical benefit.

34.4.1 Aspirin

Low-dose aspirin is used extensively in cardiovascular diseases and has been studied extensively in the context of preventing pre-eclampsia. The rationale for its use is that impaired trophoblastic invasion in early pregnancy is thought to lead to activation of platelets and the clotting system, leading to an imbalance of the ratio between thromboxane A2 and prostacyclin. Low-dose aspirin, as opposed to higher doses, can diminish thromboxane synthesis while maintaining prostacyclin synthesis. Multiple studies and meta-analyses have shown that in women at increased risk for pre-eclampsia, low-dose aspirin given from 12 weeks of gestation can reduce the risk of preterm and severe pre-eclampsia, preterm births and intrauterine growth restriction. As such, NICE, the World Health Organization (WHO) and ACOG recommend its use in women with risk factors as outlined in Table 34.1.

Table 34.1 From the National Institute for Clinical Excellence (NICE) Quality Statement on Antenatal Assessment of pre-eclampsia risk, 2013

"High"-risk factors	"Moderate"- risk factors
Previous hypertensive disease in pregnancy	First pregnancy
Chronic kidney disease	Age 40 years or older
Autoimmune disease, e.g. SLE, APS	Pregnancy interval of >10 years
Type 1 or 2 diabetes	Body mass index ≥35 kg/m^2
Chronic hypertension	Family history of pre-eclampsia
	Multiple pregnancy

Women are considered at increased risk if they have one high-risk factor or more than one medium-risk factor

34.4.2 Calcium and Vitamin D Supplementation

Calcium supplementation has been extensively studied over the years, since it has long been noted that areas with high dietary calcium intake had lower rates of pre-eclampsia. Multiple studies over the years have been undertaken; while calcium supplementation has been shown to be of benefit in areas with dietary deficiencies, its effects are limited in areas with normal dietary calcium intake. Vitamin D is a key determinant of calcium metabolism and is thought to play a role in reducing oxidative stress and increasing vascular endothelial growth factor (VEGF) gene transcription. Although low levels of Vitamin D are associated with an increased risk of pre-eclampsia, a recent meta-analysis failed to show a clear relationship between vitamin D supplementation and disease risk [4].

34.4.3 Diet and Exercise

The role of weight loss in overweight and obese women has been studied in recent years, and small studies of women having had bariatric surgery prior to pregnancy appear to reduce the risk of pre-eclampsia. The role of exercise is also important- historically women were advised to increase calorific intake and reduce physical exercise in pregnancy. While studies showing a benefit of exercise in preventing pre-eclampsia have been inconsistent, the Royal College of Obstetricians and Gynaecologists (RCOG), in keeping with other national bodies, recommends that pregnant women undertake 30 min of moderate exercise 4–7 times weekly.

Statin therapy has been proposed as a preventative measure for prevention of pre-eclampsia based upon preclinical studies using pravastatin but remains contraindicated in pregnancy. Metformin, a predominantly insulin-sensitising agent commonly used to treat gestational diabetes in the UK, has also been studied for its effects on pre-eclampsia. When used in overweight and obese mothers along with dietary and lifestyle interventions, it has a modest effect on maternal gestational weight gain but no effect on pre-eclampsia or other pregnancy outcomes [5]. Other potential preventative measures include anti-oxidants (vitamins C and E and fish oils), dietary salt restriction and anticoagulants; despite initial promising studies, these measures have not been shown in large meta-analyses to be of overall benefit.

34.5 Predictive Biomarkers

As outlined above, a number of maternal risk factors can be used to identify which women might be at increased risk of developing pre-eclampsia. The problem with these risk factors, particularly obesity, maternal age and nulliparity, is that as well as being largely unmodifiable, they are very common in the overall pregnant population and have a low predictive value. As a result extensive work has been undertaken in recent years to attempt to identify biomarkers that can be used to predict pre-eclampsia prior to the onset of clinically detectable disease.

Uric acid levels remain the most common blood test used by obstetricians to detect pre-eclampsia. Although levels correlate with disease severity and with the presence of adverse pregnancy outcomes, it is of limited benefit in predicting the onset of disease. Other markers of inflammation including CRP, markers of abnormal lipid metabolism and markers associated

with the renin-angiotensin-aldosterone (RAAS) pathway have similarly been shown to be of limited benefit in predicting disease.

34.5.1 Anti-angiogenic Factors

Attention has turned, therefore, to markers of angiogenesis, the formation of new blood vessels. Abnormal angiogenesis is associated with increased capillary permeability and endothelial damage, which are pathological hallmarks of pre-eclampsia development. Several studies in recent years have shown that pre-eclampsia is associated with increased levels of anti-angiogenic factors, such as soluble FMS-like tyrosine kinase 1 (sFLT-1) and soluble endoglin (sEng), and with reduced levels of pro-angiogenic factors such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF). PlGF in particular has been highlighted as the most promising biomarker currently available for early prediction of disease. PlGF levels in the second trimester are lower in women who go on to develop pre-eclampsia compared to controls. Meta-analysis showed that PlGF levels are significantly lower before 30 weeks of gestation but are less reliable when measured before 16 weeks, a time point when they would be more clinically useful [6]. The sFLT-1: PlGF ratio can also be used as a predictive biomarker, with a recent meta-analysis of 15 studies revealing a sensitivity of 80% and specificity of 92% in predicting pre-eclampsia, a positive likelihood ratio of 10.5 (95% confidence interval, 6.2–18.0) and a negative likelihood ratio of 0.22 (95% confidence interval, 0.13–0.35) in both high-risk and low-risk patients [7]. These biomarkers, however, along with other predictive markers, have yet come into routine clinical practice; which women to test and at what point in pregnancy to test remain unanswered questions.

34.6 Pre-eclampsia as a Placental Disorder?

Traditional teaching dictates that the origins of pre-eclampsia lie in the placenta. A number of factors support this theory – since pre-eclampsia only occurs in pregnancy, it can occur in the absence of a viable foetus, for example, in molar pregnancy, and since it resolves after delivery of the placenta. In normal pregnancy the spiral arteries transform into high-flow, low-resistance vessels allowing supply of nutrition and oxygen to the developing foetus. In pre-eclampsia this transformation, which starts as early as the time of implantation, is impaired. Trophoblastic invasion is restricted to the peripheral spiral arteries, remodelling is incomplete, and the spiral arteries remain highly resistant. Blood supply to the foetus is therefore restricted, and as pregnancy progresses, the uterine vessels are unable to keep up with fetal demands. It is thought that the resultant stressed and under-perfused placenta releases a number of factors into the maternal circulation, leading to an exaggerated maternal inflammatory response.

Placental pathological changes such as atherosis, thrombosis and focal necrosis are well described in the literature. The placental origins of pre-eclampsia can be detected clinically, using the ever-improving field of ultrasound. Impaired remodelling of the spiral arteries can be detected by the persistence of a diastolic "notch" in the uterine arcuate vessels and by elevated pulsatility index. The utility in predicting pre-eclampsia has been extensively reported and appears to be most reliable when undertaken in the second trimester, particularly in high-risk women. Although non-invasive, uterine artery Doppler studies, however, are time-consuming and are associated with a high false-positive rate, with increased associated healthcare costs and potential patient anxiety. Their reliability for disease prediction on their own may be limited, and when added to a model of traditional risk factors, they do not appear to improve disease prediction [8]. When combined, however, with PlGF, maternal risk factors and clinical factors such as blood pressure, it is reported that 75% of cases of early-onset pre-eclampsia can be detected in the first trimester, with a false-positive rate of 10% [9]. Although these figures are promising, the majority of research in the uterine artery Doppler field has been undertaken in large centres with high levels of expertise, and whether they are as applicable in smaller hospitals remains unknown.

34.7 Pre-eclampsia as a Cardiovascular Disorder?

More recently, there has been an increasing argument that rather than being a fundamentally placental disorder, pre-eclampsia is in fact a cardiovascular disease. Pre-eclampsia has similar risk factors to cardiovascular diseases, as well as shared potential therapies including aspirin and shared underlying pathological processes. Women with a family history of myocardial infarction are at increased risk of pre-eclampsia, as are women with elevated lipid levels. One proposed theory is that women destined to develop pre-eclampsia already have impaired generalised endothelial function and that this in turn leads to impaired placental perfusion and its associated problems.

There are also arguments against pre-eclampsia being a placental disorder; the placental pathological abnormalities and Doppler ultrasound appearances outlined above are most closely associated with early-onset pre-eclampsia, itself usually in association with fetal growth restriction. Eighty to 90% of cases of pre-eclampsia, however, are late-onset, when the placental changes are less well described [10]. Doppler findings reported in pre-eclampsia may in fact be indicative of generalised vascular upset and can be detected in other vascular beds; Doppler of the ophthalmic artery, for example, is reported to be abnormal in women with pre-eclampsia, perhaps as early as the first trimester.

34.8 Future Maternal Cardiovascular Disease

Further evidence for the cardiovascular nature of pre-eclampsia is its link with future cardiovascular disease. As outlined above pregnancy puts an enormous strain on maternal physiology. Even without complications, having multiple pregnancies is itself associated with cardiovascular disease; women with six or more pregnancies have been reported to have an increased risk of future coronary heart disease compared to nulliparous women, even after correction for maternal obesity and metabolic risk factors.

Women with pre-eclampsia are often described as having failed the "stress test" of pregnancy, indicating subclinical disease and a predisposition to future metabolic syndrome. The association between pre-eclampsia and future maternal health was first described in a population from Aberdeen, UK, nearly 60 years ago. This study showed that women with a history of pre-eclampsia were at increased risk of going on to develop hypertension in later adult life, findings that have been replicated in multiple studies since then. The more recent linkage of birth records with registers of morbidity, mortality and hospitalisation has allowed several meta-analyses to examine the relationship between pre-eclampsia and future maternal health and to quantify that risk.

In general women with a history of pre-eclampsia have at least double the risk of coronary artery disease compared to women with normotensive pregnancies, and similar risks are reported for future peripheral vascular disease and cerebrovascular disease.

Renal involvement is key to diagnosis of pre-eclampsia, and it is perhaps not surprising that women with a history of pre-eclampsia are also at increased risk of kidney disease in later life. Epidemiological studies have shown that a history of pre-eclampsia is associated with an increased risk of requiring a renal biopsy in later life and a nearly fivefold increased risk of developing end-stage renal disease compared to those with normotensive pregnancies [11]. Glomerular endotheliosis is a hallmark of pre-eclampsia, but since renal biopsy is rarely undertaken in pregnancy, establishing the natural history can be difficult. The renal changes associated with pre-eclampsia resemble focal segmental glomerulosclerosis, highlighting the need for careful follow-up of renal function in the years after a pre-eclamptic pregnancy.

As outlined above, there can be significant variation in the onset and severity of pre-eclampsia, and this in turn affects their long-term cardiovascular risk. Women with early-onset pre-eclampsia are reported to have an increased long-term cardiovascular risk compared to those with late-onset disease, while having a low birth weight baby, severe disease or disease occurring in recurrent pregnancies appears to increase the long-term risk further still.

Whether a pregnancy complicated by pre-eclampsia affects the maternal vasculature leading

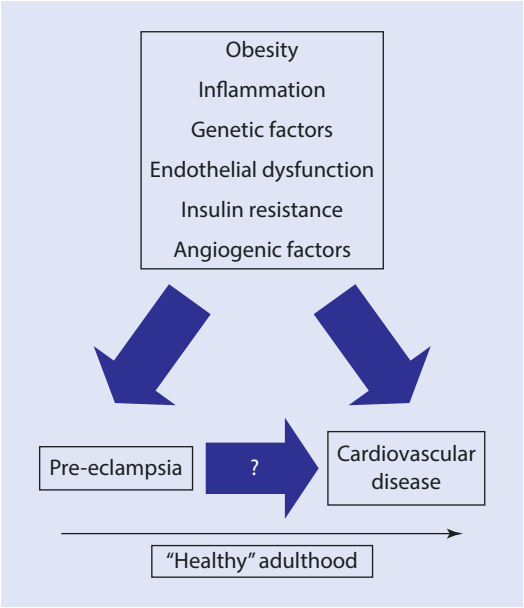

Fig. 34.1 Adapted from Carty et al. [11]

to future problems, perhaps mediated by angiogenic factors such as sFLT-1, or whether a pregnancy simply unmasks underlying predisposition to cardiovascular risk, remains unknown (Fig. 34.1). Whatever the interaction, it is vital that communication between healthcare professionals improves. After a pregnancy, care of the mother and baby will revert back to the primary care physician, who may not appreciate or act upon the long-term risks of pre-eclampsia. Communication is vital to ensure that proteinuria resolves; that blood pressure, lipids and other cardiovascular risk factors are addressed; and that women have appropriate counselling if further pregnancy is desired.

34.9 Fetal Risk

Pre-eclampsia is associated with a heritability of around 50%, and both sisters and daughters of affected women are known to be at increased risk of themselves developing the condition. As well as shared genetic risk, however, it has been proposed that anti-angiogenic placental factors released into the maternal circulation will also cross into the fetal circulation, adversely affecting the fetal vasculature, leading to future hypertensive disorders [12]. Supporting this hypothesis, increased pulmonary artery pressure and impaired flow-mediated dilatation have been demonstrated in children born to a pregnancy affected by pre-eclampsia – siblings born to normotensive pregnancies were not affected [13]. Animal studies also support this concept; when pregnant mice are injected with an adenovirus carrying sFLT-1, sustained hypertension is seen in the offspring, evident from the 1st day of life [14]. Renal dysfunction and impaired sympatho-adrenal response have also been suggested as potential mechanisms for increased offspring risk. These findings have parallels with the fetal programming hypothesis, where a stressful intrauterine environment is thought to contribute to future adverse health in the offspring.

Conclusions and Clinical Perspectives

Even with all the advances in modern medicine, pre-eclampsia continues to be a major source of maternal morbidity and mortality and to be the subject of major scientific research. Many diseases nowadays are defined by their underlying pathological basis. In contrast pre-eclampsia is defined by the hypertension and proteinuria that occur in late pregnancy; it is clear that it is a heterogeneous condition with a number of different underlying pathophysiological mechanisms. One school of thought is that there are two distinct diseases. The first "placental" disease begins in early pregnancy, affects women without underlying medical conditions and causes an early-onset disease, with placental dysfunction and a generally more severe phenotype. The second "maternal" disease affects women who are more likely to have underlying medical conditions and is associated with late-onset less severe disease, with less of a significant role for the placenta. In reality this distinction probably oversimplifies the condition, and for the majority of women, there is an overlap between the two entities. Similarly, the placental versus cardiovascular argument is likely to continue. In gestational diabetes, the maternal pancreas fails to keep up with demands of pregnancy; does pre-eclampsia signify pre-existing subclinical disease with an inability of the maternal cardiovascular system to keep up with these demands [15]? An ideal study would be one in which women are studied before they embark upon pregnancy, to identify

whether pre-pregnancy, subclinical signs of disease can be identified and used to stratify pre-eclampsia risk.

At present the only effective proven preventative measure for pre-eclampsia that can be offered is aspirin, with calcium supplementation for women who are deficient. It is hoped that by improving our understanding of underlying pathophysiological mechanisms, we will be able to better predict the disease and to offer better treatments to our patients.

34.10 Summary

Gaps in Knowledge

- Can women who are at risk of developing pre-eclampsia be identified by vascular or biomarker testing before pregnancy?
- What is the optimal method for diagnosing pre-eclampsia before the onset of clinical symptoms?
- How should pre-eclampsia be treated; what are the benefits of interventions including early delivery versus expectant management?

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Stable Coronary Syndromes

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_35

Key Concepts

- Angina pectoris may result from epicardial (macrovascular) coronary stenosis and disorders of coronary vascular function (microvascular disease and endothelial dysfunction).
- Invasive and non-invasive diagnostic tests allow for comprehensive disease endotyping and provide clinically relevant insights into patients with microvascular and vasospastic angina.
- Disease-modifying therapeutic options are limited in patients with disorders of coronary vascular function.

35.1 Introduction

Ischaemic heart disease (IHD) is the leading global cause of death and lost life years in adults. Stable IHD (also referred to as stable coronary artery disease (CAD)) describes the syndrome of recurrent, transient episodes of chest pain reflecting demand-supply mismatch, i.e. angina pectoris. In clinical practice, the diagnostic management of patients with angina pectoris focuses on the detection of obstructive epicardial CAD. In this stenosis-centred concept of myocardial ischaemia, angina is synonymous with obstructive CAD [1]. There are well-established treatment options for patients with epicardial CAD, namely, optimal medical therapy and myocardial revascularisation by either percutaneous coronary intervention or coronary artery bypass grafting [1]. However, the paradigm of angina pectoris resulting only from obstructive epicardial CAD fails to account for the approximately one third of patients who suffer from angina in whom obstructive epicardial stenosis is excluded [2].

The underlying aetiology of chest pain symptoms and a 'negative' coronary angiogram is varied. Coronary microvascular dysfunction (CMD) and/or coronary vasospasm are potential causes of angina and nonobstructive coronary artery disease (ANOCA). These patients present a diagnostic and therapeutic challenge [3]. Management is heterogeneous, and many patients go undiagnosed, receive no further work-up and are left untreated.

In this chapter, we review the causes of ischaemia based on the underlying coronary pathophysiology. We focus on disorders of coronary vascular function, their clinical significance and the potential for stratified medicine to bring future benefits to patients. The investigation and management of patients with obstructive epicardial CAD have been discussed in ▶ Chap. 33.

35.2 The Clinical Conundrum of Angina

The traditional diagnostic work-up of patients with angina pectoris focuses on the detection of obstructive epicardial CAD alone (◘ Fig. 35.1). In patients with ANOCA or refractory angina following revascularisation of epicardial CAD, myocardial ischaemia secondary to a disorder of coronary vascular function may be relevant. In routine clinical practice, specific disease endotypes are not usually tested for.

Microvascular dysfunction may result from coronary structural abnormalities, whereby decreased capillary luminal size and number result in increased microvascular resistance to myocardial blood flow and reduced vasodilatory capacity [4]. Functional abnormalities of the coronary epicardial vessels and microvasculature may result in either abnormal vasoconstriction or impaired vasodilatation, and these abnormalities may be secondary to either endothelium-dependent or endothelium-independent mechanisms [5]. These conditions are clinically relevant, as abnormalities of coronary vascular function portend a worse prognosis in patients with both obstructive epicardial CAD and ANOCA [6]. Therapeutic interventions are lacking in patients with ANOCA, and historical therapeutic studies have been performed in heterogeneous patient cohorts due to a lack of diagnostic tests to appropriately define endotypes of disease [7, 8].

The term stable coronary syndrome (SCS) describes a clinically relevant classification that incorporates disorders of epicardial and microvascular coronary circulation (◘ Fig. 35.2) [3]. Microvascular dysfunction and vasospastic disease may result in ANOCA and myocardial ischaemia and are recognised as a condition of unmet clinical need [9]. Rather than interrogating the epicardial coronary compartment alone in patients presenting with angina, comprehensive testing strategies incorporating tests of coronary pressure, flow, resistance and endothelial function, in addition to

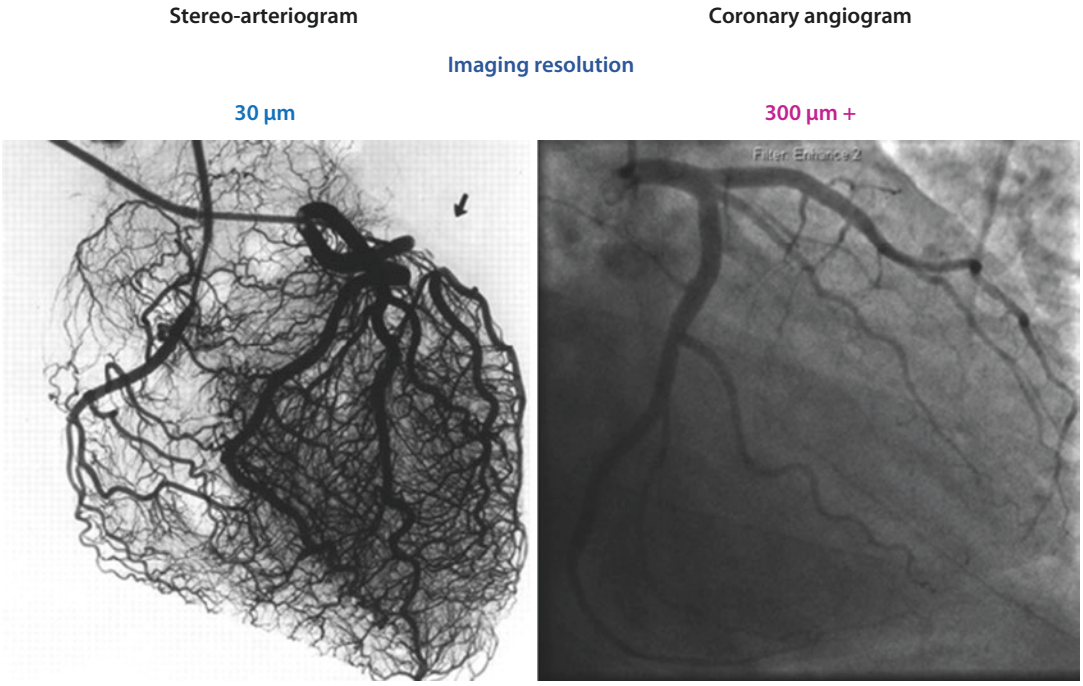

Fig. 35.1 Ex vivo coronary stereo-arteriogram. Invasive coronary angiographic cine still image (right) in comparison to an ex vivo stereo-arteriogram image (left). (William Fulton, MD thesis, University of Glasgow, 1963)

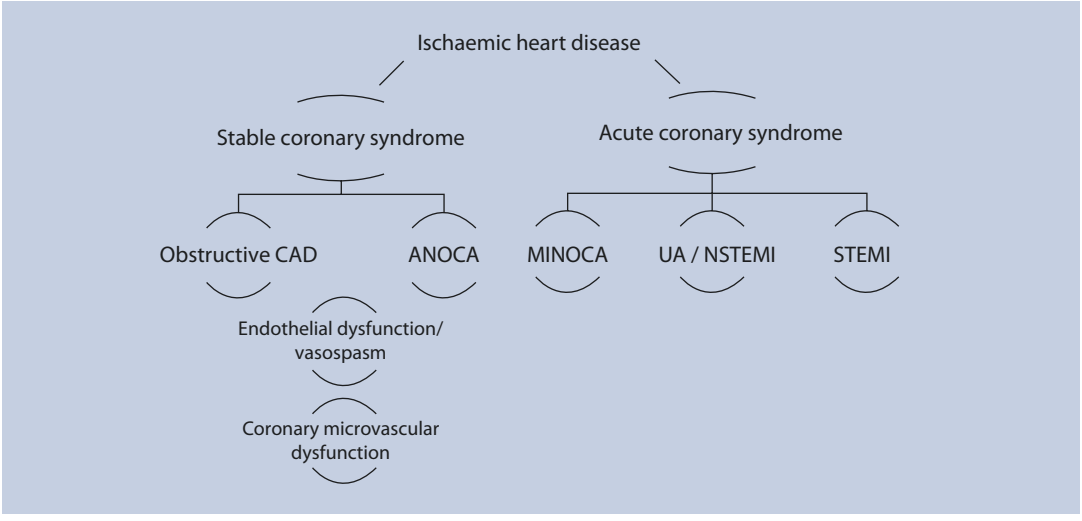

Fig. 35.2 Taxonomy of ischaemic heart disease. (Adapted from Berry et al. [7]). *ANOCA* = angina and nonobstructive coronary artery disease, *MINOCA* = myocardial infarction with nonobstructive coronary arteries, *UA* = unstable angina, *NSTEMI* = non-ST-elevation myocardial infarction, *STEMI* = ST-elevation myocardial infarction

the assessment of objective evidence of myocardial ischaemia. These invasive tests provide clinically relevant insights [10]. However, there remains a missing link between the use of diagnostic tests of coronary vascular function, therapeutic agents with proven efficacy and health outcomes of patients with angina secondary to disorders of coronary vascular function.

35.3 Pathophysiology of Coronary Vascular Dysfunction

Coronary vascular dysfunction may result from several pathophysiological conditions. Exclusion of epicardial coronary abnormalities other than stenosis (such as fistulae and anomalous vessels) is usually apparent from the invasive coronary angiogram. Abnormalities of coronary vascular function may be considered as either structural or functional, and both may result in reduced myocardial blood flow and angina, due to either increased microvascular resistance, impaired vasodilatation or inappropriate vasoconstriction.

Traditional major risk factors for atherosclerotic epicardial CAD (i.e. smoking, hypertension, dyslipidaemia and diabetes mellitus) are also implicated in coronary vascular dysfunction and are prevalent in patients with ANOCA. However, many patients with ANOCA do not have risk factors for vascular disease, and other pathophysiological factors (e.g. adipocyte dysfunction and inflammation) may be relevant but have not yet been fully elucidated.

35.3.1 Anatomical Abnormalities in the Coronary Circulation

Coronary vascular dysfunction may result from coronary structural abnormalities [4]. Decreased capillary luminal size and number result in increased microvascular resistance to myocardial blood flow (as per Poiseuille's law). These structural changes have been demonstrated in patients with arterial hypertension and hypertrophic cardiomyopathy, and these conditions are frequently cited as exemplar of microvascular dysfunction. Both conditions result in pathological left ventricular hypertrophy (LVH), and although the relative resting myocardial blood flow per gram of myocardium may remain constant, the increases in left ventricular mass necessitate an increase in the absolute level of resting flow (ml/min) through the coronary artery [11]. Therefore, LVH results in reduced CFR for a given arterial blood pressure, with an inverse relationship between CFR and LVH, and patients with both conditions may present with ANOCA secondary to coronary microvascular dysfunction [12].

Anatomical diagnosis of coronary vascular dysfunction is limited, and there is no diagnostic test that enables direct visualisation of coronary microcirculation in vivo. Invasive coronary angiography has a resolution of ~500 μm, and therefore the pre-coronary arterioles and arterioles are not visualised. Endomyocardial biopsy includes vessels less than 200 μm, therefore not sampling larger microvasculature. Additionally, only those vessels in the endocardial surface that can be reached by endomyocardial bioptome and the sample may not be representative of the global microvasculature. In small studies of heterogeneous patient cohorts, structural microvascular abnormalities including capillary rarefaction, perivascular fibrosis and myocardial hypertrophy have been demonstrated. However, myocardial biopsy is not a feasible diagnostic option particularly as disease-modifying therapies are lacking. Therefore, currently, the diagnosis of CMD is empirical when specific tests of coronary function are not used.

35.3.2 Functional Microvascular Abnormalities

Functional abnormalities of the epicardial arteries and microvessels relate to (1) enhanced vasoconstriction, (2) impaired vasodilatation secondary to endothelium-independent or endothelium-dependent mechanisms and (3) a combination of these disorders. Disorders of coronary vasomotion include epicardial and/or microvascular coronary spasm, impaired coronary artery vasorelaxation and endothelial dysfunction-related reduced myocardial blood flow [13]. Various vasoactive substances may be implicated (e.g. endothelin-1 concentrations are elevated in patients with microvascular dysfunction).

The coronary endothelium regulates vascular tone and myocardial blood flow via nitric oxide (NO)-dependent mechanisms [12]. Abnormal vasoconstrictive responses to acetylcholine infusion, consistent with impaired endothelial function, occur in patients with angina and nonobstructive epicardial CAD. Abnormal endothelium-independent vasodilator function may involve resistance to NO, adenosine and prostacyclin.

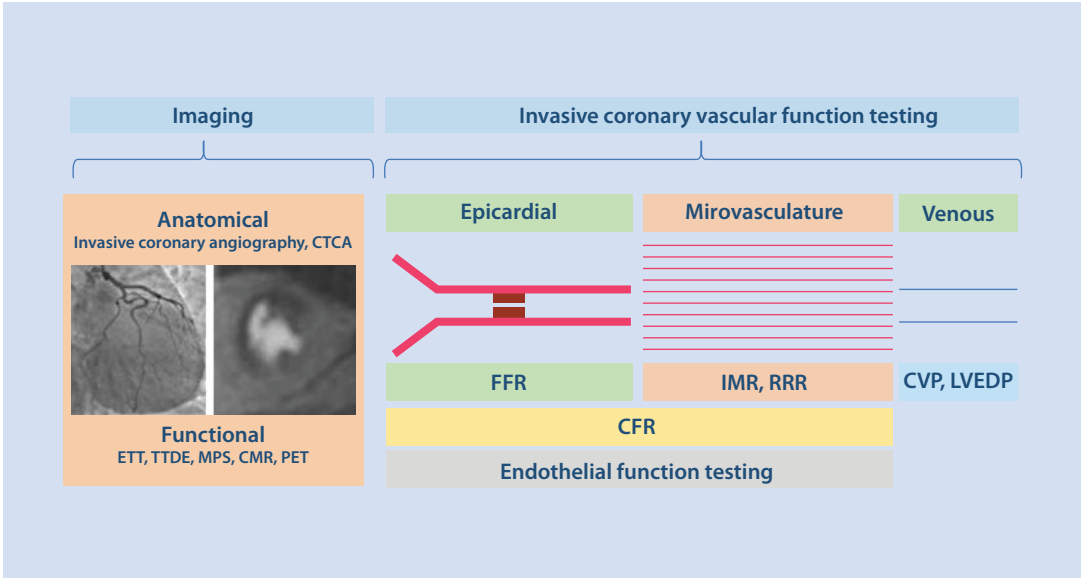

Fig. 35.3 Summary of the available diagnostic tests for coronary vascular dysfunction. *CTCA* = cardiac tomography coronary angiography, *ETT* = exercise treadmill testing, *TTDE* = transthoracic Doppler echocardiography, *MPS* = myocardial perfusion scintigraphy, *CMR* = cardiac magnetic resonance imaging, *PET* = positron emission tomography, *FFR* = fractional flow reserve, *IMR* = index of microcirculatory resistance, *RRR* = resistance reserve ratio, *CFR* = coronary flow reserve, *CVP* = central venous pressure, *LVEDP* = left ventricular end-diastolic pressure

35.4 Diagnosis of Coronary Vascular Dysfunction

Diagnosis of coronary microvascular and vasomotor dysfunction is challenging due to the limited spatial resolution of current diagnostic tests, the patchy distribution of disease throughout the myocardium and the heterogeneity of underlying disease processes. There is no available in vivo technique for imaging the coronary microcirculation, and anatomical tests are fundamentally limited by their spatial resolution and the small size of the coronary microvasculature. Therefore, the diagnosis of microvascular angina and vasospastic angina is predominantly made with functional tests (Fig. 35.3).

The COVADIS group have published a guideline for the diagnosis (rule-in / rule-out) of microvascular angina and vaspastic angina [10]. Invasive coronary angiography combined with adjunctive tests of coronary vascular function represents the reference diagnostic approach for disorders of coronary vascular function. In contrast, non-invasive imaging involves less discomfort for patients, is safer than invasive procedures and is generally less expensive and more widely available. Recent developments with cardiovascular magnetic resonance (CMR) imaging now enable measurement of myocardial blood flow with high spatial and temporal resolution [14]. A summary of the disease endotypes in patients presenting with angina is shown in Table 35.1.

35.4.1 Invasive Assessment of Coronary Vascular Function

Invasive testing is the reference standard for assessing coronary vascular dysfunction. The first step in the diagnosis of ANOCA is to exclude epicardial disease. Traditionally this is performed with visual interpretation of invasive coronary angiography alone. However, invasive physiological assessment to determine the functional significance of epicardial stenosis is recommended as anatomical-physiological mismatch is frequent (i.e. a significant number of lesions may be incorrectly classified as functionally significant or not on the assessment of percentage diameter stenosis alone) [15]. The use of invasive diagnostic tests at the time of coronary angiography allows comprehensive assessment of coronary vascular function [16].

Table 35.1 Summary of stable coronary syndrome disease endotypes

Disease endotype	Mechanism	Invasive diagnostic test
Microvascular angina	↑ Microvascular resistance	IMR ≥25
	↓ Coronary vasorelaxation	CFR <2.0
	↓ Microvascular vasodilator capacity	RRR <2.0
	Microvascular spasm	ACh testing: Angina, ischaemic ST segment deviation, epicardial coronary vasoconstriction <90%
Vasospastic angina	Epicardial spasm	ACh testing: Angina, ischaemic ST segment deviation, >90% epicardial coronary vasoconstriction
Mixed microvascular and vasospastic angina	CMD and epicardial vasospasm	Epicardial vasospasm and either ↑ microvascular resistance, ↓ coronary vasorelaxation or ↓ microvascular vasodilator capacity
Obstructive epicardial CAD	Epicardial stenosis	>50% lesion by diameter stenosis in epicardial artery >2.5 mm or FFR ≤0.80
Noncardiac pain	Nil	Exclusion epicardial (FFR >0.8), microvascular (CFR >2.0, IMR <25, RRR >2.0) vasospasm (normal ACh response)

The Coronary Vasomotion Disorders International Study Group (COVADIS) working group diagnosis requires (1) symptoms of myocardial ischaemia ((a) effort and/or rest angina and/or (b) angina equivalents (i.e. shortness of breath)), (2) absence of obstructive epicardial CAD (<50% diameter reduction or FFR >0.80), (3) objective non-invasive evidence of myocardial ischaemia and (4) evidence of impaired coronary microvascular function. Definitive microvascular angina is only diagnosed if all four criteria are present and probable if only three criteria are met [10]
IMR index of microcirculatory resistance, *CFR* coronary flow reserve, *RRR* relative resistance ratio, *CAD* coronary artery disease, *FFR* fractional flow reserve, *ACh* acetylcholine

Fractional flow reserve (FFR) is an invasive pressure-based test which assesses the functional significance of an epicardial stenosis. Other pressure-derived indices, such as fractional flow reserve (FFR), contrast-enhanced FFR, non-hyperaemic pressure ratio (NHPR), may be used to guide revascularisation decisions. Further discussion of the investigation of epicardial CAD is made in ▶ Chap. 33.

The coronary wire, used to measure FFR, may be simultaneously used to interrogate the microvasculature (by indicator thermodilution). This allows for a more complete assessment of a patient's coronary vascular function during invasive coronary angiography and can aid in the differentiation of symptoms due to focal or diffuse epicardial disease, microvascular disease or both [17]. Invasive coronary vascular function testing allows for specific disease endotypes to be diagnosed, namely, with testing of microvascular resistance (index of microcirculatory resistance, IMR), microvascular vasodilatory capacity (resistance reserve ratio, RRR) and epicardial and microvascular vasodilatory capacity (coronary flow reserve, CFR). Equivalent metrics may also be measured using intracoronary Doppler wire interrogation.

In addition to coronary pressure, flow and resistance, a comprehensive invasive coronary function testing protocol should include interrogation of endothelial function [18]. This is most commonly performed with intracoronary acetylcholine provocation, testing for endothelial dysfunction and vasospasm [19]. These tests may guide management, and prognostic information is obtained [6, 20]. These metrics are considered further in ▶ Chap. 25.

35.4.2 Non-invasive Assessment of Coronary Vascular Function

The available non-invasive ischaemia tests were all validated for the detection of obstructive epicardial CAD. Traditional non-invasive isch-

aemia tests (e.g. exercise electrocardiogram testing, myocardial perfusion scintigraphy and stress echocardiography) have poor diagnostic accuracy to detect inducible ischaemia in patients with disorders of coronary vascular function. However, non-invasive methods, namely, stress perfusion positron emission tomography (PET) and CMR which image earlier in the ischaemic cascade and have greater spatial resolution, may provide new insights into the burden of myocardial ischaemia. The reference-standard non-invasive assessment of myocardial blood flow is stress PET imaging, which permits quantitative flow derivation in ml/g/minute. In real-world practice, the use of PET is limited by its availability (including radioisotopes), cost and exposure to ionising radiation. CMR imaging holds most promise as a preferred non-invasive imaging option. Although CMR is also comparatively expensive, it has clear benefits including lack of ionising radiation, high spatial resolution, high sensitivity and specificity for perfusion abnormalities and multi-parametric imaging techniques (reference standard LV volumes and function, myocardial tissue characterisation with late gadolinium enhancement imaging and parametric mapping) [21].

Coronary microvascular disease may be revealed by a deficit in myocardial blood flow during stress CMR. The spatial distribution of this abnormality typically involves the subendocardium, which is the location of the microvascular plexus. In contrast, vasospastic angina occurs due to spontaneous spasm of the epicardial and microvascular vasculature. Vasospastic angina may not be detected by conventional stress testing which routinely use adenosine (an endothelial-independent vasodilator). Microvascular disease may be a generalised process resulting in diffuse myocardial perfusion abnormalities rather than gross defects as seen in obstructive epicardial CAD. Therefore, traditional non-invasive ischaemia tests may be normal in patients with CMD due to the absence of regional perfusion abnormalities typically seen in obstructive CAD. The relationships between the reference standard invasive metrics of coronary vascular function and non-invasive estimates of myocardial blood flow are uncertain and an area of current research.

35.5 Therapeutic Agents for Coronary Vascular Dysfunction

The optimal management strategies in patients with ANOCA or confirmed coronary vascular dysfunction are undefined. There is a lack of adequately powered studies investigating the effect of therapeutic agents on long-term health outcomes and no routinely used treatment algorithm. The available evidence base largely comes from small randomised studies with variable inclusion criteria, differing diagnostic test thresholds for inclusion, and there are discordant results [22]. The treatment effect of these studies is likely diluted by the enrolment of heterogeneous groups of patients with distinct endotypes of coronary vascular dysfunction, and therapies have predominantly been investigated in patient cohorts without a precise disease endotype coronary vascular dysfunction.

Therapeutics in coronary vascular dysfunction may be considered as symptom-modifying (i.e. non-endotype specific) or disease-modifying agents (i.e. endotype specific). In current practice, symptom-modifying therapy with standard antianginal agents and secondary prevention is empirical and based on the paradigm of evidence-based therapies for obstructive epicardial CAD [15]. Of equal importance is the proportion of patients with ANOCA who may have their antianginal therapy discontinued following exclusion of obstructive epicardial CAD and may therefore have an untreated and significant symptom burden.

35.5.1 Pharmacological Symptomatic Therapy

Clinical practice guidelines recommend ß-blockers as first-line therapy (class 1) [1], and ß-blocker therapy is associated with reduced angina burden and improved ischaemic threshold. A calcium channel blocker should be considered if ß-blockade is not tolerated or is not symptomatically beneficial (class I). In patients with proven vasospastic angina, calcium channel blockers are associated with reduced vasospastic episodes and may have prognostic benefit. Oral nitrates result in venodilatation and coronary vasodilatation. Unlike in patients with angina and obstructive CAD, oral nitrate therapy may paradoxically result in worsening myocardial

ischaemia in patients with ANOCA. Third-line symptomatic agents (nicorandil, ranolazine, ivabradine) have also been found to have symptomatic benefit in patients with ANOCA.

35.5.2 Disease-Modifying Therapies

There is a lack of evidence for endotype-specific therapy in patients with ANOCA [9], but novel therapeutics are being investigated: (1) endothelin is a potent vasoconstrictor, and its abnormalities in the endothelin pathway are associated with microvascular dysfunction. Abnormal upregulation of endothelin-mediated vasoconstriction may be associated with abnormal myocardial perfusion heterogeneity, and endothelin receptor antagonists are being investigated in patients with microvascular dysfunction [23]. (2) The Rho-kinase signalling pathway is implicated in abnormal vasoconstriction in patients with vasospastic angina. Targeted Rho-kinase inhibition may reduce myocardial ischaemia in patients with vasospastic angina [24]. (3) Phosphodiesterase-5 inhibitors block the cGMP-specific phosphodiesterase type 5 enzyme, thereby increasing cGMP availability. This mechanism promotes vasodilatation via the NO-soluble guanylate cyclase signalling pathway, and phosphodiesterase-5 inhibitors are being investigated in ANOCA patients.

35.5.3 Secondary Prevention

Analogous to obstructive epicardial CAD, treatment of modifiable vascular risk factors is an important adjunct to disease-specific therapy in patients with ANOCA [15]. There are no specific therapeutic agents for the secondary prevention of coronary vascular dysfunction, but therapeutics validated for epicardial CAD are often used empirically. Clinical practice guidelines support the use of secondary prevention with aspirin and statin therapy [1]: (1) intravascular ultrasound imaging studies in patients with coronary microvascular dysfunction demonstrate that most have evidence of epicardial atherosclerosis, and therefore aspirin is recommended [16]. (2) Statin therapy reduces cardiovascular risk via low-density lipoprotein reduction but has pleiotropic effects including improvements in vascular inflammation and enhanced endothelial function. In patients with ANOCA, statin therapy has been associated with improved exercise tolerance, enhanced endothelial function and improved coronary flow reserve. (3) Angiotensin II is a potent vasoconstrictor formed by ACE from angiotensin I. Angiotensin II may modulate coronary microvascular tone. ACEi are associated with reduced angina burden, enhanced endothelial function and improved CFR in patients with ANOCA. ACEi improve endothelial dysfunction and vasoreactivity via NO stimulation helping to reverse vascular hypertrophy and improve vascular compliance.

Conclusion and Clinical Perspective

- Angina pectoris may result from epicardial (macrovascular) coronary stenosis and disorders of coronary vascular function (microvascular disease and endothelial dysfunction).
- The role of diagnostic testing of coronary vascular dysfunction in patients with angina and stable coronary syndromes is expanding. The stratified medicine approach to a disease describes the identification of specific disease endotypes within a heterogeneous population, and diagnostic testing in ANOCA patients allows for patients to be carefully phenotyped.
- Personalising care for patients should target therapy to the underlying disease endotypes with novel disease-modifying agents, rather than the current dogma of empirical therapy. This approach also applies to patients with refractory anginal symptoms following revascularisation.

Gaps in Knowledge

- There is a need for an evidence-based diagnostic algorithm to guide clinicians in the work-up of patients with disorder of coronary vascular function.
- There are uncertain relationships between the results of invasive diagnostic tests and the differing non-invasive ischaemia methods and their relation to downstream therapeutic agents and long-term health outcomes.
- Large adequately powered health outcome trials are needed to determine whether hard morbidity and mortality endpoints may be improved by targeted therapy.

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Heart Failure with Reduced Ejection Fraction

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_36

Key Concepts

- Heart failure with reduced ejection fraction is driven by the complex interplay between a number of haemodynamic, neurohumoral and cellular pathophysiological processes.
- Neurohumoral modulation forms the basis of treatment for the condition.
- Novel therapeutic approaches targeting the natriuretic peptide system have opened up new avenues for treatment.
- In a number of patients, device therapy can further improve prognosis.

36.1 Introduction

Heart failure is defined as a clinical syndrome of signs and symptoms that occur as a consequence of reduced cardiac output or raised intracardiac pressures [1]. It results from a series of pathophysiological processes that are triggered by structural or functional changes to the myocardium. The prevalence of heart failure is 1–2% in the developed world and doubles with each decade of life; amongst those 80 years or older, more than 10% have heart failure [2]. Worldwide, it is estimated that 37.7 million are living with the condition [2]. Amid the many classifications of heart failure, left ventricular ejection fraction provides a means of discerning whether the disease is systolic or diastolic in nature. Over half of patients with heart failure have left ventricular systolic dysfunction, or heart failure with reduced ejection fraction (HF-REF), which in clinical practice refers to patients with a left ventricular ejection fraction of 40% or less. HF-REF carries with it a high burden of morbidity and mortality, despite marked progress in managing the condition. This chapter focuses on the pathophysiology of chronic HF-REF and the current evidence-based treatments available.

36.2 Aetiology and Pathophysiology

In every case, the primary cardiac insult driving the disease process should be sought and, in some cases, may be reversible. Coronary artery disease accounts for the underlying aetiology in approximately 60–70% of patients in clinical trials over the past decade. Other causes include diabetes, hypertension, primary valvular disease, arrhythmias, cardiomyopathies secondary to systemic diseases and inherited cardiomyopathies. Dilated cardiomyopathy can be either genetic or acquired, the latter resulting from external factors such as alcohol, viral infection and drugs (e.g. cardiotoxic chemotherapy).

In response to a decline in stroke volume, haemodynamic and neurohumoral mechanisms attempt to provide compensatory support to the malfunctioning heart. While initially effective in maintaining cardiac output, over time these are maladaptive and, if left untreated, lead to pathological remodelling of the left ventricle with dilatation and increased sphericity, worsening of cardiac function and development of the heart failure syndrome. Cardiac dilatation is a marker of poor prognosis in HF-REF, and, conversely, regression of left ventricular remodelling is associated with improved outcomes for such patients [3].

During the early stages of left ventricular systolic dysfunction, the interplay between left ventricular end-diastolic volume and stroke volume, depicted by the Frank-Starling curve, results in preservation of cardiac output [4]. However, as the degree of cardiac dysfunction worsens and contractility declines, the myocardium is unable to generate the force required to maintain an adequate stroke volume despite an increase in left ventricular end-diastolic volume. Eventually, the end-diastolic volume expands to such a point that any further increase in stroke volume is blunted and the Frank-Starling curve plateaus before eventually falling.

Systemic responses to a reduction in cardiac output ensue, most notably activation of neurohumoral pathways. A resultant surge of catecholamines, following activation of the sympathetic nervous system, has direct effects on the heart to increase heart rate and contractility, and on the peripheral vasculature to cause vasoconstriction, thereby maintaining tissue perfusion [5]. The renin-angiotensin-aldosterone system (RAAS) is triggered by both the sympathetic nervous system and by renal hypoperfusion. Ultimately, increased levels of angiotensin II lead to vasoconstriction, sodium and chloride reabsorption in the renal tubules, the production of aldosterone from the adrenal gland and vasopressin secretion from the posterior pituitary gland [5]. The neurohumoral pathways are illustrated in Fig. 36.1.

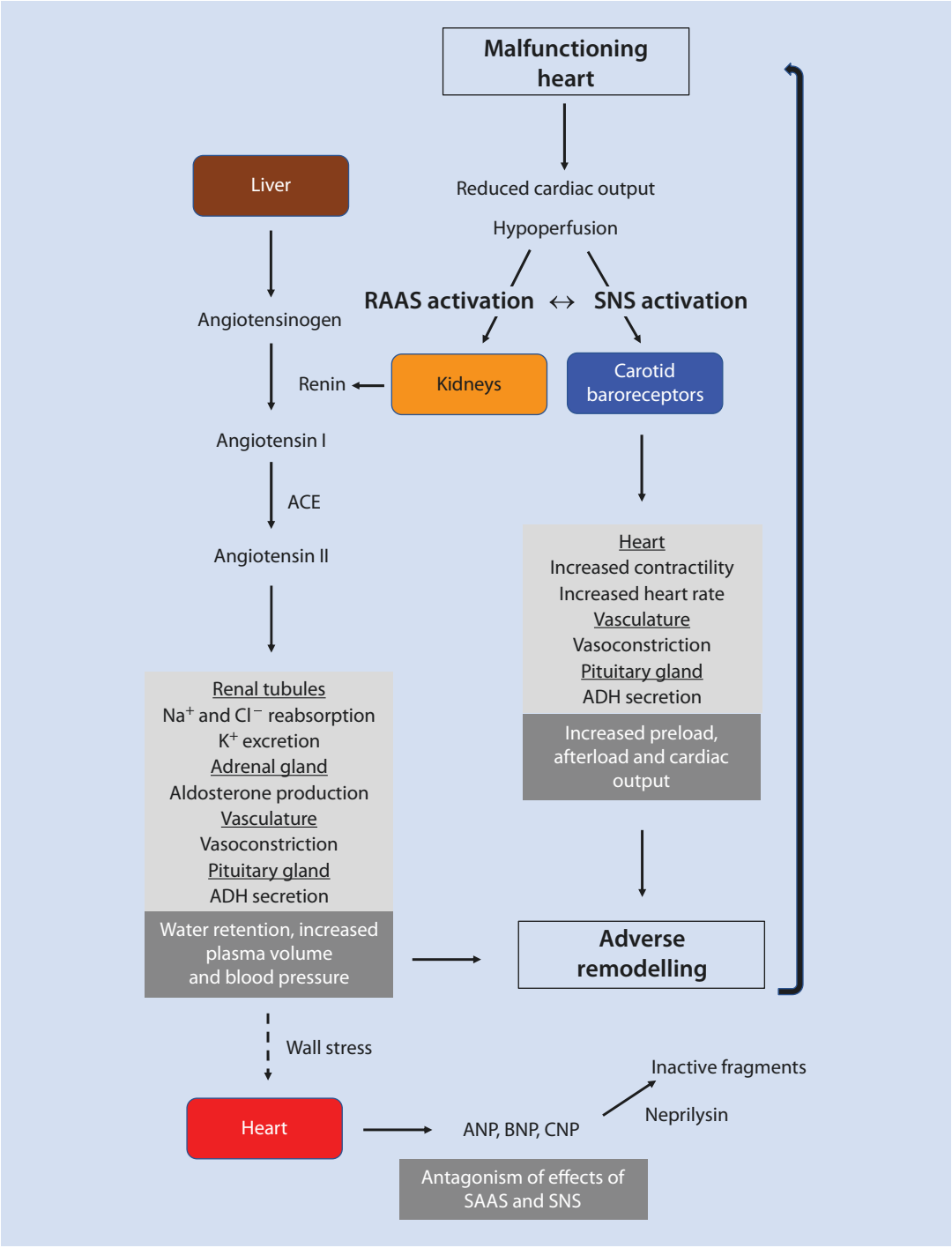

Fig. 36.1 Neurohumoral pathways in HF-REF. *RAAS* renin-angiotensin-aldosterone system, *SNS* sympathetic nervous system, *ACE* angiotensin-converting enzyme, *ADH* antidiuretic hormone, *ANP* A-type natriuretic peptide, *BNP* B-type natriuretic peptide, *CNP* C-type natriuretic peptide

At a histological level, myocytes and other cardiac cell types undergo a series of changes induced by the initial insult to the heart. Structural transformations attempt to compensate for overall loss of contractile function and maintain cardiac output. This process of cellular remodelling

encompasses hypertrophy, apoptosis, fibroblast proliferation and interstitial fibrosis [3]. Factors other than those related to the RAAS and sympathetic nervous system that are thought to contribute to cardiac remodelling include endothelin, cytokines, nitric oxide, oxidative stress and mitochondrial damage [6].

In HF-REF, the maladaptive upregulation of both the RAAS and the sympathetic nervous system is counterbalanced by the natriuretic peptide system. Sodium and water retention, resulting from higher levels of angiotensin II, aldosterone and circulating catecholamines, leads to expansion of the left ventricular end-diastolic volume and increased wall stress [7]. In response, pre-proB-type natriuretic peptide is released and cleaved to B-type natriuretic peptide (BNP: the active peptide) and N-terminal proBNP (NT-proBNP: the inert by-product). BNP exerts its effects through promotion of natriuresis and vasodilatation [7]. A-type natriuretic peptide, which has similar properties to BNP, and C-type natriuretic peptide, released from endothelial cells, are also important in protecting against the effects of volume overload [7].

Uninhibited, the sympathetic and RAAS pathways have detrimental systemic effects that manifest clinically as congestion, lethargy, arrhythmias, progressive pump failure and, eventually, death. Over the last 30 years, pharmacological modification of these deleterious processes, and more recently augmentation of those that are beneficial, has resulted in a substantial reduction in mortality, as well as improvements in symptom burden and quality of life. Median survival for patients with heart failure is steadily improving [8].

36.3 Diagnosis

Clinical assessment should prompt further workup in patients with suspected HF-REF. Symptoms such as breathlessness and fatigue and signs such as peripheral oedema, pulmonary oedema and elevated jugular venous pressure are cardinal features of the disease. Investigations are used to determine the presence and severity of cardiac dysfunction, to discern the underlying aetiology, to establish the presence of end-organ dysfunction and to identify relevant comorbidities such as anaemia, diabetes and renal impairment [1]. Patients with suspected HF-REF should undergo routine investigation with an electrocardiogram, biochemical and haematological blood tests (to identify potentially reversible causes and also sequelae of cardiac dysfunction) and natriuretic peptide levels [1]. A number of co-existing clinical conditions can impact natriuretic peptide levels; for example, chronic kidney disease is associated with an elevated concentration of these biomarkers, while the opposite is true for people with obesity. However, a normal natriuretic peptide level excludes a diagnosis of heart failure in the majority of patients. Chest radiography is often undertaken and may provide information about pulmonary congestion and heart size, but it is unable to detect changes in cardiac function. Abnormalities of clinical assessment, electrocardiography or natriuretic peptide testing should prompt further corroborative investigation. Assessment of cardiac structure and function using transthoracic echocardiography will confirm the diagnosis, provide quantification of disease severity and expose other abnormalities such as the presence of left ventricular thrombi and valvular disease [1]. Cardiac magnetic resonance imaging can, in most cases, distinguish ischaemic from non-ischaemic aetiologies and is particularly useful when tissue characterisation is important, such as in myocarditis or infiltrative cardiomyopathies [1]. Other tests might be used primarily to establish the aetiology of left ventricular systolic dysfunction; these include stress echocardiography, myocardial perfusion imaging, coronary angiography (using computed tomography or invasive techniques) or specific biochemical blood tests such as thyroid function, creatine kinase and ferritin [1].

36.4 Treatment

Pharmacotherapy is the mainstay of treatment in HF-REF and has been shown to be effective at lessening symptom burden, enhancing quality of life, reducing hospitalisation and improving survival. For select patients, implantable devices or surgical intervention may be beneficial. Advanced heart failure management with mechanical circulatory support (such as ventricular assist device) is generally reserved for younger patients with no contraindications to cardiac transplantation, or for those in whom recovery of left ventricular systolic function is expected. Positive randomised drug and device trials in HF-REF are summarised in Table 36.1.

Table 36.1 Landmark randomised trials of drug and device therapy showing benefit in HF-REF (placebo-controlled unless specified otherwise)

Trial name and year by drug class	Number of patients	NYHA class	Treatment	Follow-up (years)	Primary endpoint	Relative risk reduction (%)
ACE inhibitors						
CONSENSUS, 1987 [14]	253	IV	Enalapril 20 mg bd	0.5	Death	40
SOLVD-T, 1991 [15]	2569	II–IV	Enalapril 20 mg bd	3.5	Death	16
V-HeFT II, 1991 [16]	804	–	Enalapril 10 mg bd vs hydralazine 75 mg qds/ISDN 40 mg tds	2.3	Death	28
ATLAS, 1999 [48]	3164	II–IV	Lisinopril 32.5–35 mg od vs lisinopril 2.5–5.0 mg od	3.8	Death or hospitalisation	12
ARBs						
CHARM-Alternative, 1999 [18]	2028	II–IV	Candesartan 32 mg od	2.8	CV death or HF hospitalisation	23
Val-HeFT, 2001 [17]	5010	II–IV	Valsartan 160 mg bd	1.9	CV death or morbidity	13
CHARM-Added, 2003 [19]	2548	II–IV	Candesartan 32 mg od	3.4	CV death or HF hospitalisation	15
HEAAL, 2009 [49]	2846	II–IV	Losartan 150 mg od vs losartan 50 mg od	4.7	Death or HF hospitalisation	10
Beta-blockers						
CIBIS-II, 1999 [20]	2647	III–IV	Bisoprolol 10 mg od	1.3	Death	34
MERIT-HF, 1999 [21]	3991	II–IV	Metoprolol CR/XL 200 mg od	1.0	Death	34
COPERNICUS, 2001 [22]	2289	IV	Carvedilol 25 mg bd	0.9	Death	35
COMET, 2003 [50]	3029	II–IV	Carvedilol 25 mg bd vs metoprolol 50 mg bd	4.8	Death	17
SENIORS, 2005 [51]	2128	II–IV	Nebivolol 10 mg od	1.8	Death or CV hospitalisation	14
MRAs						
RALES, 1999 [24]	1663	III–IV	Spironolactone 25 mg od	2.0	Death	30

(continued)

Table 36.1 (continued)

Trial name and year by drug class	Number of patients	NYHA class	Treatment	Follow-up (years)	Primary endpoint	Relative risk reduction (%)
EMPHASIS-HF, 2011 [25]	2737	II	Eplerenone 50 mg od	1.8	CV death or HF hospitalisation	37
ARNIs						
PARADIGM-HF, 2014 [26]	8399	II–IV	Sacubitril-valsartan 200 mg bd vs enalapril 10 mg bd	2.3	CV death or HF hospitalisation	20
Vasodilators						
V-HeFT, 1986 [29]	459	–	Hydralazine 75 mg qds/ISDN 40 mg qds	2.3	Death	34
A-HeFT, 2004 [30]	1050	III–IV	Hydralazine 75 mg tds/ISDN 40 mg tds	0.8	Composite score	–
Digitalis glycosides						
DIG, 1997 [31]	6800	II–IV	Digoxin (individualised dose)	3.1	Death	0
HCN channel blockers						
SHIFT, 2010 [32]	6558	II–IV	Ivabradine 7.5 mg bd	1.9	CV death or HF hospitalisation	18
ICDs						
SCD-HeFT, 2005 [52]	1676	II–III	ICD	3.8	Death	23
MADIT-II, 2002 [41]	1232	I–III	ICD	1.7	Death	31
CRT						
COMPANION, 2004 [42]	1520	III–IV	CRT-P vs OMT	1.2	Death or hospitalisation	19
			CRT-D vs OMT			20
CARE-HF, 2005 [43]	813	III–IV	CRT vs OMT	2.5	Death or CV hospitalisation	37
MADIT-CRT, 2009 [44]	1820	1-II	CRT-D vs ICD	2.4	Death or nonfatal HF event	34
RAFT, 2010 [45]	1798	II–III	CRT-D vs ICD	3.3	Death or HF hospitalisation	25

NYHA New York Heart Association, *od* once daily, *bd* twice daily, *tds* three times daily, *qds* four times daily, *CV* cardiovascular, *HF* heart failure, *ISDN* isosorbide dinitrate, *HCN* hyperpolarisation-activated cyclic nucleotide-gated, *ICD* implantable cardioverter defibrillator, *CRT* cardiac resynchronisation therapy, *CRT-P* CRT-pacemaker, *CRT-D* CRT-defibrillator, *OMT* optimal medical therapy

Initiation and escalation of therapy is guided by both the degree of left ventricular systolic dysfunction and to what extent the patient is symptomatic. Symptoms are quantified using the New York Heart Association (NYHA) functional classification – a system used in almost all randomised controlled trials in HF-REF and also in clinical practice. Patients with no symptoms are classified as NYHA class I, while patients with symptoms at rest or on slight exertion are classified as NYHA class IV. Those with mild and moderate symptoms are classified as NYHA class II and III, respectively.

The care of patients with HF-REF is best coordinated by a multidisciplinary team. Involvement of specialist heart failure nurses has been shown to reduce hospital admissions, shorten hospital stays and decrease mortality [9]. Education is crucial and should support the patient and carers in understanding the disease process, recognising signs of clinical deterioration and adhering to treatment.

36.4.1 Treatment of Symptoms

Diuretics are used to alleviate fluid retention, which in turn leads to a reduction in breathlessness and peripheral oedema. Diuretic dose can be increased or decreased depending on clinical response, and the goal is to render the patient euvolaemic. Regular weights can be useful to help guide dosing adjustments. The preferred class of diuretic in HF-REF is a loop diuretic such as furosemide or bumetanide. These drugs act on the ascending limb of the loop of Henle in the renal nephron to inhibit the reabsorption of sodium and chloride ions. A thiazide diuretic such as bendroflumethiazide or metolazone can be added to a loop agent to augment diuresis in cases of refractory congestion. These drugs act in a similar fashion to a loop diuretic but target the distal convoluted tubule of the renal nephron. If the combination of a loop and thiazide diuretic is used, close monitoring of renal function and serum electrolyte levels is required because of the risk of derangement of both. In patients with oedema resistant to oral treatment, hospital admission for a course of intravenous diuretic therapy may be required. There is evidence to suggest that a strategy of continuous infusion confers no more benefit than that of a bolus regime in this situation [10].

36.4.2 Disease-Modifying Agents

36.4.2.1 Angiotensin-Converting Enzyme Inhibitors

For many years, angiotensin-converting enzyme (ACE) inhibitors have formed the foundation of HF-REF treatment. ACE inhibitors slow the rate of ventricular dilatation, promote small increases in ejection fraction and improve functional capacity [6, 11, 12]. They are also effective at reducing progression to heart failure, heart failure hospitalisation and death in patients with asymptomatic left ventricular systolic dysfunction and should therefore be used regardless of NYHA class [13]. Two large randomised controlled trials in patients with NYHA class II–IV symptoms showed that treatment with enalapril resulted in a 16–40% reduction in the risk of death when compared to placebo [14, 15]. ACE inhibitors have also been shown to be more effective than the combination of hydralazine-isosorbide dinitrate at reducing death in a head-to-head trial in men with heart failure (relative risk reduction of 28%) [16].

36.4.2.2 Angiotensin Receptor Blockers

Angiotensin receptor blockers (ARBs) should be used in patients with unacceptable side-effects from ACE inhibitors, including cough or angioedema, which occur in a dose-independent manner. The mortality benefit of ARBs is comparable to that of ACE inhibitors. In one of the earliest large trials of ARB use in chronic heart failure, valsartan did not reduce mortality compared to placebo when added to standard therapy in patients with NYHA class II–IV symptoms, 93% of whom were already treated with an ACE inhibitor [17]. For the combined mortality and morbidity endpoint, treatment with valsartan led to a 13% relative reduction in risk. In a post hoc subgroup analysis of patients being treated with both an ACE inhibitor and a beta-blocker, there was suggestion of a trend towards harm with the addition of valsartan, prompting a further dedicated study to address this concern. Shortly afterwards, a programme of trials examined the efficacy of candesartan used as an alternative to an ACE inhibitor or added to an ACE inhibitor in patients with HF-REF [18, 19]. These showed that an ARB in place of an ACE inhibitor resulted in a benefit

of similar magnitude to that seen in the former enalapril trials (23% reduction in risk of cardiovascular mortality and heart failure hospitalisation) [18]. The combination of ARB plus ACE inhibitor resulted in a further 15% reduction in cardiovascular mortality and heart failure hospitalisation but with higher rates of renal dysfunction and hyperkalaemia [19]. We have since learned that the addition of a mineralocorticoid receptor antagonist to an ACE inhibitor and beta-blocker confers more benefit and is therefore favoured (see mineralocorticoid receptor antagonists).

36.4.2.3 Beta-Blockers

It has been shown that modulation of the sympathetic nervous system results in greater reversal of adverse left ventricular remodelling and a larger increase in ejection fraction than ACE inhibition alone [6]. As such, beta-blockers should also be used first line in patients with HF-REF [1]. Three large randomised controlled trials of bisoprolol, metoprolol and carvedilol in HF-REF showed a 34–35% reduction in mortality when compared to placebo in patients with NYHA class II–IV symptoms [20, 21, 22]. In all trials, the rate of ACE inhibitor or ARB use was approximately 96%. Due to their negative inotropic effects, beta-blockers should be initiated when the patient is compensated and up-titrated gradually. In a decompensated patient, new beta-blockade should be postponed until clinical stability and euvolaemia is achieved. Unless clear evidence of organ hypoperfusion exists, continuation of beta-blocker therapy during periods of decompensation appears to be safe and results in higher rates of treatment in the months following hospital discharge [23].

36.4.2.4 Mineralocorticoid Receptor Antagonists

Mineralocorticoid receptor antagonists (MRAs) were first examined in patients with HF-REF and severe symptoms (NYHA class III–IV) and, in this demographic, spironolactone resulted in a 30% reduction in risk of all-cause mortality [24]. Later, the benefit of MRAs was shown to extend to patients with milder symptoms (37% reduction in risk of cardiovascular death of heart failure hospitalisation for eplerenone versus placebo) [25]. The rate of ACE inhibitor or ARB and beta-blocker use was 94% and 87%, respectively. MRAs are therefore recommended for patients with HF-REF and ongoing symptoms despite treatment with an ACE inhibitor and beta-blocker [1]. The choice of MRA is guided not only by NYHA class but also by side-effect profile; due to its selectivity for aldosterone receptors, eplerenone results in fewer anti-androgenergic side-effects, namely gynaecomastia.

36.4.2.5 Angiotensin Receptor-Neprilysin Inhibitors

The landscape of pharmacological therapy in HF-REF was recently transformed by a large randomised controlled trial comparing treatment with an angiotensin receptor-neprilysin inhibitor (ARNI) to an ACE inhibitor in patients with NYHA class II–IV symptoms [26]. In a prior study, treatment with a recombinant form of natriuretic peptide was not found to improve outcomes for patients with acute heart failure [27]. It was subsequently hypothesised that an alternative approach, via inhibition of endogenous natriuretic peptide degradation, would prove successful. The neprilysin inhibitor sacubitril, when combined with valsartan, reduced the occurrence of the primary endpoint (cardiovascular death or hospitalisation for heart failure) by 20% and all-cause mortality by 16% over the gold standard, enalapril [26]. As a result, treatment with sacubitril-valsartan is recommended in place of an ACE inhibitor or ARB in treatment of symptomatic patients with HF-REF [1]. In the trial, hypotension was more common in patients treated with sacubitril-valsartan, but renal dysfunction, hyperkalaemia and cough occurred more frequently in the enalapril group. When switching from an ACE inhibitor to sacubitril-valsartan, a washout period of at least 36 hours is necessary to diminish the risk of angioedema. Despite the success of targeting the natriuretic peptide system in the treatment of HF-REF, therapy guided by natriuretic peptide levels has not been found to be more effective than usual care [28].

36.4.2.6 Other Medications

Some of the earliest drug trials in heart failure examined the effects of vasodilators which, at one time, were the mainstay of treatment for the condition. In the era before beta-blockers and modulators of the RAAS, hydralazine and isosorbide

dinitrate used together were shown to reduce 2-year mortality in men with heart failure when compared with placebo [29]. Later, a more convincing survival benefit was seen amongst African-American patients with NYHA class III–IV symptoms [30]. On the basis of these studies, guidelines suggest using this combination therapy on top of treatment with an ACE inhibitor or ARB in black patients with moderate to severe symptoms, or in patients of any ethnicity who cannot tolerate treatment with an ACE inhibitor or ARB [1].

Digoxin is a potent inhibitor of cellular sodium-potassium ATPase. It causes a rise in intracellular sodium, which triggers transport of calcium into the cell across a sodium-calcium exchanger system. In the only large randomised controlled trial of digoxin versus placebo in HF-REF, though hospitalisation for heart failure was reduced, there was no reduction in overall mortality [31]. In this study, an inclusion criterion was the presence of sinus rhythm; the effect of digoxin on hard endpoints in patients with HF-REF and atrial fibrillation remains unknown. As a positive inotrope with no blood pressure-lowering action, digoxin is the preferred mode of initial ventricular rate control in patients with rapidly conducted atrial fibrillation and decompensated heart failure.

The If current inhibitor, ivabradine, acts directly on the sinoatrial node to slow the heart rate in patients in sinus rhythm. A trial of ivabradine versus placebo in patients with HF-REF, NYHA class II–IV symptoms and a heart rate of 70 beats per minute or greater in sinus rhythm resulted in an 18% reduction in the combined risk of cardiovascular death or hospitalisation for heart failure [32]. The benefit was driven by a reduction in heart failure hospitalisation and death due to heart failure. In clinical practice, ivabradine can be considered for a patient with ongoing symptoms who is on the maximum tolerated dose of beta-blocker and remains in sinus rhythm with a high heart rate [1].

Other studies in HF-REF have shown no benefit of anticoagulation [33, 34], direct renin inhibition [35], endothelin antagonism [36], vasopressin blockade [37] or treatment with the dihydropyridine calcium channel blocker, amlodipine [38]. Drugs that have been shown to result in harm include thiazolidinediones, nonsteroidal anti-inflammatory drugs, non-dihydropyridine calcium channel blockers and most antiarrhythmic drugs (with the exception of amiodarone) [1].

36.4.3 Device Therapy

36.4.3.1 Implantable Cardioverter Defibrillators

Across clinical trials in HF-REF, which generally provide more detail about mode of death than do observational studies, sudden death accounts for an average of approximately 40% of deaths [39]. Sudden deaths are thought to be due predominantly to arrhythmias such as ventricular tachycardia. Implantable cardioverter defibrillators (ICDs) have been shown to reduce the risk of sudden death in patients with HF-REF, but, historically, their role in patients with a non-ischaemic aetiology has been less certain. More recently, a trial investigating the effect of primary prevention ICD added to conventional therapy versus conventional therapy alone in patients with non-ischaemic cardiomyopathy, in whom disease-modifying pharmacotherapy use was comprehensive, failed to demonstrate a reduction in all-cause mortality [40]. Moreover, over half of the patients received cardiac resynchronisation therapy. When the effect of ICD use was examined across subgroups, there was a suggestion that younger patients (those under the age of 68 years) might derive benefit in terms of death from any cause. The lower rate of death from non-cardiovascular causes in such patients is likely to contribute to this finding. In the future, better prediction of those at greatest risk of sudden death may help further refine the role of ICDs in HF-REF due to non-ischaemic causes, particularly as the risk of sudden death in clinical trial patients is declining [39]. The evidence for primary prevention ICDs in patients with HF-REF due to coronary artery disease is more robust, and, as such, they should be considered in optimally treated patients with NYHA class II–IV symptoms and a left ventricular ejection fraction of 35% or less whose functional status and anticipated survival are reasonable [1, 41]. An episode of sustained ventricular tachycardia or cardiac arrest due to ventricular arrhythmia mandates implantation of a secondary prevention ICD to reduce the risk of death [1].

36.4.3.2 Cardiac Resynchronisation Therapy

In patients with left ventricular systolic dysfunction and an interventricular conduction delay (evidenced by prolonged QRS duration on electrocardiogram), cardiac output is further compounded by the presence of electrical and mechanical dyssynchrony. Cardiac resynchronisation therapy (CRT), or biventricular pacing, augments stroke volume, minimises mitral regurgitation and promotes left ventricular reverse remodelling by improving intraventricular, atrioventricular and interventricular synchrony. Clinically, this translates into improvements in symptoms and functional capacity, a reduction in heart failure hospitalisation and increased survival. The benefit of CRT has been proven in, and should thus be considered for, patients with a left ventricular ejection fraction of 35% or less, QRS duration of 130 milliseconds or greater and ongoing heart failure symptoms (NYHA class II–IV) despite optimal medical therapy [1, 42–45]. Longer QRS duration, non-ischaemic causes of heart failure and female sex appear to predict a better response to therapy. For all patients, a rate of biventricular pacing that exceeds 98% should be sought. The term 'nonresponder' has been applied to patients who fail to demonstrate improvement, but there exists heterogeneity in the criteria used to define response. The efficacy of CRT in patients with atrial fibrillation is less certain, but if stringent ventricular rate control can ensure sufficient biventricular pacing, then implantation of the device should be considered [1].

36.4.4 Surgical Intervention

Coronary artery bypass grafting (CABG) has been evaluated against standard medical therapy in patients with coronary artery disease and left ventricular systolic dysfunction in a large randomised study with extended follow-up to 10 years [46]. In this trial, there was a 16% reduction in the risk of death from any cause in the patients randomised to surgery, though the benefit only began to accrue after 2 years. The benefit of CABG in terms of all-cause mortality appears to lessen with age, likely explained by the higher proportion of non-cardiovascular deaths in older patients. Adding surgical ventricular reconstruction to CABG has not been shown to improve clinical outcomes, despite a reduction in left ventricular volume [47].

Other invasive procedures in HF-REF largely fall under the umbrella of advanced heart failure management. Cardiac transplantation in chronic heart failure is reserved for patients who are deteriorating despite optimal medical and device therapy and in whom no contraindications, such as older age or active malignancy, exist. Bridging strategies, such as a ventricular assist device, can provide mechanic circulatory support to patients waiting for a donor heart to become available. These devices are also used universally as a bridge to recovery or a bridge to transplant candidacy, but only in some countries as an alternative to transplantation.

36.4.5 Other Approaches

Improved physical conditioning through structured exercise training is encouraged in patients with HF-REF in order to enhance exercise capacity and reduce hospitalisations [1]. Lifestyle measures (such as restricting dietary salt intake) are a common component of patient education, but there is little evidence to support an associated impact on morbidity or mortality [1]. A multidisciplinary approach (the patient is cared for by a team of physicians, specialist heart failure nurses and pharmacists) to the treatment of heart failure is recommended by the guidelines as it has been shown to improve morbidity and mortality [1].

Despite the success of treatment advances in slowing disease progression (and, in some, inducing recovery of left ventricular systolic function), the trajectory for many patients with HF-REF remains one of a downward slope, associated with interspersed hospital admissions, worsening symptoms and deteriorating cardiac function. A patient-centred palliative care approach should be offered to those with advancing disease despite appropriate therapy, with a view to maintaining quality of life during the later stages of the condition.

Conclusion and Clinical Perspectives

- HF-REF is a complex condition, the development of which is driven by a number of overlapping haemodynamic, neurohumoral and structural processes.

- Huge advances in treatment over the past 30 years have translated into substantial benefits for patients living with the disease, both in terms of symptoms and survival.
- Research in HF-REF continues to thrive, and sustained progress is likely.
- The new challenge will lie in better prevention of heart failure as the population ages and risk factors become more prevalent.

Gaps in the Knowledge

The evidence base underpinning the management of chronic HF-REF has grown exponentially since the late 1980s. However, there remain a number of areas in need of further definitive research and development. At present these include, but are not limited to:

- Contemporary epidemiology worldwide
- The efficacy of novel targeted pharmacotherapies (such as glucose-lowering drugs, intravenous iron, soluble guanylyl cyclase inhibitors and agents directed at augmenting cardiomyocyte function)
- Better selection of the population most likely to benefit from device therapy
- The role of percutaneous intervention for co-existing valvular disease (particularly functional mitral regurgitation)
- A better understanding of the disease and potential therapeutic options in specific populations, including those underrepresented in clinical trials (such as the elderly or those with severe kidney disease)

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Heart Failure with Preserved Ejection Fraction

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_37

Key Concepts

- Heart failure with preserved ejection fraction (HFpEF) is now reported to be the predominant form of heart failure.
- HFpEF is associated with considerable morbidity and mortality, and outcomes have not improved over recent decades.
- Heart failure with mid-range ejection fraction (HFmrEF) is not likely to survive more iterations of definitions in international guidelines.
- Identification of HFpEF can be challenging, and there remains inconsistency and debate regarding which diagnostic criteria should be used.
- HFpEF is characterised by a high burden of associated cardiovascular and non-cardiovascular comorbidities.
- The pathophysiology of HFpEF is not well understood and is unlikely to be explained by a single unifying paradigm.
- To date, no treatments for HFpEF have been demonstrated to have any convincing prognostic benefit.
- Large randomised trials of promising drugs and devices in HFpEF with hard clinical endpoints will report soon.

37.1 Introduction

Heart failure with preserved ejection fraction (HFpEF) is the clinical syndrome of heart failure (HF) in the presence of a normal or near-normal left ventricular ejection fraction (LVEF). HF is characterised by typical symptoms (i.e. dyspnoea, fatigue, ankle swelling) which may be associated with signs of congestion (i.e. elevated jugular venous pressure, lung crepitations, peripheral oedema). As many patients without HF have these non-specific symptoms and signs, the 2016 European Society of Cardiology (ESC) guidelines mandated the presence of both an abnormality of cardiac structure on cardiac imaging and elevated natriuretic peptides before a diagnosis of HFpEF can be made [1]. Controversy exists over the LVEF cut-off considered to represent preserved left ventricular systolic function. Contemporary guidelines classify patients with HF in the presence of an LVEF ≥50% to have HFpEF and those with a LVEF <40% to have HF with reduced ejection fraction (HFrEF) [1, 2]. Some patients fall into an intermediate group, i.e. those with an LVEF of 40–49%. The ESC defines this group as a distinct clinical entity, termed HF with mid-range ejection fraction (HFmrEF) [1]. These patients appear to have a similar response to pharmacological therapies as those with HFrEF [3]. The HFmrEF classification is currently of unclear clinical relevance and is likely to be superseded by an alternative nomenclature. It should be noted that the measurement error of LVEF can mean that a patient can be reclassified between the current definitions of HFpEF and HFmrEF by serial echocardiographic measurements within hours [4].

37.2 Epidemiology and Outcomes

In Europe and North America, HF affects 1–3% of the population. Epidemiological studies suggest that the prevalence of HFpEF in relation to HFrEF has increased over recent years, with some reporting that HFpEF now accounts for over 50% of HF [5]. The proportion of patients with HFpEF is, however, uncertain as most of these studies are population-based and lack the rigour necessary to accurately categorise these patients.

Patients with HFpEF are generally older, more frequently female, and have a higher burden of comorbidities than those with HFrEF. The ageing population is thought to represent a major reason for the female preponderance, higher prevalence of associated comorbidities and the increasing prevalence of HFpEF compared to HFrEF [6].

Outcomes of populations with HFpEF vary depending on study design, clinical setting and the biomarker and LVEF thresholds used to define HFpEF. Patients with HFpEF have significantly poorer outcomes when compared with populations with similar age and comorbidity profiles without HF [7]. Although the prognosis in HFpEF and HFrEF was thought to be similar, more recent studies suggest that patients with HFpEF have better outcomes than those with HFrEF [8]. Despite this, hospitalisation and mortality rates in HFpEF remain high, and, in contrast to HFrEF, outcomes have not improved over recent decades [9].

37.3 Diagnosis

The diagnosis of HFpEF is frequently challenging, especially in ambulatory patients. The diagnosis can be obvious in hospitalised patients with abnormal cardiac imaging (usually echocardiography), pulmonary congestion and elevated cardiac biomarkers. In ambulatory outpatients the diagnosis can be more uncertain. Patients with obesity and/or lung disease are often thought to be breathless because of these conditions, but they may also have HFpEF. Elevated biomarkers are necessary to confirm the diagnosis of HFpEF.

The diagnosis of HFpEF must be confirmed by cardiac imaging findings (e.g. preserved LVEF, left atrial [LA] enlargement, left ventricular hypertrophy [LVH], evidence of increased left ventricular [LV] filling pressures and/or pulmonary hypertension [PH]) together with elevated natriuretic peptides. Ambulatory patients frequently experience symptoms only on exertion and often have no clinical signs of fluid overload. Recent invasive and non-invasive studies have reported that, in HFpEF patients, LV filling pressures may be normal at rest but increase dramatically with exercise [10]. Stress testing may, therefore, be required to confirm or exclude the diagnosis of HFpEF in patients presenting with unexplained exertional dyspnoea. Some investigators make a diagnosis of HFpEF based on haemodynamic changes, even in the absence of elevated biomarkers.

An alternative presentation of HFpEF is that of a breathless patient with preserved LVEF and evidence of PH on echocardiography. This HFpEF subgroup represents an advanced stage of the condition, and these patients appear to have a poor prognosis [11].

37.4 Comorbidities in HFpEF

Epidemiological studies and randomised controlled trials (RCTs) describe a higher burden of hypertension, atrial fibrillation (AF), obesity, arthritis, stroke, chronic lung disease, anaemia and diabetes mellitus in patients with HFpEF versus those with HFrEF. The high frequency of comorbidities, many of which can cause similar symptoms and signs to HF, has led some to suggest that these patients do not have HF at all [12]. However, a comparison of randomised controlled trial data of patients with HFpEF versus those with cardiovascular conditions without HF found that the poor outcomes associated with HFpEF did not appear to be explained on the basis of age or comorbidities [7].

The failure of RCTs in HFpEF to find effective therapies has led to attempts to divide the HFpEF population into distinct clinical sub-phenotypes, with the aim of identifying more homogenous groups which may respond to targeted therapies. Patients with HFpEF can be phenotyped based on the presence or absence of several important comorbidities for which specific treatments exist (e.g. hypertension, AF, coronary artery disease [CAD]). More extensive phenotyping techniques have also been explored. Shah and colleagues used phenomapping to identify 3 distinct HFpEF phenotypes in a prospective study of 397 patients: younger patients with lower natriuretic levels ('early HFpEF'); obese patients with diabetes mellitus and obstructive sleep apnoea; and older patients with chronic kidney disease, high natriuretic peptide levels and PH ('advanced HFpEF') [13]. Whether trials directed at testing therapies in distinct HFpEF phenotypes will be successful remains to be seen.

37.5 Pathophysiology

The pathophysiology of HFpEF is not well understood, in large part due to the heterogeneity of the HFpEF population. The major underlying pathological cardiac mechanism is thought to be LV diastolic dysfunction. However, various other cardiovascular processes have been implicated, including subtle LV systolic dysfunction, ventricular-arterial stiffening, LA dysfunction and AF, right ventricular (RV) dysfunction and pulmonary vascular disease, ventricular interdependence, chronotropic incompetence, peripheral factors and endothelial dysfunction (Figs. 37.1 and 37.2).

37.5.1 LV Diastolic Dysfunction

Almost all patients with HFpEF have evidence of diastolic dysfunction at rest [14]. However, in HFpEF patients with exertional symptoms, diastolic dysfunction may only become apparent with exercise. Conversely, elderly patients without HF frequently have echocardiographic evidence

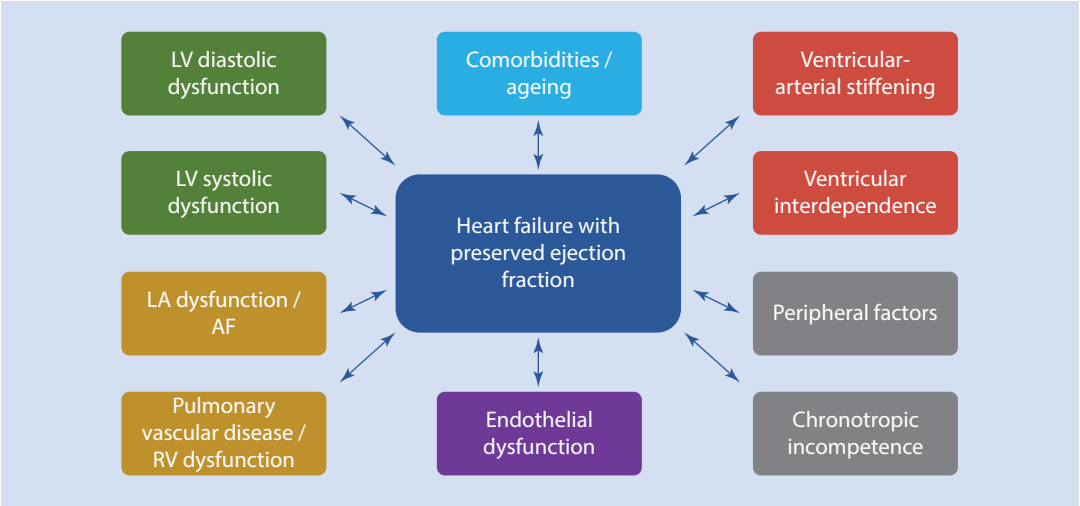

Fig. 37.1 Pathophysiology of heart failure with preserved ejection fraction

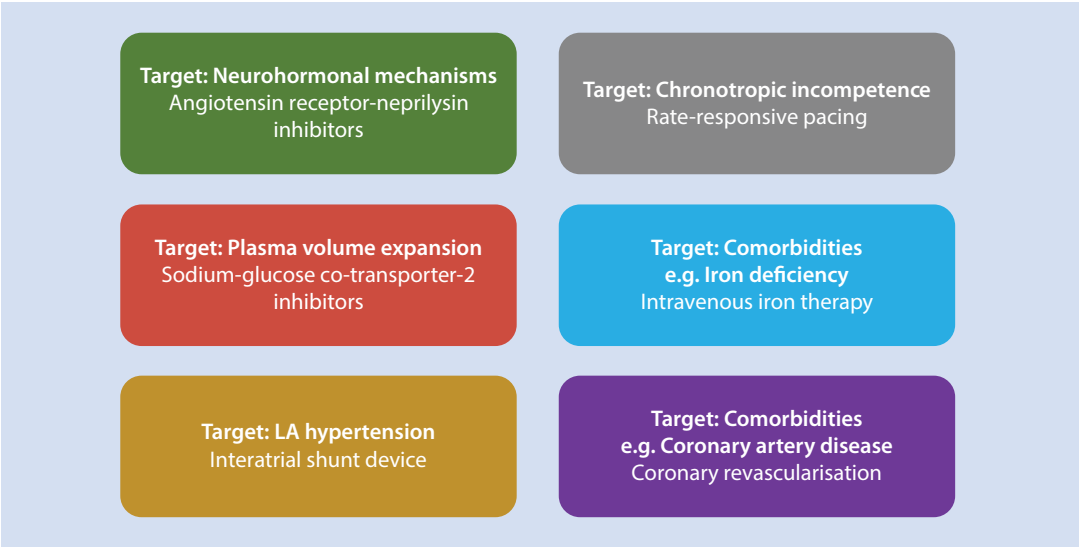

Fig. 37.2 Potential treatments for heart failure with preserved ejection fraction

of diastolic dysfunction at rest [15]. Diastolic dysfunction, therefore, may play a central role in HFpEF, but there are clearly other important pathophysiological mechanisms involved.

Two major processes determine LV diastolic function: active relaxation and passive filling [14]. Active relaxation is regulated by calcium homeostasis and the phosphorylation state or levels of specific proteins (e.g. phospholamban) that modify the sarcoplasmic reticulum calcium ATPase pump (SERCA2a). Active relaxation requires the removal of cytosolic calcium during diastole, primarily by SERCA2a, which is inhibited by phospholamban in its unphosphorylated state. Because active relaxation is an energy-dependent process, it is vulnerable to ischaemia, and, therefore, impaired diastolic filling occurs early in the ischaemic cascade [16].

Diastolic function is also determined by the passive elastic properties of the LV. LV diastolic stiffness increases with age and myocardial injury. Increased passive stiffness was previously thought to be due to myocardial fibrosis and changes in extracellular matrix composition; however, diastolic stiffness is frequently elevated in patients without fibrosis, and acute changes to diastolic

stiffness are seen in the context of ischaemia or changes in the compliance of the large sarcomeric protein, titin [17].

Cardiomyocyte resting tension is highly dependent on the function of titin, which acts as a physiological molecular spring. The properties of titin can be altered by the expression of different isoforms and by post-translational phosphorylation [17]. Due to differential splicing, titin exists in two isoforms: N2B (shorter, stiffer) and N2BA (longer, more compliant). There appears to be a shift towards expression of the N2B isoform in patients with HFpEF [18]. Phosphorylation of titin can occur at various sites in the molecule, and several pathways are involved. Protein kinase A (PKA) and protein kinase G (PKG) improve titin compliance and, therefore, decrease cardiomyocyte resting tension. Endomyocardial biopsy studies have revealed low PKA and PKG activity in HFpEF, and their administration has been shown to acutely reduce resting cardiomyocyte stiffness in vitro [19, 20].

37.5.2 LV Systolic Dysfunction

Despite having a normal or near-normal LVEF, studies using sensitive measures of LV contractility (e.g. speckle tracking echocardiography) demonstrate that HFpEF patients have subtle LV systolic dysfunction [21]. Furthermore, patients with HFpEF exhibit an inability to increase their LVEF and cardiac output with physiological stress, which may contribute to exercise intolerance.

37.5.3 Ventricular-Arterial Stiffening

Patients with HFpEF have increased LV systolic stiffness and arterial stiffness when compared with healthy (but not hypertensive) controls [22]. Elevated LV and arterial stiffness result in a steep end-systolic pressure-volume relationship. This leads to an augmented blood pressure response to changes in preload or afterload, predisposing to severe hypotension and hypertensive crises in patients with HFpEF. Normally, there is a decrease in the arterial stiffness/LV systolic stiffness ratio with exercise. However, this decrease is attenuated in HFpEF, resulting in an impaired cardiac output response to exercise.

37.5.4 LA Dysfunction and AF

Few patients with HFpEF have normal LA volumes [23]. The LA plays an important role in LV diastolic filling, both as a conduit for blood flow from the pulmonary circulation and via atrial contraction. Patients with HFpEF have chronically elevated LA pressure with resulting atrial dilatation, loss of atrial contractile reserve and electrical remodelling. This predisposes to AF, which affects two-thirds of HFpEF patients [24]. It is unclear whether AF merely represents a more advanced stage of the condition or if it plays a role in the progression of HFpEF.

37.5.5 RV Dysfunction and Pulmonary Vascular Disease

In a community-based study of 244 HFpEF patients, the prevalence of PH (defined as an echocardiography-derived pulmonary artery systolic pressure >35 mmHg) was 83% [25]. Chronic LA pressure overload results in passive pulmonary venous hypertension and postcapillary PH. However, the severity of PH observed in HFpEF patients does not appear to be explained by this alone, suggesting an element of precapillary PH. It is unclear whether this is a result of reactive changes to the pulmonary vasculature due to long-standing pulmonary venous hypertension or whether other processes (e.g. primary pulmonary arterial dysfunction) are involved. RV dysfunction in HFpEF is associated with male sex, concomitant AF and CAD [11]. It can be a consequence of chronic PH; however, there is also evidence of increased RV diastolic stiffness and abnormal RV-PA coupling ventricular-arterial coupling. Both PH and RV dysfunction are independent predictors of poor outcomes in HFpEF.

37.5.6 Ventricular Interdependence

The pericardium contributes around 40% to the LV end-diastolic pressure under resting conditions [26]. As described above, HFpEF is frequently associated with LA and RV dysfunction and dilatation. This leads to an increase in cardiac size, which may augment ventricular interdependence in

patients with HFpEF. The role of pericardial constraint and ventricular interdependence in the pathophysiology of HFpEF is currently unknown.

37.5.7 Chronotropic Incompetence

Studies suggest that over half of patients with HFpEF have evidence of chronotropic incompetence, suggestive of autonomic dysfunction [27]. Beta-blockers and ivabradine have failed to show benefit in HFpEF [3, 28].

37.5.8 Peripheral Factors

Various studies have suggested that skeletal muscle abnormalities may contribute to exercise intolerance in some patients with HFpEF. Patients with HFpEF have lower lean body mass, increased intramuscular fat content, fewer type I (slow-twitch) fibres and microvascular rarefaction when compared with healthy controls [29]. Interestingly, the benefits of exercise training in HFpEF appear to be mediated via peripheral, rather than central, mechanisms (i.e. improved skeletal muscle and/or microvascular function) [30].

37.5.9 Endothelial Dysfunction

That endothelial dysfunction plays a pivotal role in the pathogenesis of HFpEF has attracted a great deal of attention (see below). Some studies have reported more peripheral endothelial dysfunction in patients with HFpEF compared to hypertensive and healthy controls [31]. However, this finding has not been observed in all HFpEF studies, and whether the observed endothelial dysfunction could be explained by concomitant atherosclerosis or diabetes has not been investigated.

37.5.10 Is There a Unifying Pathophysiological Paradigm of HFpEF?

Hypertension is extremely common in the HFpEF population, and, traditionally, it has been thought to be central to the pathogenesis of HFpEF. Long-standing hypertension causes activation of the renin-angiotensin-aldosterone system and afterload excess, with resultant LV remodelling, hypertrophy and diastolic dysfunction. This leads to LA hypertension and dilatation, with pulmonary venous hypertension and, eventually, to right heart dysfunction. However, most patients with HFpEF do not have a history of chronic poorly controlled hypertension, and at least a third does not have LVH [32]. Whilst hypertension undoubtedly plays an important role in HFpEF, it is inadequate to solely explain the underlying pathophysiology in the majority of patients.

In 2013, a much-cited paradigm was proposed suggesting that endothelial dysfunction plays a central role in the pathophysiology of HFpEF [33]. This hypothesises that multimorbidity induces a systemic inflammatory process, with coronary microvascular endothelial inflammation and dysfunction. This results in reduced nitric oxide (NO) bioavailability, cyclic guanosine monophosphate (cGMP) content and PKG activity in adjacent cardiomyocytes. Low PKG activity favours cardiomyocyte hypertrophy and increases resting tension via hypophosphorylation of titin. Both stiff cardiomyocytes and interstitial fibrosis result in LV diastolic dysfunction and HF. This concept is based on findings from five studies of human endomyocardial biopsies. Aside from the small numbers, the patients studied represent a highly selected group. The majority of patients were referred for endomyocardial biopsy because of suspicion of infiltrative cardiomyopathy, and, in 1 study, 5 out of 12 of the patients included were cardiac transplant recipients. The mean age of patients was considerably younger than the typical HFpEF population, and, in all but one of the studies, men comprised a majority. Furthermore, patients with important comorbidities, such as CAD and AF, were frequently excluded. Consequently, extrapolating the findings from small studies of highly selected patients not representative of the general HFpEF population to a universal pathophysiological paradigm for such a vast and heterogeneous condition as HFpEF should be considered with caution.

An autopsy series found greater coronary microvascular rarefaction in HFpEF patients when compared with controls, independent of epicardial CAD [34]. A recent prospective observational study of 202 HFpEF patients reported evidence of coronary microvascular dysfunction (using adenosine stress Doppler echocardiography) in 75%

of patients [35]. Both studies hypothesise that their findings could be the result of coronary microvascular endothelial dysfunction; however, this is speculative. Various studies report evidence of peripheral endothelial dysfunction in HFpEF [36]; however, no human studies have yet assessed coronary endothelial function in vivo.

Given the heterogeneity of the HFpEF population, it is unlikely that an overarching pathophysiological model will be identified. As described above, HFpEF is a complex and diverse condition characterised by multimorbidity and abnormalities in many aspects of cardiovascular structure and function. Patients with HFpEF may exhibit a number of functional impairments, and the relative contributions of each may differ between patients.

37.6 How Do You Treat HFpEF?

To date, no treatment has been shown to provide prognostic benefit in patients with HFpEF. International guidelines for HFpEF are currently based on expert consensus opinion. These recommend the use of diuretics to improve symptoms and signs of fluid retention (if present) and the optimal treatment of associated comorbidities (e.g. hypertension, CAD) [1, 2].

37.7 Clinical Trials in HFpEF

37.7.1 Therapies Targeting the Renin-Angiotensin-Aldosterone System in HFpEF

Randomised trials testing the effect of renin-angiotensin-aldosterone system antagonists in HFpEF have consistently failed to show benefit. One moderately large randomised trial (PEP-CHF) showed that treatment with the ACE inhibitor perindopril had no effect on the primary composite endpoint of all-cause mortality or HF hospitalisation in elderly patients with HFpEF [37]. Two large RCTs also failed to demonstrate benefit in composite primary endpoints with the angiotensin receptor blockers candesartan (CHARM-Preserved) and irbesartan (I-PRESERVE) [38, 39]. Although the mineralocorticoid receptor antagonist spironolactone improved LV diastolic function, it did not affect exercise capacity, symptoms or quality of life in a small randomised trial (Aldo-DHF) [40]. In a much larger multicentre RCT (TOPCAT), spironolactone had a neutral effect on the composite primary outcome of cardiovascular death, aborted cardiac arrest or HF hospitalisation [41]. Post hoc analyses have demonstrated marked regional variations in TOPCAT, with patients enrolled in Russia and Georgia having much lower event rates in the placebo group than those enrolled in the Americas [42]. The majority of patients enrolled in Russia and Georgia were included on the basis of a previous HF hospitalisation, rather than elevated natriuretic peptide levels, raising concerns that a significant proportion of patients in the trial did not have HFpEF. In an analysis restricted to those enrolled in the Americas, treatment with spironolactone appeared to be beneficial. The post hoc nature of this analysis means this should be considered with caution; however, to date, the evidence for spironolactone is the most convincing of any therapy in HFpEF.

37.7.2 Therapies Targeting NO-cGMP-PKG Signalling in HFpEF

Despite much enthusiasm, several studies assessing therapies targeting the systemic inflammatory paradigm of HFpEF have failed to show any convincing benefit. In this hypothesis, low cGMP activity is thought to play a central role in the pathophysiology of HFpEF; however, several studies of therapies which increase cGMP levels have failed to meet their primary endpoints. The soluble guanylate cyclase stimulator vericiguat did not meet its primary outcome in SOCRATES-PRESERVED [43]. When compared with placebo, the phosphodiesterase-5 inhibitor sildenafil did not improve exercise capacity or clinical status in HFpEF patients in the RELAX trial [44]. In NEAT-HFpEF, treatment with the organic NO donor isosorbide mononitrate reduced activity levels [45]. However, in a small study, inorganic nitrate appeared to improve submaximal exercise endurance in patients with HFpEF [46], but a larger multicentre trial of inhaled inorganic nitrite failed to demonstrate benefit (INDIE-HFpEF, [47]).

Neprilysin inhibition prevents the breakdown of biologically active natriuretic peptides, leading to increased intracellular cGMP. A phase II study of the neprilysin inhibitor sacubitril in combination with the angiotensin receptor blocker valsartan (sacubitril/valsartan) suggested benefit over valsartan alone in HFpEF [48], and a large multicentre pivotal RCT is in progress and will report within 2 years (PARAGON-HF, NCT01920711).

37.7.3 Therapies Targeting Renal Glucose Reabsorption in HFpEF

Sodium-glucose co-transporter-2 (SGLT-2) inhibitors prevent renal glucose reabsorption and reduce HF hospitalisations in patients with type 2 diabetes and established cardiovascular disease [49–51]. Three large RCTs in HFpEF are underway in patients with and without diabetes (DELIVER, NCT03619213; EMPEROR-Preserved, NCT03057951 [HFpEF]; and SOLOIST-WHF, NCT03521934 [HFrEF and HFpEF with diabetes]). These trials are due to report within 2–3 years.

37.7.4 Therapies Targeting Heart Rate and Exercise Intolerance in HFpEF

Lower heart rate increases the duration of diastole and can facilitate greater LV filling. On the other hand, it may exacerbate chronotropic incompetence which is prevalent in HFpEF (discussed above). The beta-blocker nebivolol was assessed in a prespecified subgroup analysis of an RCT including patients with both HFrEF and HFpEF (SENIORS), showing a neutral effect on a composite of all-cause mortality and cardiovascular hospitalisation [52]. However, beta-blockers have yet to be evaluated in an adequately powered RCT. The effect of digoxin in HFpEF was assessed in a moderately large RCT (the ancillary DIG trial) with no effect on the primary endpoint of HF mortality or HF hospitalisation [53]. There was a trend towards a reduction in HF hospitalisations. The I_f current blocker ivabradine has also been evaluated in phase II HFpEF trials with mixed results. A small study is currently underway to assess the effect of rate-responsive pacing in HFpEF patients with chronotropic incompetence (RAPID-HF, NCT02145351).

37.7.5 Therapies Targeting LA Hypertension in HFpEF

Elevated LA pressure is thought to be one of the central pathophysiological findings in HFpEF. Reducing LA pressure by creating an interatrial shunt has been studied in an observational cohort of 64 patients with improved haemodynamics and quality of life [10]. A small, sham-controlled, blinded RCT trial found reduced exercise pulmonary artery wedge pressure with this technique [54], and a large, sham-controlled, blinded RCT is in progress (REDUCE LAP-HF II, NCT02600234). Trials of pharmacological therapies have thus far been neutral in HFpEF. Device therapy has primarily focussed on HF with reduced ejection fraction but may be beneficial in HFpEF.

37.8 Future Directions

37.8.1 Clinical Trial Design

The consistently neutral outcomes of HFpEF trials have led to much discussion and debate. The designs of previous HFpEF trials have used inclusion criteria that might allow some patients without HFpEF to be enrolled. They have employed inconsistent definitions of HFpEF. Recruitment of elderly, frail patients with HFpEF to clinical trials is extremely challenging, meaning that they are unlikely to be representative of the general HFpEF population.

37.8.2 HFpEF Sub-phenotypes

The heterogeneity in the HFpEF population has led many to argue that any therapy targeting a single pathophysiological mechanism is unlikely to demonstrate benefit in unselected patients with HFpEF. Trials focusing on HFpEF sub-phenotypes (e.g. those with a specific cardiovascular abnormality or comorbidity) could assess therapies in more homogeneous groups that are more likely to respond to targeted interventions.

Conclusion and Clinical Perspective

- HFpEF represents a large and growing unmet clinical need.
- It is characterised by a complex interplay of various cardiovascular mechanisms and associated comorbidities, with marked phenotypic variation between patients.
- The 'one-size-fits-all' approach to randomised controlled trials (RCTs) in HFpEF has so far failed to demonstrate any clinically meaningful benefit.
- Several promising therapies are currently under investigation in large, well-designed clinical trials with hard endpoints which will report soon.
- Future RCTs in HFpEF are likely to focus on assessing targeted therapies in sub-phenotypes of HFpEF.

Gaps in Knowledge

- The pathophysiology of HFpEF remains poorly understood. The contribution of cardiac and vascular abnormalities as well as comorbidities is unknown.
- There are no evidence-based therapies for HFpEF.
- Whether or not therapies targeting single pathophysiological mechanisms will demonstrate clinical benefit is unknown.

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Renal Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_38

Key Concepts

- Chronic kidney disease (CKD) regardless of its aetiology is increasing in prevalence globally.
- The progressive nature of CKD, resulting renal fibrosis and eventual end-stage kidney disease (ESKD) places a significant burden on patients, their carers and healthcare services.
- CKD, even with only mild renal dysfunction, is an independent cardiovascular risk factor.
- Current management of CKD is based on controlling known risk factors, e.g. hypertension, which can slow down progression.
- There is a clear and substantial unmet clinical need to develop novel treatments, which can prevent progression of renal dysfunction.

38.1 Kidney Disease: Background

Disorders leading to the loss of renal function may present acutely, leading to acute kidney injury (AKI), requiring urgent treatment or may follow a less rapid course, so-called chronic kidney disease (CKD). The clinical, therapeutic and cellular aspects of AKI are complex and out with the remit of this review, as is the management of renal failure, by dialysis and/or transplantation. CKD is a term that encompasses the majority of renal diseases that lead to impaired function of the kidney. CKD is common and can be asymptomatic, and worldwide prevalence of CKD is increasing. Within the UK studies report between 6% and 18% of the UK population have CKD depending on population and definition used [1]. CKD is staged by estimated glomerular filtration rate (eGFR), and patients are classified into stages 1–5 based on this (Table 38.1). eGFR is widely used in clinical practice to identify and monitor renal function. It is calculated using an estimating equation, either the MDRD or CKD-EPI formula [2], which uses serum creatinine, age, race and gender to derive estimated kidney function.

Table 38.1 GFR categories as determined by GFR measurements based on creatinine levels and their correlation to kidney function

CKD stage	GFR (mL/min/1.73 m^2)	Kidney function
1	>90	Normal function
2	60–89	Mildly decreased function
3a	45–59	Mildly to moderately decreased function
3b	30–44	Moderately to severely decreased function
4	15–29	Severely decreased function
5	<15	Kidney failure

GFR glomerular filtration rate

38.2 Clinical Management of CKD: Overview

Current management of CKD targets four broad areas. Interventions should be targeted to prevent progressive damage to the kidneys leading to an inevitable decline in kidney function. Second, therapy should address the complications of CKD, such as renal anaemia, bone disease and oedema. When available, treatment should target the specific cause of CKD, such as prevention of cyst formation in autosomal dominant polycystic kidney disease, glycaemic control in diabetic nephropathy or immunosuppression in vasculitis. Again, specific disease management is out with the scope of this chapter. The progressive nature of CKD, resulting fibrosis and eventual end-stage kidney disease (ESKD) places a significant burden on the NHS with total CKD costs of £1.45 billion/year [3]. Current management of CKD is based on controlling known risk factors, e.g. hypertension (which slow down progression); however, ~14% of patients have resistant hypertension, and the nature of CKD is a steady decline towards ESKD [4]. Regardless of the cause of the renal dysfunction, the pathophysiology of chronic renal disease is consistent. The hallmark of chronic renal disease is the loss of nephrons and

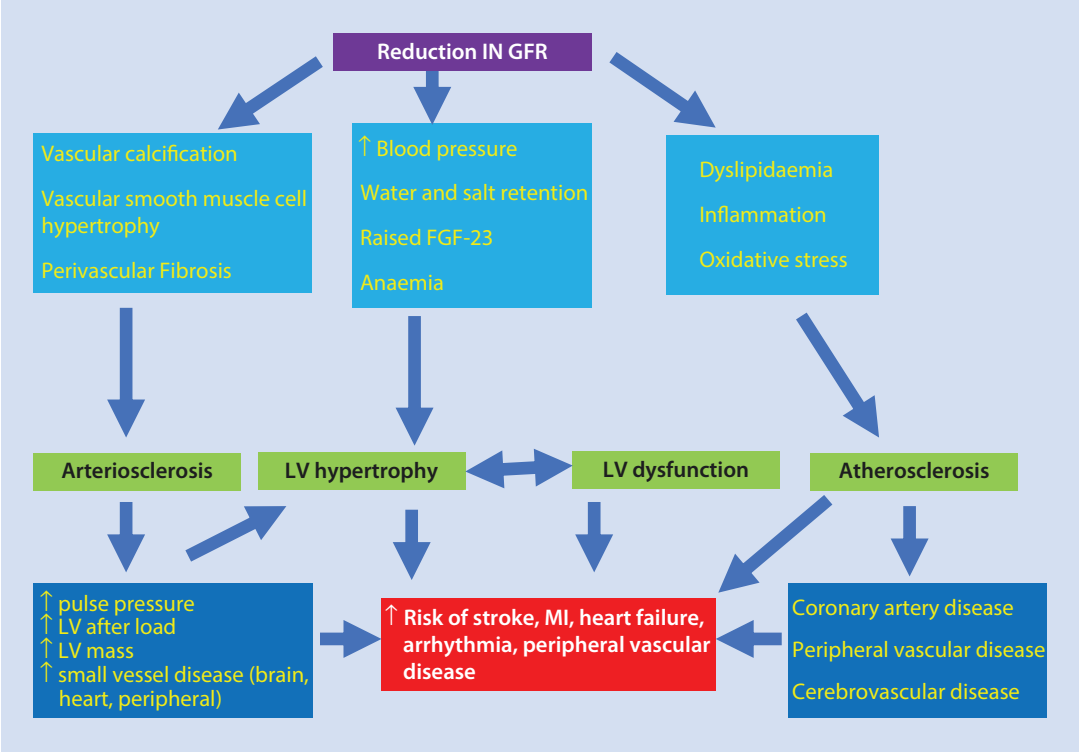

Fig. 38.1 Schematic diagram of the mechanisms by which cardiovascular risk is increased in patients with chronic kidney disease

replacement of functioning renal tissue with fibrotic scar, which decreases the capacity of the kidneys to filter. Reduced kidney function, as an indicator of established kidney fibrosis (glomerulosclerosis or tubulointerstitial fibrosis (TF)), is one of the strongest predictors of development of kidney failure in kidney disease. The histological features of tubulointerstitial fibrosis are excess deposition of interstitial extracellular matrix, interstitial inflammatory cells, tubular loss and rarefaction of microvasculature. However, despite the recognition of these key features, the interplay between these hallmarks of TF and the priority of each amongst the varying contributing pathways remains unresolved.

Finally, in patients with progressive decline in kidney dysfunction despite these measures, preparation should be made for management of ESKD, with dialysis or kidney transplantation. Once ESKD has been reached, the only treatment option for patients is either dialysis or transplantation. Neither of these treatment options is optimal. There is an unmet clinical need to develop new therapies that reduce/block/reverse progression of CKD, thereby reducing numbers of patients requiring expensive treatments and potentially improving the CVD-related premature deaths in CKD patients (Fig. 38.1).

38.3 CKD and Cardiovascular Risk

Importantly, CKD is an independent risk factor for premature cardiovascular disease (CVD). The CVD risk increases incrementally with the decline in eGFR, and once patients have ESKD and are requiring dialysis, the risk is 20–100 times that of the general population, depending on the age of the patient [5]. CKD is a state of both accelerated atherosclerosis and arteriosclerosis although the commonest presentations of CVD in ESKD are heart failure and sudden cardiac death rather than myocardial infarction. This reflects the impact of abnormalities in cardiac structure and function, rather than atheromatous coronary heart disease.

Table 38.2 Conventional cardiovascular risk factors, which are present in many patients with CKD, and cardiovascular risk factors specific to CKD

CV risk factors present in patients with CKD	
'Conventional' risk factors	**'CKD-specific' risk factors**
Hypertension	Bone and mineral disorder
Diabetes mellitus	Anaemia
Hyperlipidaemia	Dialysis adequacy
Physical inactivity	Malnutrition
Smoking	Dialysis access
Prior ischaemic heart disease	Immunosuppressive drugs (e.g. ciclosporin)
Inflammation (raised C-reactive protein)	Uraemic toxins
Left ventricular hypertrophy	Salt and volume overload

The risk factors for developing progressive CKD are similar to those for CVD and include diabetes mellitus, hypertension, smoking and hyperlipidaemia (Table 38.2). However, some risk factors for CVD are either more prominent in CKD and include proteinuria, inflammation and left ventricular hypertrophy or specific to the effect of loss of kidney function such as impaired calcium-phosphate homeostasis (parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23) and anaemia. These contribute to the CVD risk in this population (Table 38.2). Treatment of CVD in CKD is challenging as historically, patients with CKD were excluded from clinical trials which aimed at CVD risk reduction. However, more recent data suggest that patients with CKD benefit from interventions targeting lipid lowering and dysglycaemia [6]. However, once established on dialysis, the evidence is less clear. Well-established interventions, such as statins, do not impact on CVD risk in the dialysis population.

38.4 CKD: Principles of Management and Targets for Therapy

Conventional CVD risk factors are prevalent in patients with CKD and ESKD and contribute to the progression of CKD. However, certain risk factors, which either are specific to CKD or have an augmented effect on patient outcomes, have a dominant effect in this population. The management of CKD currently is based on reducing the risk factors for progression of CKD and reducing CVD risk factors. No treatment regime targets the underlying common cause of the impairment of renal function whereby functioning nephrons are replaced with fibrotic scar. Specific risk factors for renal progression or indicators of patients at high risk of cardiovascular complications of CKD are discussed.

38.5 Hypertension

Hypertension is common in CKD patients and is a risk factor for progressive kidney disease as well as being a major risk factor for CVD. The pathophysiology of hypertension in CKD initially involves the activation of the renin-angiotensin system (RAS), sympathetic nervous system and endothelial dysfunction, but as the disease advances, it becomes more dependent on sodium and water retention and is complicated by vascular calcification, which increases systolic pressure. Good blood pressure control is recommended in patients with CKD as this is associated with a reduced rate of decline of renal function and potential benefits for CVD [7]. Treatment with inhibition of the RAS has been demonstrated to retard the progression of CKD, independent of its blood pressure effect [8]. However, caution must be exhibited in using RAS inhibition in patients with CKD secondary to renal artery stenosis, as the associated reduction in glomerular perfusion pressure induced by RAS inhibition may lead to a dramatic decline in eGFR in the presence of renal artery stenosis. In patients at higher risk of CKD progression (proteinuria >1 g/day, diabetes), target SBP of <130 mmHg has been demonstrated to be associated with reduction of risk of progression to ESKD [9].

38.6 Proteinuria and Albuminuria

The leak of protein from the kidneys is one of the hallmarks of glomerular damage. Albumin is the major component of protein in the urine. Data from the Framingham studies have shown that proteinuria is a risk factor for cardiovascular mortality

in the general population. Similar findings have emerged from other large epidemiological studies that either proteinuria or microalbuminuria is not only predictive of progression of renal disease but also of future cardiovascular events [10]. Analyses from the Prevention of Vascular and End-Stage Renal Disease (PREVEND) study show clearly that urinary albumin acts as a continuous risk factor for cardiovascular events with no lower limit [11]. Microalbuminuria and proteinuria have been accepted to represent a consequence of renal damage. It has also been proposed that albumin leakage reflects widespread vascular damage, with the kidney as a 'window' to the vasculature. This hypothesis has been reinforced by studies demonstrating that microalbuminuria is associated with changes in endothelial dysfunction in patients with diabetes [12]. The Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria (IRMA-2) study, evaluating the effect of the angiotensin II antagonist irbesartan, demonstrated that reduction of albuminuria patients with type II diabetes was associated with renal protection and some degree of cardiovascular protection [13]. Recent meta-analysis of several trials in CKD suggests that for each 30% reduction in albuminuria, the risk of ESKD is reduced by 23.7% [14].

38.7 Diabetic Kidney Disease

Diabetes mellitus (both types 1 and 2) can lead to diabetic nephropathy, and this accounts for 20–40% of ESKD patients. The classic history is of microalbuminuria preceding the development of macroalbuminuria and consequent development of reduced kidney function. However, there is a distinct overlap between classic diabetic nephropathy and impaired renal function related to ischaemia, hypertension and arteriosclerosis in patients with diabetes, whereby the patient has advanced CKD in the absence of proteinuria. Mirroring general trends in society, the proportion of patients with CKD and diabetes is rising with the increase in incidence of type 1 and particularly type 2 diabetes. It is expected that approximately one-third of all patients will develop CKD, but our abilities are still not optimal for identification of those at highest risk before urinary albumin excretion increases and/or glomerular filtration rate declines.

Good glycaemic control is recommended and reduces the progression of nephropathy, whereas tight blood pressure control reduces progression of CKD and CV events [15]. However, it is well established that despite optimal BP and glycaemia management with RAS inhibition, many patients still progress to ESKD, and alternatively treatment strategies are necessary. Amongst the several potential strategies for diabetic nephropathy in clinical or preclinical trials include endothelin receptor antagonism, pentoxifylline, Nox 1/4 inhibition and chemokine receptor inhibition [16].

Recent clinical trials of sodium-glucose transport protein 2 inhibitors (empagliflozin, canagliflozin) as a glucose lowering therapy have demonstrated impressive reduction in rate of progression of diabetic kidney disease, including reducing the incidence of the traditional end point used in renal trials of a combination of doubling of serum creatinine, requirement for renal replacement therapy or death [17, 18]. It appears that this class of drug restores tubuloglomerular feedback in the setting of diabetic kidney disease, which in turn reduces intraglomerular pressure and proteinuria. Further large clinical trials are in progress with these agents in both diabetic and nondiabetic kidney diseases.

38.8 Lifestyle Modification

Cigarette smoking is a risk factor for progression of CKD and CVD. This risk persists in patients receiving maintenance dialysis and even following transplantation [19]. Patients with CKD are strongly advised to stop smoking. Dietary intervention to reduce salt intake is important and has specific benefits in CKD as it reduces the risk of fluid retention. Salt restriction also augments the benefits of conventional therapy for hypertension; restricting dietary sodium intake to <5 g salt per day provides additional antihypertensive and anti-proteinuric effects of RAS antagonism. Reduction in dietary sodium intake has been shown to reduce proteinuria, independently of a reduction in blood pressure [20]. These results provide powerful argument for salt restriction in patients with CKD. Obesity is an additional risk factor for CKD progression. It is thought that obesity induces glomerular hyperfiltration and subsequently leads to proteinuria and kidney

damage. However, many obese patients are hypertensive and may have diabetes, thereby increasing the risk of CKD by several mechanisms. Therefore, appropriate lifestyle modification to avoid obesity may have secondary benefits on nephropathy.

38.9 Left Ventricular Abnormalities in CKD

Left ventricular abnormalities are common in CKD and strongly associated with adverse outcomes in ESKD. Echocardiographic studies report three patterns of cardiomyopathy – left ventricular hypertrophy (LVH), LV dilatation and left ventricular systolic dysfunction – that affect up to 90% of patients starting dialysis therapy. Each is associated with reduced survival compared with unaffected patients; effects that persist even after successful transplantation [21]. LVH develops early in the course of chronic kidney disease and is associated with stiffening of the LV wall, a precursor to diastolic heart failure [22]. Hypertension, anaemia, hyperparathyroidism and hypoalbuminaemia have all been associated with its development [23].

Histological analysis of the heart in animal models of experimental renal failure shows that LVH is associated with an increase in cardiomyocyte volume. This, in turn, results in increased oxygen diffusion distance and subsequent cardiomyocyte ischaemia. Cardiomyocyte number may increase initially but subsequently is reduced. In sub-totally nephrectomised rats, reduced capillary density per volume of myocardium is seen [24]. Electrophysiological abnormalities in calcium handling have been demonstrated with abnormal sarcoplasmic calcium uptake and increased cytosolic calcium concentrations in diastole [25]. These electrophysiological changes lower the threshold for arrhythmia and underpin the evolution of diastolic heart failure.

38.10 Abnormal Calcium and Phosphate Metabolism and CKD

During the progression of CKD, patients develop chronic hyperphosphataemia and hypocalcaemia, which result in hyperparathyroidism. Hyperphosphataemia is almost universal in ESKD and results from impaired excretion of phosphate. Conventionally it is thought that phosphate promotes vascular calcification and induces the transformation of vascular smooth muscle cells to an osteoblast-like phenotype leading to arterial calcification [26]. Coronary artery calcification is highly prevalent in ESKD and a marker for the future for CVD and mortality. Serum parathyroid hormone (PTH) is also involved in vascular calcification and is increased in experimental uraemia and promotes cardiac fibrosis, arteriolar thickening and raised PTH in patients which is a risk factor for mortality in ESKD [27]. However, recent studies have suggested that phosphate itself can have a direct effect on the vasculature and nitric oxide synthase-mediated vasodilatation, which contributes to the vascular dysfunction. Phosphate levels are modifiable through the use of phosphate binders so targeting phosphate could result in improved vascular function and a decrease in CV-related events in the CKD population. In the last few years, fibroblast growth factor 23 (FGF-23) has been identified as playing a pivotal role in ESKD-associated CVD. FGF-23 is a phosphaturic hormone, which rises as eGFR falls to compensate for phosphate retention. Off-target (non-renal) effects of FGF-23 are to promote LV hypertrophy and cardiac fibrosis, consequently associated with increased mortality [28]. Emerging data support two pathophysiological mechanisms in the contribution of bone mineral disorders/FGF-23/phosphate to the CV complications of CKD: first, vascular calcification and dysfunction – and the direct vascular effects of hyperphosphataemia – and second, fibrotic LVH, to which FGF-23 and PTH contribute and which leads to an increased risk of heart failure and sudden, arrhythmic, cardiac death. These studies identify FGF-23 both as a biomarker and a potential future therapeutic target.

38.11 Novel Therapeutic Targets in CKD

With better understanding of the various signalling pathways in CKD, novel therapeutic targets are emerging, which may be exploited to reduce the progression of CKD. We focus on

Table 38.3 Novel targets for the treatment of renal fibrosis with potential for clinical translation to man

Novel target	Effect	Therapeutic translation
miRNAs – small noncoding RNA	Blockade though anti-miRs blocks fibrosis and improves renal structure and function	Blockade though anti-miRs blocks fibrosis and improves renal structure and function
Relaxin – small peptide in the insulin family	Genetic deletion of relaxin results in renal hypertrophy, dysfunction and fibrosis. Administration of recombinant peptide reverses functional decline and fibrosis	Recombinant relaxin peptide
BMP-7 – bone morphogenic protein 7, member of TGF-β superfamily	BMP-7 is reduced in renal disease. Administration of recombinant BMP-7 reduces renal damage and fibrosis in a large number of preclinical models	Recombinant BMP-7 protein
Alk-3 receptor – activin-like kinase receptor type III is a bone morphogenetic protein receptor and member of the TGF-β superfamily	Agonists for this receptor include BMP-7. Activation of ALK-3 prevents renal damage and fibrosis	THR-123 is a synthetic agonist of ALK-3 receptor
TG-2 – transglutaminase 2, Ca2+-dependent enzyme which binds extracellular matrix and induces ECM stabilisation	Blocking TG-2 prevents fibrosis and improves renal function in a variety of renal injury models	Blockade of irreversible chemical inhibitors or monoclonal antibody against TG-2

tubulointerstitial fibrosis (TF), the final common pathway in 'irreversible' renal scarring, a histological finding common to many progressive kidney diseases.

38.12 Tubulointerstitial Fibrosis

Despite advances in the understanding of the molecular mechanisms driving fibrosis and a variety of preclinical strategies to inhibit/reverse TF, no therapies, which specifically target TF, are in therapeutic use. Finding effective targets for future therapeutic design which can be translated from preclinical to clinical use depends on having a deep understanding on the molecular signals that modulate fibrogenic events in the kidney. Recently miRNAs have been shown to be implicitly involved in renal dysfunction. Two miRNAs, specifically miR-21 and miR-214 [29, 30], have shown to be upregulated in experimental models of renal fibrosis, and blockade of these miRNAs prevents fibrosis indicating that targeting these miRNAs could have potential as a novel therapeutic [29]. TGF-β is a profibrotic cytokine which is critical in the progression of CKD and fibrosis. Blockade of TGF-β directly by antibody-based therapies showed utility in preclinical models, but this has not translated to man. More novel blockers of TGF-β signalling which have been investigated preclinically include BMP-7 mimics, ALK-3 agonists [31] and transglutaminase-2 antagonists [32] but as yet have not entered clinical trials (Table 38.3).

Conclusion and Clinical Perspectives

CKD is common and is increasing in prevalence in both the Western and developing world. Although kidney failure requiring dialysis and/or transplantation is less common, CKD and its complications place an enormous burden on patients, carers and the wider NHS. Better treatments are required for treatment of both CKD and its complications to both minimise progression of CKD to kidney failure and reduce the dramatically elevated cardiovascular risk associated with CKD.

Gaps in Knowledge

- Better understanding of molecular mechanism involved in renal fibrosis will identify novel targets for interventions to reduce progression of CKD.
- Strategies are required to rapidly move therapeutic agents which may retard progression of CKD into clinical trials which are able to assess their efficacy.
- Better surrogate markers of progression or CKD (imaging, biomarkers, etc.) are required to facilitate these trials.
- Therapeutic agents to regress vascular calcification are required.

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Obesity

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_39

Key Concepts

- Adipocytes (cells in adipose tissue) store dietary triglyceride, and new adipocytes are formed once cells are full.
- In some individuals, new adipocytes cannot be formed, and existing adipocytes have to expand in size, becoming insulin resistant and leading to cardiometabolic disease.
- Obesity is associated with adverse cardiovascular risk factors such as hypertension, increased lipids and type 2 diabetes.
- Cardiac structure and function are altered by obesity, even in children, with higher left ventricular mass and diastolic dysfunction.
- Weight loss improves adverse cardiovascular risk factors, and bariatric surgery has been shown to reduce the risk of cardiovascular events.

39.1 Introduction

Originally thought of as an inert storage site, the adipose tissue is now considered to have a central role in the development of insulin resistance and its associated pathology. The adipocyte has roles in lipid storage, in thermogenesis and in endocrine/paracrine signalling.

39.2 Types of Adipose Tissue

Human white adipose tissue can be classified according to anatomical location such as subcutaneous adipose tissue and visceral adipose tissue. Eighty percent of white adipose tissue is located in subcutaneous compartments that are distributed throughout the body, whereas around 10–20% is located in the visceral compartment around the mesentery and omentum. Visceral adipose tissue can deliver fatty acids directly to the liver via the splanchnic circulation and is closely linked to insulin resistance and an adverse metabolic and inflammatory profile [1, 2]. There are also small amounts of perivascular adipose tissue around blood vessels, and adipose tissue can also be found in the liver, muscle, joints and bone marrow. Brown adipose tissue stores are minimal in humans, and significant amounts are found only in infants or in adults who have undergone cold adaptation. Brown adipose tissue promotes non-shivering thermogenesis via the expression of uncoupling protein 1 (UCP1) in its mitochondrial membranes and may have an important role in energy homeostasis [3]. White adipocytes can adapt to take on some of the features of brown adipocytes, and beige adipocytes with intermediate features are produced [3].

39.2.1 Cell Types in Adipose Tissue

Adipose tissue is comprised mainly of adipocytes. Excess fatty acids are stored in lipid droplets within adipocytes as triglyceride and in this way prevent lipotoxicity resulting from excessive circulating fatty acids or triglyceride storage in other organs. The other cells found in adipose tissue are called adipose-derived stromal cells [4]. These stromal cells are a mixture of pre-adipocytes, endothelial cells, fibroblasts, lymphocytes, macrophages, myeloid cells, pericytes, smooth muscle cells and mesenchymal stem cells. Adipose tissue stromal cells support the proliferation and the differentiation of pre-adipocytes into adipocytes and in addition secrete a variety of cytokines and growth factors with potential paracrine effects. Adipose-derived mesenchymal stem cells are multipotent and can differentiate into a variety of cell types, i.e. adipocytes, osteoblasts, chondrocytes and myocytes.

39.3 Adipose Tissue Expansion

After a meal or when an individual is in positive energy balance, small subcutaneous adipocytes take up free fatty acids released by lipoprotein lipase from triglyceride contained within circulating plasma lipoproteins. These fatty acids are re-esterified as triglycerides resulting in the production of larger, mature adipocytes [4]. Later release of fatty acids from adipose tissue triglyceride stores is regulated by insulin. The accumulation of subcutaneous adipose tissue with plenty of capacity to store fat is regarded as a benign process. Some anatomical sites of storage, i.e. lower body subcutaneous adipose tissue, appear to be superior at regulating fatty acid storage, whereas

central subcutaneous adipose tissue appears to be more insulin resistant, and there is less net storage and more net release of fatty acids into the circulation.

If there is insufficient capacity to store triglycerides in the current mature adipocytes, new adipocytes are formed from pre-adipocytes in order to increase the storage capacity of the adipose tissue [5]. The formation of new adipocytes (adipogenesis) occurs in two steps [3]. The first step is commitment to differentiation and involves the production of white pre-adipocytes from mesenchymal stem cells. Committed pre-adipocytes are no longer multipotent and can only differentiate into adipocytes or proliferate. The second step is terminal differentiation to form mature white adipocytes which have stored lipid and have the characteristic appearance of the mature adipocyte containing one single lipid droplet that occupies almost all the space within the cell. Expansion of adipose tissue to increase the number of adipocytes is termed hyperplasic expansion.

39.3.1 Failure of Adipose Tissue Expansion

In some individuals there appears to be a limited ability to produce mature adipocytes from pre-adipocytes, i.e. to undergo hyperplasia, and instead excess fatty acids are stored in existing mature adipocytes leading to an increase in their size [6]. This is termed hypertrophic expansion, and the larger adipocytes formed tend to be dysfunctional triggering pathological consequences. Hypertrophic adipocytes are insulin resistant leading to increased lipolysis due to resistance to the anti-lipolytic effects of insulin and release of fatty acid from the adipocytes results [3]. Failure of angiogenesis and provision of an adequate blood supply to hypertrophic adipocytes leads to necrosis, macrophage infiltration into adipose tissue and inflammation and adipokine release. The "spillover" of fatty acids unable to be retained in hypertrophic subcutaneous adipocytes leads to an increase in the visceral fat compartment. Visceral adipose tissue is a marker for insufficient storage capacity in subcutaneous depots and can be regarded as a primary ectopic site of triglyceride accumulation.

39.3.2 Development of Systemic Insulin Resistance and Increased Risk of Metabolic Disease

Failure of adipocyte expansion leads to insulin resistance and accumulation of fatty acids, stored as triglyceride in the liver as described above. Liver fat accumulation inhibits insulin suppression of glucose production and results in increased liver triglyceride-rich very low density lipoprotein (VLDL) production. As the ability of subcutaneous adipocytes to store the triglycerides carried in VLDL is compromised, other tissues start storing the fat. This causes the dedifferentiation of beta cells in the pancreas and eventual failure of insulin secretion. Muscle triglyceride accumulation leads to decreased muscle glucose utilisation contribution further to insulin resistance. Accumulated ectopic fat causes lipotoxicity in the tissues concerned and can contribute further to the pro-inflammatory environment already generated by inflamed adipose tissue. Thus ectopic fat accumulation in liver is closely linked to the development of insulin resistance and type 2 diabetes mellitus [3, 7]. It is notable that ethnic groups with an increased propensity to have visceral fat, such as South Asians, are at increased risk of type 2 diabetes which is proposed to result from limited adipocyte expansion [8]. Recently it has been shown that very low calorie diet interventions that are associated with reductions in liver fat and pancreatic content have been shown to reverse type 2 diabetes [7]. Since these pathways also increase cardiometabolic risk, it is not surprising that visceral fat accumulation is also associated with atherosclerosis [9].

39.4 Epidemiology of Obesity and Cardiovascular Disease

39.4.1 Epidemiology Support for Obesity Links to CVD

Most people might think obesity is a strong risk factor for cardiovascular disease (CVD) so that rates of heart attacks ought to be rising as population obesity levels continue to rise. However, the truth is that obesity per se is a modest CVD risk factor at least as compared to type 2 diabetes,

where the link is much stronger. Thus, whilst diabetes incidence rates have escalated over the last two to three decades, in part due to rising obesity levels in many countries, CVD rates have in fact declined in high-income countries due to better treatment of "downstream" risk factors. Nevertheless, rising BMI from a younger age is causing increasing concern since if individuals become obese before other risk factors are often measured and so targeted (as occurs in the younger adults and adolescents), then a reversal in the population declines in CVD may occur over the forthcoming decades. Moreover, rising population BMI brings with it a decline in quality of life and higher risks for many other chronic conditions. For those researchers and students interested in CVD, it is important to understand better the pattern and the nature of the links of obesity to CVD.

The best evidence linking BMI to future CVD outcomes is from a paper led by the Emerging Risk Factor Collaboration (ERFC) [10]. This study collated individual participant data from over 58 cohorts of individuals without prior CVD to assess the links between three measures of obesity (BMI, waist circumference and WHR) and incident coronary heart disease (CHD) and incident ischaemic stroke (Fig. 39.1). In this work, it was noted that in those with BMI of 20 kg/m^2 or higher, hazard ratios for CVD were 1·23 (95% CI 1·17–1·29) with BMI, 1·27 (1·20–1·33) with waist circumference, and 1·25 (1·19–1·31) with waist-to-hip ratio, after adjustment for age, sex, and smoking status. These hazard ratios were calculated for a one-standard deviation higher baseline value of each of these three adiposity measures, equating to 4·56 kg/m^2 higher BMI, 12·6 cm higher waist circumference and

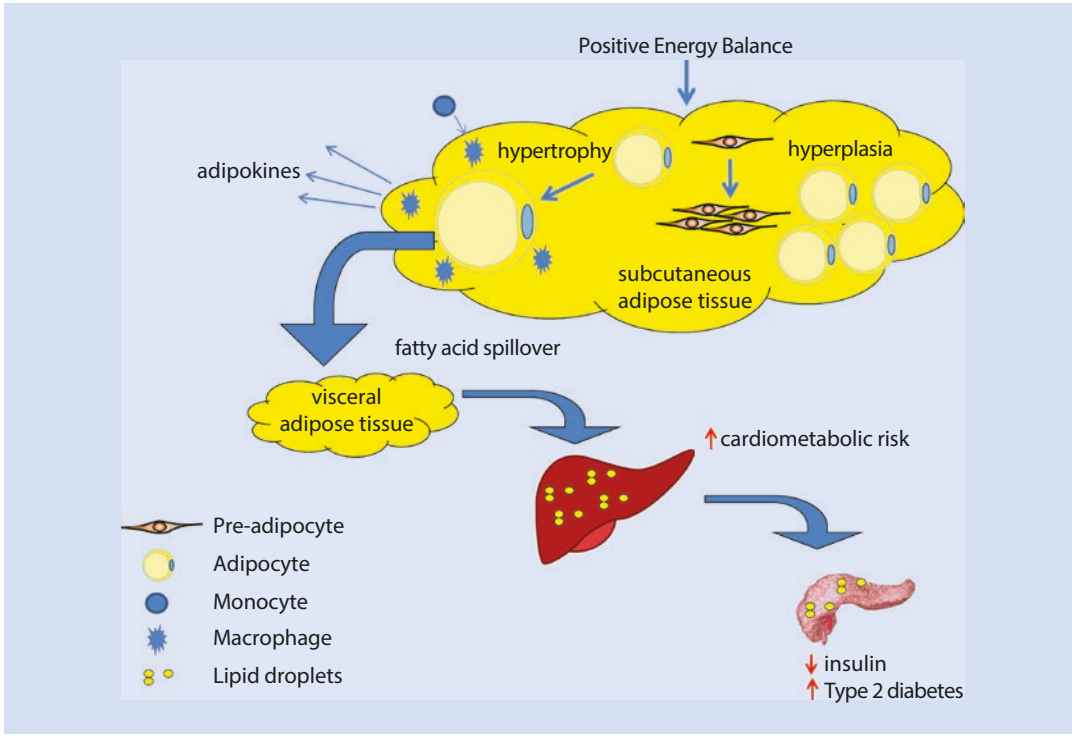

Fig. 39.1 Excess calories from the diet can be stored in one of two ways in subcutaneous adipose tissue. The first, benign, way (hyperplasia) is for preadipocytes to differentiate and increase the number of adipocytes in which to store the excess fat as triglyceride. The second way (hypertrophy) occurs when the pre-adipocytes fail to expand and the triglyceride is stored instead in the same number of larger size adipocytes. Larger adipocytes are more insulin resistance and begin to "spill out" fatty acids. Furthermore they tend to become hypoxic and necrotic, thus attracting macrophage infiltration and the development of adipose tissue inflammation. Fatty acids not retained by hypertrophic adipocytes are stored first in the visceral adipose tissue, and when the ability of that depot to expand is exceeded, triglycerides are stored ectopically in the liver leading to insulin resistance and the development of a cardiometabolic risk phenotype and/or in the pancreas leading to beta cell toxicity and failure

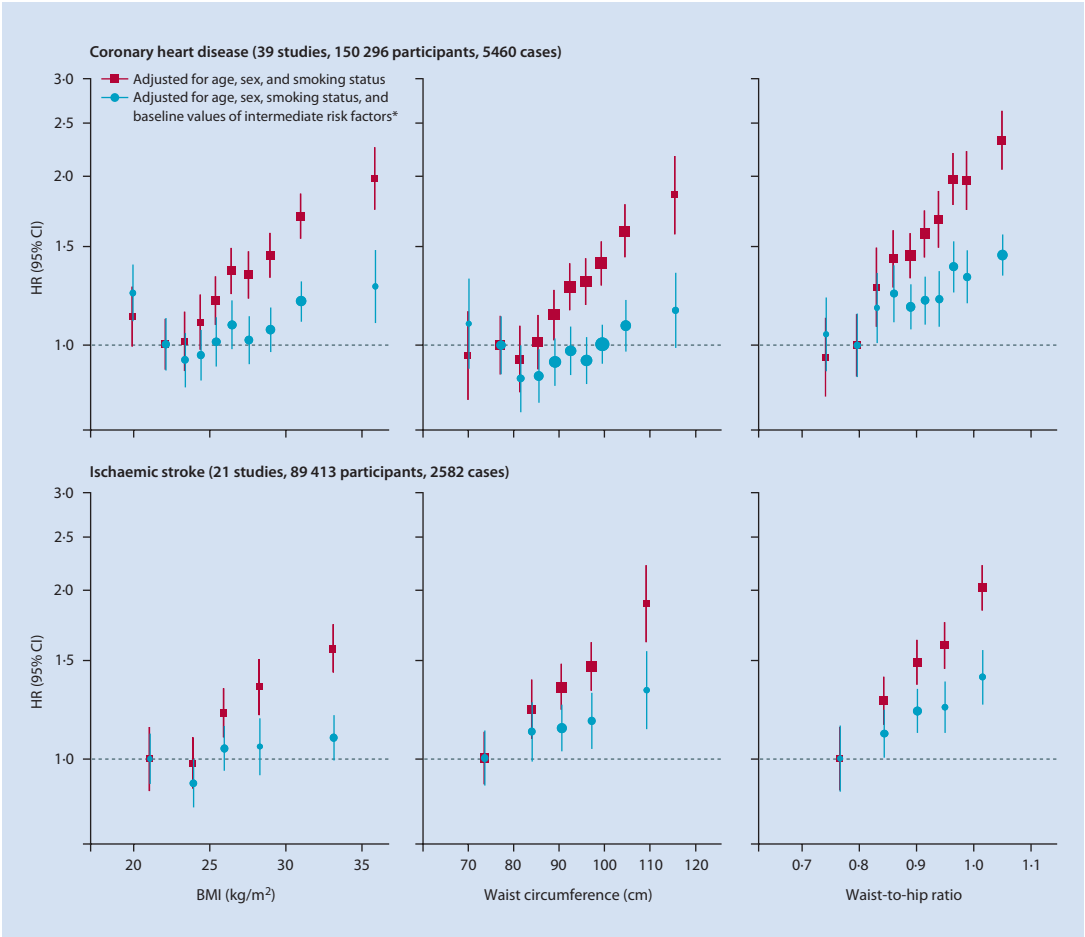

Fig. 39.2 HRs for coronary heart disease and ischaemic stroke across quantiles of baseline BMI, waist circumference and waist-to-hip ratio. (Taken from [10], with permission). Regression analyses were stratified, where appropriate, by sex. Adjusted study-specific log HRs were combined by multivariate random effect meta-analysis. Y-axes are shown on a log scale. Reference groups are the second deciles in the plots for coronary heart disease and the first quintiles in the plots for ischaemic stroke. HR hazard ratio, BMI body mass index. *Intermediate risk factors were systolic blood pressure, history of diabetes and total and HDL cholesterol

0·083 higher waist-to-hip ratio. It was also noted that following further adjustment for baseline systolic blood pressure, history of diabetes and total and HDL cholesterol, corresponding hazard ratios were significantly attenuated by over half, as shown in Fig. 39.1, so that independent associations of adiposity with CVD outcomes were rendered more modest. The other point of note is that the relationship of increasing adiposity measures with both incident CHD and ischaemic stroke seemed linear for waist circumference and WHR, whereas there was a slight J-shape for BMI. These findings suggest that if reported associations are causal, at a population level, keeping weight and adiposity levels down should lessen risk for adverse CVD outcomes (Fig. 39.2).

On a separate point, in this same ERFC paper, there was a hint that obesity is more strongly linked to risk for fatal than non-fatal CVD events, a finding also confirmed in the WOSCOPS study [11], although why this should be so needs further study. Furthermore, an increase in BMI appears to more strongly linked to risk for incident heart failure hospitalisation than incident acute myocardial infarction [12], at least in younger men. This surprising finding suggests that pathways linking obesity to heart failure go well beyond atherosclerosis, an area ripe for further investigation.

Table 39.1 Established and novel risk factors linking obesity to cardiovascular disease

	Risk pathways
Established	Hypertension
	Dyslipidaemia
	Diabetes
Novel	Inflammation
	Vascular dysfunction
	Haemostatic changes
	Cardiac remodelling
	Intravascular volume changes
	Renal/glomerular hyperfiltration

39.4.2 Risk Factors Linking Obesity to CVD

Rising BMI is linked to several established risk factors (Table 39.1). These include a rising blood pressure, abnormal lipid profile (rising levels of triglyceride and lower HDL-c, more so than an elevation in LDL-c levels) and type 2 diabetes. Thus, obesity must in part contribute to CVD risk via these "downstream" causal risk pathways. This is not to say the CVD risk from obesity (i.e. BMI) is fully captured by these risk factors. We know from the QRISK3 risk score [13], a risk score used in England and Wales to estimate 10-year risk for CVD events, that BMI adds to CVD risk even if its independent contribution is relatively modest when the above established risk factors are factored in.

Why might obesity influence risks for hypertension? The mechanistic explanations are only partially explored. Obesity is linked to greater ectopic fat including around blood vessels and might, via vasocrine signals, impair endothelial function [14]. Also, with rising insulin levels, there is a tendency to sodium retention and fluid retention in obesity, but multiple other mechanisms also likely operate to increase peripheral resistance and cardiac output.

In terms of diabetes, we know now very well that with rising BMI, different people at different thresholds put their fat into metabolic sensitive ectopic tissues like the liver and pancreas, as well into muscle, with the results that the liver and muscle become less responsive to insulin, whereas the pancreas's production of insulin may be impeded. The result is a tendency to hyperglycaemia which once the glucose or HbA1c levels reach specific thresholds becomes frank diabetes, a condition well established as a strong independent risk factor for CVD outcomes and heart failure.

Of course, there are many other pathways which may link obesity to CVD. These include a low-grade systemic inflammation, demarcated by slightly higher levels of circulating CRP and IL-6 levels, with evidence to suggest the latter but not former being causal for CVD, altered haemostatic pathways, renal adverse effects as well as ectopic cardiac fat. Other researchers also often evoke the presence of fatty liver as a link between obesity and CVD, but this area remains contentious. In truth, no one knows to what extent these factors mediate risks, and it is difficult to determine cause from association for many of these putative factors.

Finally, with respect to risk pathways, there is now overwhelming evidence that cardiac structure and function are altered by obesity, even in children, although initial changes can be subtle. For example, in a cross-sectional study of heart structure and function in 612 adolescents and young adults (aged 10–24 years), left ventricular mass (indexed to body surface area) was greater in the obese individuals than in lean controls [15]. Furthermore, diastolic function was also impaired in the obese group suggesting adverse effects on several aspects of heart function. This finding in younger adults together with other evidence from our group [16] suggests that the overall lifetime exposure to higher obesity levels matters to CVD risk more so just becoming obese at one time in point in later life.

39.4.3 Genetics Epidemiology Establishing a Link Between Obesity and CVD

In recent years, researchers have realised that genetic polymorphisms which are relatively common and which determine lifelong differences in phenotypic characteristics such as BMI can aid the search for causal risk factors and novel drug targets. This type of work is called Mendelian ran-

domisation and is now popular way to distinguish causation from simple association. Using this information, researchers have recently shown that a genetic predisposition to being heavier lifelong (based on a genetic risk score comprising 93 single-nucleotide polymorphisms associated with BMI from previous studies) is associated with worse CHD outcome as well as relevant risk factors, systolic blood pressure and type 2 diabetes [17]. These genes were associated with adverse risks after adjusting for obvious confounders. Of course, one must caution that this type of work is not always without potential caveats, but, if done properly, it can help prioritise likely causal pathways.

39.4.4 The Treatment of Obesity

The main treatment of obesity is through weight loss using a combination of diet, physical activity and behavioural techniques [18]; this is known as a lifestyle intervention and achieves around 5–10% weight loss in total. These are generally an energy-reduced diet (the standard is a 600 kcal deficit per day) and the use of techniques such as goal setting, self-monitoring, relapse prevention and peer support to aid weight loss and promote weight loss maintenance. Physical activity has a limited role in weight loss as the amount needed for a sufficient energy deficit is very high, but it has a more established role in helping maintain weight loss.

Bariatric surgery is a surgical procedure with the main purpose of inducing weight loss. The three most commonly used types are sleeve gastrectomy, Roux-en-Y gastric bypass and laparoscopic adjustable gastric band. All three types alter the anatomy of the stomach and upper gastrointestinal track, and they achieve a 20–40% weight loss depending on the type used. The risk of death as a result of the operation is very low if patients are selected well, but there is a risk for longer-term complications and the need for reoperation, and lifelong nutritional supplements and monitoring is required.

Several medications have been available for the treatment of obesity, but many have become unavailable due to side effects, and the availability of newer medications is limited as they are not generally any more effective than a good-quality lifestyle programme.

39.4.5 The Effect of Weight Loss on Cardiovascular Risk Factors

There is good evidence from many randomised controlled trials that weight loss of 5–10% of body weight can significantly improve cardiovascular risk factors [18]. A 10% weight loss is associated with a 0.25 mmol/l drop in total cholesterol, a 6.1 mmHg drop in systolic blood pressure and a 3.6 mmHg drop in diastolic blood pressure. Given the strong link between type 2 diabetes and cardiovascular disease, it is important to note that a 7% reduction in weight can reduce the risk of progressing from impaired fasting glucose (a state where the fasting glucose is above the normal rage but not yet at the diabetes range) by over 50%. This has been shown in numerous randomised trials, most famously the US-based Diabetes Prevention Programme, and such programme has been implemented in clinical practice worldwide.

39.4.6 The Effect of Weight Loss on Cardiovascular Events and Mortality

There have been no randomised trials of non-surgical weight loss interventions with a positive effect on cardiovascular events or mortality. The US-based LookAHEAD trial [19] randomised 5145 adults with existing type 2 diabetes to either usual care of general lifestyle advice or to a very intensive weight management programme including the options of total meal replacements, personal trainers and frequent meetings with a dietician over 4 years. The study aimed to answer the question of whether intentional weight loss reduced the incidence of fatal and non-fatal cardiovascular and cerebrovascular events. However the study was eventually stopped after nearly 10 years of follow-up as it was considered futile to continue as there was no sign of a difference in outcomes between the two groups. However it is considered that this does not mean that there is no cardiovascular benefit from weight loss. In LookAHEAD all the participants had very well-controlled diabetes, good blood pressure and lipid control, and the control arm lost weight during the trial; overall the rate of cardiovascular events in participants was far lower than that in the general population with type

2 diabetes. These factors are thought to have affected the outcome of the trial and make it very different from real-world patient care.

There is evidence of a reduction in cardiovascular events from observational studies of bariatric surgery [20], where the weight loses are far larger. Compared to people with obesity not choosing to have bariatric surgery, those having surgery have a lower risk of death after 10 years. There is a reduction in cardiovascular events though these are seen in those who had type 2 diabetes at the time of surgery. This suggests that the normalisation of glucose metabolism alongside improvements in lipids, blood pressure and other cardiovascular risk factors is the means in which this procedure affects cardiovascular risk. Bariatric surgery also has an effect on the development of heart failure, with lower rates of heart failure development in patients with obesity who chose to have surgery versus those who did not. However it should be noted that the observational bariatric surgery studies that have had sufficient length of follow-up to allow cardiovascular end points to be assessed were all started in the 1980s and 1990s when the treatment of type 2 diabetes and cardiovascular risk was far inferior to current practice. These results may not be seen in a contemporary cohort; instead it is more likely that the outcome would be that metabolic control could be achieved with less mediations.

Gaps in Knowledge

- More research is required to understand the exact mechanisms related to adipocyte dysfunction and the development of pharmacological therapies targeted towards this.
- Other than bariatric surgery, there is a lack of weight loss interventions which can induce long-lasting weight loss in a majority of individuals leaving it a chronic relapsing condition. It is hoped that in future, medications may be able to replicate the effects of bariatric surgery.

Conclusion and Clinical Perspectives

- Hypertrophic expansion leads to dysfunctional adipocytes triggering pathological consequences. Insulin resistance leads to increased lipolysis, failure of angiogenesis leading to necrosis, macrophage infiltration into adipose tissue and inflammation and adipokine release.
- Epidemiological and genetic data, together with what is known about obesity and its effects on some important risk factor pathways, as well as several putative risk factors, confirm an important link between obesity and CVD outcomes.
- Weight management either through diet and physical activity or with bariatric surgery can improve obesity-related adverse cardiovascular risk factors. There is some evidence from bariatric surgery that sustained large-scale weight loss may reduce the risk of cardiovascular events and death.

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Diabetes and Vascular Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_40

Key Points

- Cardiovascular disease (CVD), also known as macrovascular disease, is the leading complication causing morbidity and mortality in types 1 and 2 diabetes.
- In both conditions, microvascular complications (retinopathy, nephropathy, neuropathy) also carry a heavy burden: visual loss, kidney failure and foot ulceration/amputation. These are strongly related to controlling high blood glucose (hyperglycaemia) and its downstream cellular consequences.
- In type 1 diabetes, hyperglycaemia and its downstream consequences are the main drivers of CVD for the first 10–15 years after onset. Hypertension and dyslipidaemia become independent risk factors around the age of 35–40 years.
- In type 2 diabetes, insulin resistance leads to altered lipid metabolism, hyperglycaemia, pro-inflammatory signalling, oxidative stress, hypercoagulability and impaired insulin signalling in blood vessels; all these metabolic processes contribute to the pathogenesis of CVD.
- Insulin has direct actions on vascular tissues, stimulating secretion of vasoactive mediators that improve capillary recruitment and nutrient delivery to tissues and may be protective against atherosclerosis.
- A key focus of current research is identifying new and more specific drug targets to combat the pathogenesis and progression of diabetes-related vascular disease.

40

40.1 Cardiovascular Risk in Type 1 and 2 Diabetes

Diabetes is a metabolic disease defined by elevation of blood glucose (hyperglycaemia). In the 1950s it was differentiated into two main categories: type 1 (a state of insulin deficiency) and type 2 (a state of insulin resistance). Type 2 has become exceedingly common in the twenty-first century with prevalence quadrupling over the last three decades. Current global prevalence is 9.0%: e.g. 2.1% in sub-Saharan Africa, 5.4% in Scotland, 11.6% in China and 25.4% in Saudi Arabia [1]. It is approximately ten times more prevalent than type 1 diabetes. The UK (particularly Scotland) and the Scandinavian countries have the highest rates of type 1 in the world with incidence rising slowly year on year, for reasons that are unknown [2].

After insulin was discovered in the early 1920s and purified for therapeutic use, young people with type 1 began to survive into adulthood. It was then realised that many went on to develop retinopathy (blindness), nephropathy (kidney failure) and neuropathy (leading to a requirement for limb amputation following foot ulceration). It was suspected that many of these "microvascular" (small blood vessel) complications could be prevented with careful metabolic control of blood glucose, but it was not until the early 1990s that this was fully demonstrated in young adults randomised for 6.5 years either to intensive or conventional glucose control in the Diabetes Control and Complications Trial (DCCT) [3].

Follow-up of cohorts with type 1 has shown that – like type 2 – it is just as much a cardiovascular disease as a metabolic one. It is associated with an accelerated form of atherosclerosis causing high rates of premature cardiovascular disease (CVD), i.e. myocardial infarction (MI, "heart attack") and stroke. Post-trial follow-up of DCCT participants has shown that tight blood glucose control can also prevent these "macrovascular" (large blood vessel) complications [4]. However, tight control is rarely achieved with current methods of insulin delivery and blood glucose monitoring: updated estimates show that type 1 still carries a reduction in life expectancy of about 12 years, mainly due to CVD [5].

CVD has long been recognised to be the commonest cause of death in type 2 diabetes. Elevated CVD risk – two- to threefold that of the general population – is already established during the stage of "prediabetes" well in advance of the development of the condition itself [6]. The onset of type 2 is more insidious than that of classical type 1: diagnosis is based on a level of fasting blood glucose greater than or equal to a threshold value (7.0 mmol/L) associated with a future risk of retinal microvascular complications. There is often a preclinical phase during which insulin sensitivity is impaired ("insulin resistance") due to obesity during which glucose levels are compensated by

secretion of high levels of insulin from ß-cells in the pancreatic islets ("hyperinsulinaemia") [6].

The rising prevalence of type 2 particularly in high-income countries is in part due to more sedentary lifestyles and ready access to high-fat foods but also due to improved survival. Only in the last 10 years have cases of type 2 commonly been seen before the age of 40 years. As the condition develops from prediabetes towards frank diabetes, there is progressive impairment of the ability of insulin to dispose of circulating glucose and free fatty acids into target tissues (muscle and fat) for storage or utilisation. Other key actions of insulin are also impaired, including suppression of glucose production by the liver and regulation of blood flow into muscle. These complex cardiometabolic abnormalities are associated with high circulating levels of free fatty acids and triglycerides and a rise in blood pressure – a constellation of features known as "the metabolic syndrome".

The stage at which insulin secretion decompensates as type 2 diabetes develops is influenced by variation at multiple genetic loci, and there is often a family history. At this point, there may not yet be classical symptoms (thirst, polyuria and nocturia), but a "silent" risk of complications is already present. As the disease progresses, CVD risk increases until by 8 years from diagnosis the risk of MI can be as high as in a nondiabetic individual with a previous MI – "coronary risk equivalent" [7]. Hypertension (high blood pressure) and dyslipidaemia are major players in establishing this state of accelerated atherosclerosis in type 2 – not just blood glucose.

Perhaps because of these accompanying conditions, efforts to reduce CVD by targeting blood glucose have had limited success [8]. In contrast, long-term intensive control of blood glucose can reduce microvascular complications, as demonstrated over 11 years by the UK Prospective Diabetes Study [9] published in the late 1990s.

In this brief review, we summarise the mechanisms linking metabolic abnormalities with accelerated atherosclerosis (and hence CVD) in both main types of diabetes.

40.2 Insulin and the Vasculature

Classical actions of insulin are to promote and regulate storage of circulating glucose as glycogen in the muscle and liver and triglyceride in adipose tissue. To achieve this, it stimulates glucose uptake in striated muscle and adipocytes, promotes glycogenesis in liver and muscle and suppresses glucose production by the liver (gluconeogenesis). It also has marked effects on lipid metabolism, including the stimulation of fatty acid and triglyceride synthesis and suppression of lipolysis.

In addition to these well-characterised actions on carbohydrate and lipid metabolism, insulin was demonstrated in the 1990s to have additional direct actions on blood vessels. In particular, insulin regulates the synthesis of the vasoactive mediators nitric oxide and endothelin-1 by the vascular endothelium. Multiple studies have confirmed direct actions of insulin on the arterial vasculature, such that in larger conduit arteries, it increases compliance, whereas in smaller resistance arteries, it increases blood flow [10]. Furthermore, it has been proposed that insulin is able to augment its own metabolic actions by increasing the number of capillaries perfused within a target tissue (e.g. limb skeletal muscle) by dilating small arterioles ("capillary recruitment") [10, 11]. This is thought to lead to increased nutrient supply to muscle for storage as glycogen.

Insulin binds to the insulin receptor (IR) on target cells, including those of the vascular endothelium. The IR is a tyrosine kinase; insulin binding stimulates autophosphorylation, which serves to recruit scaffold proteins including the insulin receptor substrate proteins (IRSs) and stimulate diverse intracellular signalling cascades. In particular, insulin stimulates NO synthesis in cultured endothelial cells by a mechanism dependent on activation of the lipid kinase PI3K (phosphatidylinositide 3′-kinase) and the protein kinase Akt [also known as protein kinase B (PKB)] (Fig. 40.1) [10, 11].

In addition to augmenting production of the vasodilator NO, insulin can under some circumstances stimulate synthesis of the vasoconstrictor hormone endothelin-1 (ET-1) by the vascular endothelium. Insulin-stimulated secretion of ET-1 is PI3K-independent but dependent on the protein kinases ERK1/2 in endothelial cells (Fig. 40.1). Control of vascular tone by insulin is therefore likely to be regulated by the balance of NO and ET-1 secretion in response to insulin, as the vasodilator action of insulin is enhanced by ET-1 receptor blockade in humans and animals [10–13].

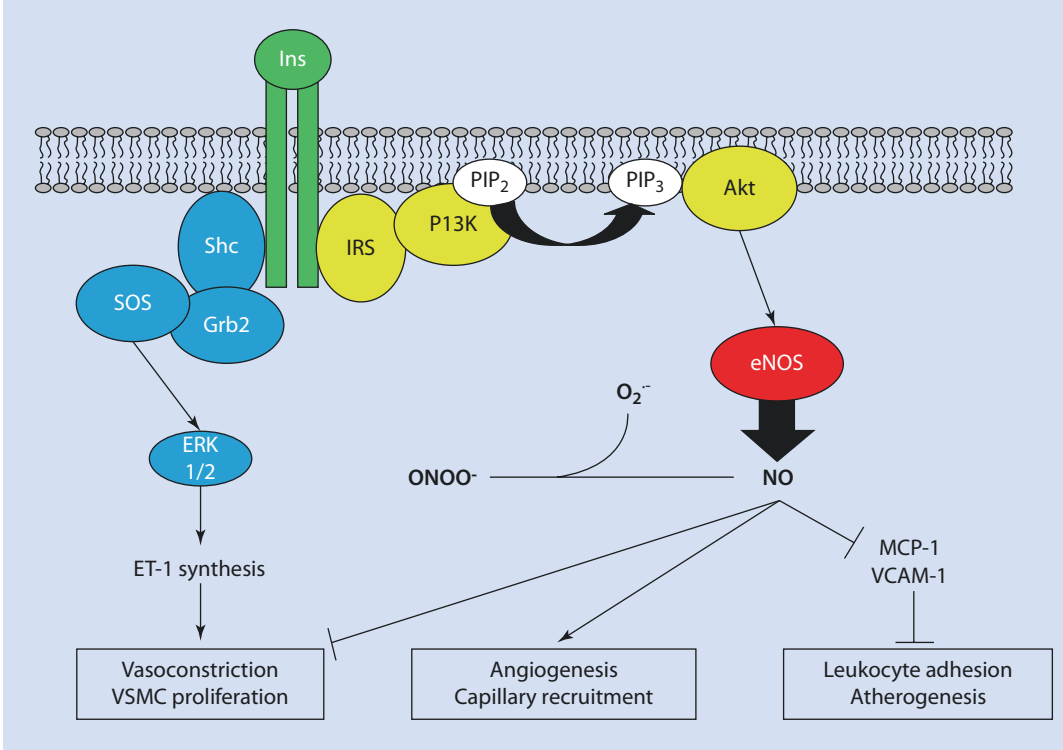


Fig. 40.1 Insulin-stimulated NO and endothelin-1 synthesis. Insulin (Ins) stimulates phosphorylation and recruitment of IRS proteins, leading to stimulation of PI3K, which synthesises PIP_3 (phosphatidylinositol-3,4,5-trisphosphate) which subsequently stimulates Akt, which phosphorylates and activates eNOS, leading to increased NO synthesis. NO stimulates vasodilation, angiogenesis and capillary recruitment and inhibits VSMC proliferation and atherogenic expression of MCP-1 and VCAM-1. NO is sequestered by elevated concentrations of superoxide, leading to production of peroxynitrite. Insulin stimulates the mitogenic protein kinases ERK1/2 through the Shc/Grb2/SOS pathway, stimulating release of endothelin-1 (ET-1) which stimulates vasoconstriction and VSMC proliferation

40.3 Insulin and Atherosclerosis

In addition to regulation of vascular tone, endothelial PI3K activation and NO synthesis suppress leukocyte adhesion to endothelial cells (Fig. 40.1), an effect mediated, at least in part, by reduced chemokine and adhesion molecule expression [including intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1)] due to attenuated pro-inflammatory signalling via the transcription factor nuclear factor κB (NFκB). Furthermore, insulin has been demonstrated to promote endothelial cell proliferation and migration and has direct effects on VSMCs, stimulating relaxation, proliferation and attenuating contractility [14].

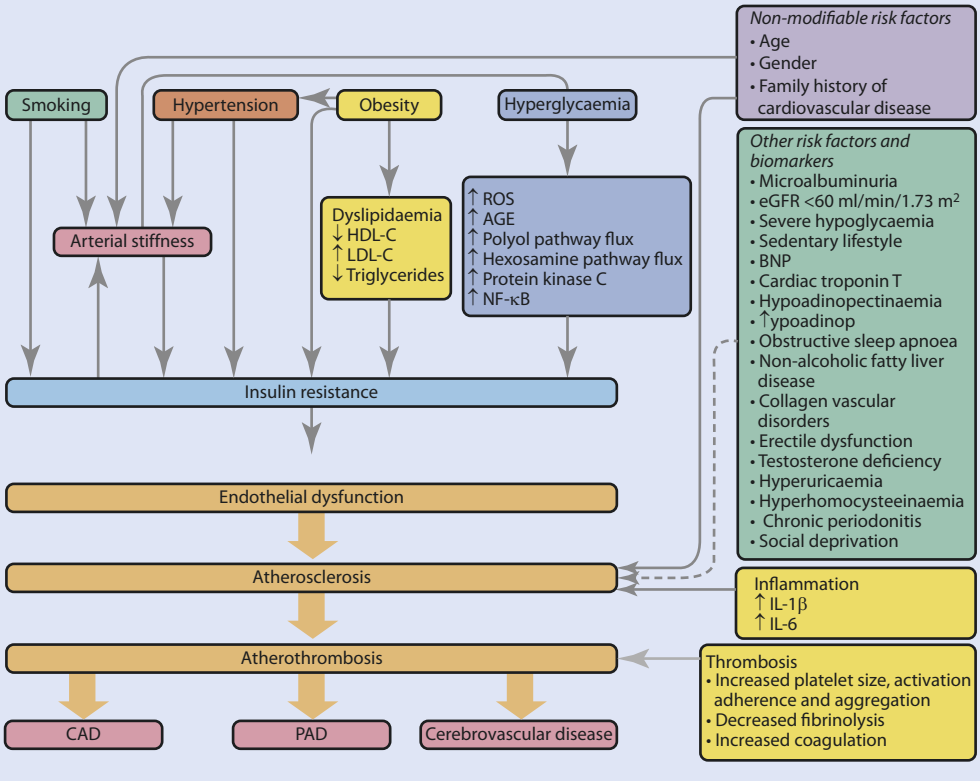
More recently, elegant studies in mice with genetic up- or downregulation of vascular insulin signalling have provided further insights into the anti-atherogenic actions of insulin. Mice with a targeted deletion of IRs in the vascular endothelium exhibit increased atherosclerotic lesion size, mononuclear cell adhesion and expression of vascular cell adhesion molecule-1 (VCAM-1). These effects are independent of whole body insulin sensitivity, glucose tolerance, plasma lipids or blood pressure, indicating that loss of insulin signalling in the vascular endothelium specifically exacerbates atherosclerosis [15]. Paradoxically, enhancing insulin sensitivity by endothelial-specific overexpression of the human IR in mice has been shown to result in a reduction in endothelial function, likely due to altered balance between NO and superoxide [16]. These findings may be explained by a balance of anti- and pro-atherogenic actions of insulin mediated by a number of insulin signalling pathway components at the post-receptor level, rather than at the level of the IR itself (see ▶ Sect. 40.4.3 below).

40.4 Mechanisms of Vascular Disease in Diabetes

40.4.1 Glucose

In type 2 diabetes, CVD risk is already elevated by the time glucose reaches the level diagnostic of diabetes, but over time individuals with the established condition are also subject to equivalent levels of glucose to those seen in type 1. Hence the multiple interrelated mechanisms by which glucose affects both microvascular and macrovascular complications are in play in both conditions (Fig. 40.2). As long-term restoration of normoglycaemia is extremely difficult to achieve with current therapies (whether in type 1 or 2), an exciting focus of research is to identify the most promising "druggable" targets in downstream pathways so that new and more specific drugs can be developed to block the molecular mechanisms linking glucose and complications.

Four pathways in particular have been studied: (i) the polyol pathway (target enzyme aldose reductase), (ii) the hexosamine pathway, (iii) protein kinase C (PKC-β) (inhibited by ruboxistaurin) and (iv) the receptor for advanced glycation end products (AGE/RAGE pathway) (targeted by "AGE-breaker" molecules and RAGE receptor blockers) [17]. Activation of these pathways by glucose, or its metabolism in these pathways, leads to oxidative stress from a number of sources [mitochondrial, endoplasmic reticulum, NADPH oxidases (Noxs)] and also chronic low-grade inflammation (e.g. via inhibition of the enzyme IKKβ which in turn activates NFκB).



Causality and directionality of relationships are not firmly established for all pathways and intermediate mechanisms shown.
AGE, advanced glycation end-products; BNP, B-type natriuretic peptide: CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein:IL- Iβ; interleukin-1β; IL-6, interleukin-6; LDL-C, low-density lipoprotein cholesterol; NF-κB, nuclear factor-κB; PAD, peripheral artery disease; ROS, reactive oxygen species.

Fig. 40.2 Schematic representation summarising the current understanding of the interaction between risk factors and the initiation and progression of atherosclerosis, with particular relevance to type 2 diabetes. (Reproduced with permission from Ref. [6])

Attempts to date to inhibit these complex interrelated individual pathways have been disappointing, probably because other pathways can compensate. Brownlee has influentially suggested that all are activated as a result of decreased activity of the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH), which occurs due to overactivity of the enzyme poly-ADP ribose polymerase (PARP) during DNA repair in response to glucose-induced oxidative stress [17]. The transketolase inhibitor benfotiamine was a candidate molecule that entered clinical trials with this rationale but did not progress to the clinic.

40.4.2 Lipids, Free Fatty Acids and Lipid Metabolites

Triglycerides and free fatty acids are elevated in type 2 as a result of insulin resistance; LDL cholesterol particles are relatively normal in quantity but small, dense and more atherogenic in quality. As cholesterol-lowering with hydroxymethylglutaryl-CoA reductase inhibitors ("statins") has been shown in large clinical trials to be effective in preventing CVD in middle-aged adults with types 1 and 2 diabetes, these are widely recommended by clinical guidelines. Free fatty acids are not directly targeted by current therapies; high concentrations in macrovascular endothelial cells result in increased oxidative stress and activation of the same four pathways as glucose, providing Brownlee's "unifying hypothesis" for the mechanisms of complications in types 1 and 2 [17].

At the molecular level, insulin resistance is associated with inhibitory phosphorylation of IRS-1 caused by activation of (i) PKC, (ii) c-Jun N-terminal kinase (JNK) and (iii) inhibitor of NFκB kinase β (IKKβ) signalling in response, respectively, to ectopic lipid metabolites (including diacyglycerol), pro-inflammatory cytokine signalling and endoplasmic reticulum stress [18]. Of course, once type 2 is established, many of these pathways are also activated by high glucose (see above); similarly, in type 1 diabetes, insulin resistance can occur over time (usually in association with insulin-induced weight gain) and act in concert with high glucose via these pathways.

40.4.3 Insulin Resistance

In states of insulin resistance, specific downregulation of the IRS/PI3K/Akt signalling pathway is detectable in the endothelium, without inhibition of other insulin-stimulated pathways, including ERK1/2 [14, 19]. This has been shown in blood vessels from rodent models of type 2/obesity and in cultured endothelial cells, with associated impairment of insulin-stimulated vasodilation and NO production [19]. Such selective molecular mechanism has been observed in endothelial cells from volunteers with type 2 diabetes and likely contributes to endothelial dysfunction via reduced bioavailability of NO and unaffected or increased ET-1 synthesis [19]. Mouse models in which the endothelial IR has been downregulated or upregulated will therefore exhibit impaired or strengthened signalling through both pathways rather than necessarily providing an accurate in vivo model of altered vascular insulin sensitivity. Overall, the evidence suggests that insulin in physiological concentrations has direct clinically relevant actions on blood vessels that inhibit the development of atherosclerosis.

40.4.4 Coagulation, Fibrinolysis and Cell Adhesion

Insulin resistance is associated with platelet activation (IRS-1-dependent), increased levels of coagulation factors (von Willebrand factor; plasminogen activator inhibitor-1), decreased fibrinolysis and increased expression of adhesion molecules (ICAM-1, VCAM-1). The resulting state of hypercoagulability is a further factor promoting accelerated atherosclerosis/CV disease in association with insulin resistance in type 2 diabetes [20].

40.5 Glucose-Lowering Therapies and CVD Risk

As mentioned above, long-term follow-up of young adults with type 1 participating in the DCCT showed that intensive insulin therapy for 6.5 years was associated with a long-term reduction in the risk of MI and stroke (the Epidemiology of Diabetes and Its Complications or EDIC study)

[3]. However, in most healthcare systems, only one around one-third of individuals with type 1 diabetes in the population achieve DCCT-type glucose control. This situation may improve in the coming years with wider use of adjunct therapy [e.g. metformin [21], SGLT2 inhibitors [22]) and/or availability of continuous glucose monitoring devices, insulin pumps and even closed-loop systems ("artificial pancreas" devices in which these two technologies work together using wireless technology and are governed by sophisticated algorithms). In adults with recently diagnosed type 2 in the UKPDS, intensive blood glucose control for 10 years using sulphonylureas and/or insulin did not clearly demonstrate a reduction in the rate of CVD, but post-trial follow-up 10 years after close-out did show a significant reduction in MI [9, 23]. Moreover, in an obese subgroup of the initial trial, CVD was prevented by the biguanide agent metformin. However, contemporary trials in people with more established type 2 – treated with antihypertensive drugs and statins (see below) – have not demonstrated a clear CVD benefit over 5 years of follow-up: the ACCORD study even suggested that overaggressive glucose lowering may actually be harmful, at least with some agents and in some individuals [8].

Until 2008, it was tacitly assumed by both the scientific community and the international regulatory authorities that drugs which reduced blood glucose would be effective not only in treating the symptoms but also in preventing complications. However, controversy surrounding a particular "insulin-sensitising" agent (rosiglitazone, a nuclear receptor agonist) radically changed the regulatory landscape. Since 2010, pharmaceutical companies have had to demonstrate that any novel agent is safe for the cardiovascular system [23, 24]. This is the environment into which newer classes of glucose-lowering agents have been evaluated for clinical use: the dipeptidyl peptidase-4 (DPP-4 inhibitors), the glucagon-like peptide-1 (GLP-1) agonists and the sodium glucose transporter-2 (SGLT) inhibitors.

As CVD develops slowly over many years, the double-blind, randomised trials required to compare newer agents with placebo (and standard of care) have had to recruit and follow up several thousand people with type 2 diabetes over several years to achieve sufficient statistical power. Some are designed purely to demonstrate safety (a specific level of "non-inferiority" in statistical terms), while others have been sufficiently large to demonstrate a moderate reduction in rates of CVD events ("superiority"). At the time of writing, all four trials with DPP-4 inhibitors have demonstrated safety (alogliptin, sitagliptin, saxagliptin, and linagliptin), three GLP-1 agonist trials have demonstrated superiority (liraglutide, semaglutide, and albiglutide), and three SGLT2 inhibitor trials have demonstrated superiority (empagliflozin, canagliflozin, and dapagliflozin) [25]. These results are starting to have impact on clinical guidelines, with the use of newer (more expensive) agents earlier in treatment pathways. In the case of rosiglitazone itself, safety trials were eventually reassuring [24], but the drug is no longer available (although a similar agent – pioglitazone – remains on the market).

In summary, in type 1 diabetes glucose lowering with insulin is effective in preventing CVD complications, but difficult to achieve. In type 2 diabetes, glucose lowering can be effective for CVD reduction when achieved with specific agents (including metformin, certain GLP-1 agonists and certain SGLT2 inhibitors). At present, metformin has maintained its position as first-line therapy in all clinical guidelines on the basis of favourable CVD prevention data from UKPDS [21] as well as low cost.

40.6 Multiple Risk Factor Reduction and CVD Risk

It was established around 20 years ago that hypertension and dyslipidaemia associated with insulin resistance in type 2 diabetes can effectively be targeted to improve cardiovascular outcomes using interventions unrelated to glucose lowering. A recent meta-analysis involving 100,354 people with type 2 diabetes confirmed that lowering systolic blood pressure by 10 mmHg (e.g. with ACE inhibitors) reduced rates of myocardial infarction by 12% and stroke by 27% [26]. Moreover, lowering low-density lipoprotein (LDL) cholesterol by 1 mmol/L with statins was shown in a 2008 meta-analysis of 14 randomised controlled trials involving 14,348 people to reduce the relative risk of cardiovascular events by 25%; the percentage reduction was almost identical to that in nondiabetic individuals but translated to a higher absolute reduction for almost all outcomes (including

myocardial infarction, stroke) due to higher baseline risk [27]. These CVD reduction strategies are generally easier to implement than glucose lowering.

Conclusions and Clinical Perspectives

Types 1 and 2 diabetes are cardiovascular as well as metabolic diseases. Early in the course of type 2 diabetes, insulin resistance in association with obesity is the predominant mechanism in the pathogenesis of vascular disease; hyperglycaemia comes into play later. Conversely, hyperglycaemia is central to the genesis of vascular disease in type 1 diabetes, but insulin resistance and obesity can play a role as the condition progresses. In both main types of diabetes, multiple molecular pathways conspire to produce a pro-atherosclerotic milieu characterised by dyslipidaemia, hypertension, oxidative stress, subclinical inflammation and hypercoagulability. Attenuation of the direct NO-mediated beneficial effects of insulin on cardiovascular cells and tissues in association with insulin resistance also plays an important role. While much progress has been made, rates of cardiovascular disease remain much higher in people with diabetes than in the general population and are falling more slowly [28]. Further investigation of the metabolic and cardiovascular signalling pathways engaged by insulin, and their modulation by factors altered in the two main types of diabetes may in the future identify better therapeutic targets to limit or prevent its devastating complications.

Gaps in Knowledge

- How can the rising prevalence of type 2 diabetes be halted, particularly in lower- and middle-income countries?
- Can impaired vascular insulin signalling be targeted by novel agents to further reduce rates of cardiovascular disease in type 2 diabetes?
- Does abnormal immune tolerance play a role in the accelerated atherosclerosis of type 1 diabetes?
- Can adjunct therapy improve long-term glucose control and cardiovascular outcomes in type 1 diabetes?

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Pulmonary Hypertension

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_41

Key Concepts

- Pulmonary arterial hypertension (PAH) is an area of unmet clinical need. More women get PAH than men, and survival is very poor even using existing drugs. Right ventricular failure is the leading cause of death in PAH patients.
- Current drugs target pulmonary vasoconstriction.
- Future drugs may target both vasoconstriction and pulmonary vascular remodelling as well as improve right ventricular function.
- Animal models are important for PAH research and constantly being refined.

Historically there was little interest in PAH until an 'epidemic' of the aminorex-induced PAH became apparent in the 1960s. Aminorex, which is an indirect serotonergic agonist, was sold as an over-the-counter appetite suppressant to induce weight loss and was withdrawn from markets in 1968 due to increased incidence of pulmonary hypertension. In patients who died from aminorex-induced pulmonary hypertension, the pulmonary vascular lesions were identical with those of PPH. Prompted by the aminorex epidemic, the World Health Organization (WHO) held its first meeting in Geneva in 1973 to assess pre-existing knowledge of PAH and to standardize the clinical and pathological nomenclature. These meetings were repeated in Evian, France, in 1998; Venice, Italy, in 2003; and Nice, France, in 2013 and the categories of PAH and algorithms for treatment updated at each of these meetings.

The population of PAH patients is changing [2]. The prevalence of PAH and high percentage of female patients (70–80%) have remained stable or increased over time. However, contemporary registry data indicate that the average age of patients diagnosed with PAH has increased, at least in the Western world. While the NIH registry reported the mean age at diagnosis to be 36 years in idiopathic PAH (IPAH) patients, contemporary registries report older populations ranging from 50 to 65 years. In addition, these older patients are diagnosed with more advanced PAH and reduced exercise capacity. They have multiple comorbidities and have even worse survival even when adjusted for age and disease (68% at 3 years) [3]. PAH involves increased pulmonary vascular resistance, which leads to high pulmonary pressures and increased load on the right ventricular (RV). This leads to RV failure (see below). It is a complex disease that involves dysfunction of several cell types within the pulmonary arteries, and RV and current therapies tend to address one or more of these, either directly or indirectly. There is increased proliferation, impaired apoptosis, and glycolytic metabolism in pulmonary artery smooth muscle, fibroblasts, and endothelial cells [4]. Endothelial dysfunction can lead to vasoconstriction, thrombosis, and mitogenesis.

41.1 Current Treatment Strategies

Significant advances have been made in the medical management of PAH in the past decade [5]. This has resulted in improved prognosis and quality of life but has also increased the complexity in making treatment decisions. A summary of the common therapeutic approaches is shown in Fig. 41.1. Therapy is now prescribed in accordance with the Nice 2013 consensus statement on the management of PAH and is based on functional class along with a number of other established prognostic factors, such as aetiology, n-terminal pro-brain natriuretic peptide, and 6 min walk distance [6].

41.1.1 Prostanoids

Prostacyclin is produced predominantly by vascular endothelial cells. It induces potent vasodilatation of all vascular beds and inhibits platelet aggregation. Additionally, it has cytoprotective and anti-proliferative effects. Reduced expression of prostacyclin synthase occurs in the pulmonary arteries of patients with PAH. Prostanoids can be administered intravenously (IV), subcutaneously (SC), by inhalation, or orally. Three parenteral prostaglandin analogues are currently approved for treatment of PAH: epoprostenol, treprostinil, and iloprost with epoprostenol improving survival [7]. Iloprost is delivered by the nebulized

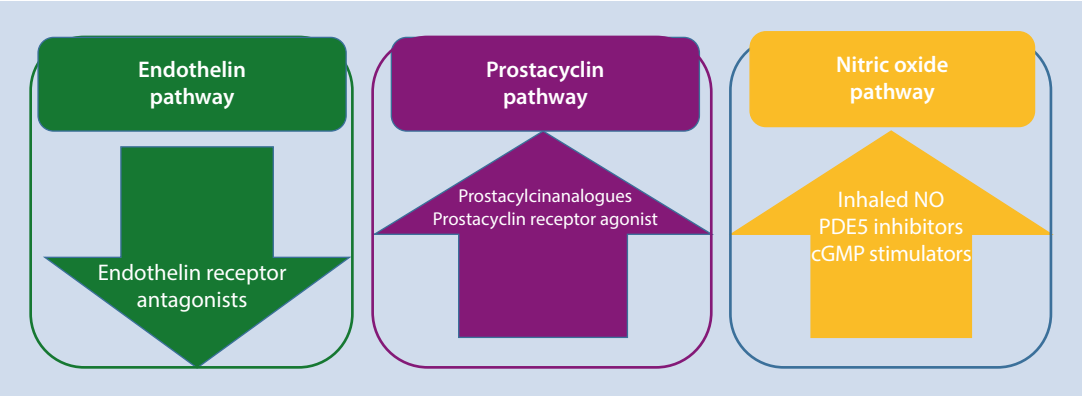

Fig. 41.1 Summary of pathways targeted by current PAH therapies

route for treatment of PAH, selectively promoting vasodilation in the pulmonary circulation in order to decrease the risk of hypotension in a population at risk of syncope due to reduced cardiac output. Treprostinil is not currently in use in the UK but is available in the USA in SC, inhaled, and IV forms. Selexipag is a novel orally active, non-prostanoid selective prostacyclin receptor agonist. It is not available as a licensed drug in the UK at present; trial data are awaited; however it holds promise as a potential future treatment with pilot data suggesting improvements in haemodynamics and exercise capacity.

41.1.2 Endothelin Receptor Antagonists (ERAs)

Activation of the endothelin system, resulting in raised levels of endothelin (ET-1), is evident in the plasma and lung tissue of PAH patients [8]. ET-1 exerts vasoconstrictor and mitogenic effects by binding to two receptors (endothelin-A and endothelin-B) in pulmonary vascular smooth muscle cells. Three ERAs are currently licensed in the UK (bosentan, ambrisentan, and macitentan) with a fourth, sitaxsentan, being withdrawn due to cases of fatal liver toxicity. Macitentan is the newest ERA and, in the randomized controlled trial (RCT) SERAPHIN, showed improved exercise capacity and significant reduction in the composite endpoint of death, atrial septostomy, lung transplantation, initiation of treatment with IV/SC prostanoid, or clinical worsening of PAH [9].

41.1.3 Nitric Oxide and Cyclic Guanosine Monophosphate Pathway

Impairment of nitric oxide (NO) synthesis and signalling through the NO-soluble guanylate cyclase (sGC)-guanosine monophosphate (cGMP) pathway is involved in the pathogenesis of PAH. Three main therapeutic targets exist:

1. Direct administration of inhaled NO
2. Enhanced effect of NO by increasing its enzymatic production: soluble guanylate cyclase (sGC) activators
3. Inhibition of NO metabolism: phosphodiesterase type 5 inhibitors (PDE5is)

41.1.3.1 Inhaled Nitric Oxide

Inhaled nitric oxide (iNO) has a short half life and acts locally and transiently in the lung as a selective pulmonary vasodilator to acutely decrease PAP and PVR. NO is absorbed systemically, where it is rapidly oxidized by haemoglobin to form nitrite, which interacts with oxyhaemoglobin, leading to the formation of nitrate and methaemoglobin (a potentially toxic effect). Currently iNO has no role in the long-term management of PAH; however it is commonly used in acute vasoreactivity testing in PAH, in persistent pulmonary hypertension of the newborn, and in other settings in adult critical illness such as acute respiratory distress syndrome.

41.1.3.2 PDE5 Inhibitors

Inhibition of the cGMP-degrading enzyme PDE5 results in vasodilation through the NO/cGMP pathway at sites expressing this enzyme.

Additionally PDE5 inhibitors exert anti-proliferative effects. Common side effects in this class are headache, GI disturbance and flushing, and nasal congestion. Three drugs exist, sildenafil, tadalafil, and vardenafil, with the former two licensed in the UK. All are administered orally.

41.1.3.3 Soluble Guanylate Cyclase Stimulators: Riociguat

Riociguat has recently been approved for the treatment of PAH, as monotherapy or in combination with ETAs for WHO Functional Class II–III PAH. Riociguat has a dual mode of action; it directly stimulates sGC independently of NO availability and acts in synergy with endogenous NO. In a recent large multicentre RCT, riociguat improved exercise capacity, haemodynamics, and WHO functional class, with reduced time to clinical worsening [10]. The most common adverse events were haemoptysis and pulmonary haemorrhage, with common side effects being headache, dizziness, and dyspepsia. Regular blood pressure monitoring is recommended due to the risk of hypotension. It has also been deemed efficacious in CTEPH, and studies are ongoing assessing its validity in pulmonary hypertension associated with idiopathic interstitial lung disease.

41.2 Recent Developments in Research

There are a number of exciting developments in the understanding of molecular pathways in pulmonary hypertension that may lead to novel therapeutic strategies for patients.

41.2.1 Sex and Oestrogen

The role of sex and oestrogen has become an important focus of research into the pathobiology of PH. Three- to fourfold more women than men develop PAH, whilst survival is poorest in men [11]. The reasons for this are unclear as oestrogens can be both pathogenic in the pulmonary circulation [12, 13] and protective when given exogenously [14]. Recent insights into this suggest an important role of the aromatase enzyme with increased expression of this in pulmonary arteries of affected patients [12]. This correlates with the findings that a polymorphism in the aromatase gene is associated with the development of porto-pulmonary hypertension [15]. A small 'proof-of-principle' clinical trial using inhibition of aromatase demonstrated the aromatase inhibitor, anastrozole, was safe and well tolerated and improved 6-minute-walk distance in patients with PAH [16].

41.2.2 BMPR2

Mutations in bone morphogenetic protein receptor-2 [BMPR2] underlie the majority of cases of heritable PAH [17]. Such mutations are associated with increased proliferation of pulmonary artery smooth muscle cells, endothelial cell apoptosis, exacerbation of vascular permeability, and altered translocation of leukocytes across the vascular wall [18]. Often the mutations present in the BMPR2 gene lead to a premature stop codon being introduced and truncation of the protein. This can be overcome by certain novel drugs (ataluren) which allow the 'run through' of this stop codon and produce an essentially normal BMPR2 protein [19]. In addition agents such as chloroquine have been shown to prevent degradation of BMPR2 protein via inhibition of the lysosomal degradation pathway [20, 21]. Recent data has suggested that BMP9 can directly and selectively stimulate the BMPR2 pathway within endothelial cells and protects these cells from apoptosis. Furthermore BMP9 has been shown to reverse pulmonary hypertension in Sugen hypoxia mice [18].

41.2.3 p38 MAP Kinase

Inflammation is recognized as important in the development of pulmonary vascular remodelling. For example, recent work has shown that inhibition of the pro-inflammatory pathway p38MAPK can reverse the pulmonary hypertension phenotype in two animal models. This suggests that this pathway could be a novel target linking inflammation and remodelling [22].

41.2.4 Epigenetics

The role of epigenetics in the development of PH is a rapidly growing field of research. Epigenetics is defined as changes in gene expression unrelated

to change in DNA sequence. Recently, the main focus of epigenetics research in relation to PH has been on microRNAs (miRNAs), and several miRNAs including miR-21, miR-204, miR-96, and the miR-143/miR-145 cluster have been found to be dysregulated in PH and contribute to the disease progression [23].

Two other epigenetic mechanisms that have received less attention are DNA methylation and histone modification. DNA methylation occurs via DNA methyltransferases (DNMTs) and usually silences genes. Histone acetylation and deacetylation are forms of post-translational modification that lead to increased and decreased transcription of genes, respectively. Acetylation occurs via histone acetyltransferases (HATs), whereas deacetylation is accomplished via histone deacetylases (HDACs). Current research suggests HDACs may be increased in PH. HDAC inhibitors (HDIs) are already used in the treatment of certain cancers making them an attractive therapeutic agent for PH. Therefore, current basic science research suggests it is likely that dysfunction of these epigenetic mechanisms may contribute to PH. The development of histone deacetylase (HDAC) inhibitors has shown reversal of pulmonary hypertension in animal models of the disease [24]. Another group of proteins involved in the reading of acetylation sites on lysine proteins is the BET proteins, and these proteins have been shown to be important in regulation of cardiac hypertrophy [25]. It is hoped that inhibitors of BET proteins may be a novel therapeutic approach.

41.2.5 Stem Cells

Regenerative therapeutic approaches represent a novel approach in the treatment of PH. These aim to repair damages of the endothelium or regenerate small blood vessels that have been occluded in the PH lung. Pluripotent stem cells have the greatest capacity for transdifferentiation and regeneration, and current research aims to establish methods to maintain their intended differentiation state and eliminate the risk of abnormal growth and tumour formation [26]. Although preliminary studies involving endothelial progenitor and mesenchymal stem cells have demonstrated promising results, considerable work still remains before clinical translation becomes a reality [27].

Lung transplantation is often the only treatment option for patients with late-stage PAH with the demand for lung transplants greatly exceeding the availability of suitable donor lungs. Moreover, patients who are fortunate enough to receive donor organs are faced with complications such as organ rejection and increased risk of death associated with chronic immunosuppressive therapies. Decellularized lung scaffolds may provide a solution to these limitations [28]. There is potential to prepare lung scaffolds from donor lungs deemed unsuitable for transplantation or from non-human species such as pigs [29]. After removal of resident lung cells by various detergents, the lung scaffold could then be recellularized using the patient's own stem/progenitor cells. Although preclinical studies provide evidence to suggest that decellularized lung scaffolds can be partially repopulated with various cell populations, and transiently supporting gas exchange when transplanted in vivo, much work remains to be done before whole lung tissue engineering becomes a therapeutic reality [29].

41.2.6 Serotonin and Growth Factors

Many cellular pathways have been implicated in the pulmonary vascular remodelling process as discussed above, and it is hoped that one day this research may translate to the clinic. The serotonin pathway was implicated in the 1960s after aminorex was shown to cause PH [30]. Since then, work on serotonin and its transporter (5-HTT) in animal models has shown involvement in vasoconstriction and remodelling in the pulmonary arteries [31, 32]. The mitogen-activated protein kinase pathway (in particular p38 MAP kinase) has been shown to control fibroblast proliferation, migration, and cytokine release in several animal models of pulmonary hypertension [33]. Vasoactive intestinal peptide (VIP) which acts through its two receptors (VPAC-1 and 2) leads to the activation of cAMP and cGMP as previously discussed. Studies have shown decreased serum levels of VIP in patients with PH. A vast amount of research has been carried out looking at the role of the TGF-beta superfamily of signalling enzymes which includes the bone morphogenic proteins (BMPs). Numerous growth factors have been implicated in the pulmonary vascular

remodelling process. Platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), basic fibroblast growth factor (BFGF), and insulin-like growth factor have all been shown to be increased in the PH lung. These growth factors interact with cells from the pulmonary vasculature and the cellular pathways resulting in vasoconstriction, migration, and proliferation of the cells, resulting ultimately in pulmonary vascular remodelling.

41.2.7 Animal Models for Pulmonary Hypertension

Animal models are an important research tool that have contributed extensively to our current understanding of pulmonary hypertension pathophysiology and function as a preclinical platform for novel experimental therapies [34].

Commonly used animal models are summarized in Table 41.1.

As with all animal models, concerns have been expressed over their ability to imitate the human condition. A shortcoming of the chronic hypoxic model is that it does not fully recapitulate the pulmonary vascular damage observed in humans with PH, displaying no obliterative pulmonary vascular lesions. However, the newer chronic hypoxia + SU5416 model, where rodents are subjected to both the vascular endothelial growth factor receptor inhibitor, SU5416, and hypoxia, results in the formation of occlusive vascular lesions allowing researchers to study the angioproliferative features of PH, in addition to haemodynamic changes adding greater clinical relevance to data generated. Furthermore, although it displays no pulmonary vascular remodelling, the pulmonary artery banding (PAB) model allows researchers to study the molecular events underlying RV hypertrophy and test the effects of novel cardioprotective experimental treatments.

Animal models have also confirmed the role of a variety of mediators including endothelin-1, serotonin, and BMPR2 in the development of PH and the remodelling process. Indeed, the monocrotaline, chronic hypoxic and chronic hypoxia + SU5416 models all demonstrate impaired expression of the BMPR2 pathway, a phenomenon that is also observed clinically, as loss of function mutations in BMPR2 are associated with the development of PH in humans. Heterozygous BMPR2 knockout mice also provide a useful tool to further study the condition. Additionally, female mice that overexpress the serotonin transporter (SERT+) spontaneously develop PH further demonstrating a role for serotonin [35]. These are useful for investigating the fact that PAH is more prevalent in women.

Table 41.1 Animal models of pulmonary hypertension and/or right ventricular dysfunction

Model	Species	Description
Monocrotaline	Rats	Monocrotaline (MCT) is a plant alkaloid. Metabolism of MCT into pyrolic derivatives initiates endothelial injury in the pulmonary vasculature. The endothelial injury is the initial trigger for obstructive pulmonary vascular remodelling that features intimal hyperplasia, medial hypertrophy, and adventitial thickening. Animals also display increased RVSP and RVH
Pulmonary artery banding (PAB)	Rats/mice	During PAB procedure a silk suture is positioned around the pulmonary artery, and the suture is tied to produce a constricted opening in the lumen of the artery. As the animal grows, the lumen narrows further, which results in increased RV afterload and RVH
Chronic hypoxia	Rats/mice	Pulmonary vascular remodelling induced by oxygen deprivation. Elevated RVSP and RVH observed. No obstructive intimal lesions in the peripheral pulmonary arteries
Chronic hypoxia + SU5416	Rats/mice	Chronic hypoxia + SU-5416 induces PH with pulmonary arterial changes resembling plexiform lesions. This model also displays increased RVH and RVSP

41.2.8 The Right Ventricle: Lessons Learned from Animal Models and the Clinic

In pulmonary arterial hypertension, angioproliferative lesions and vascular remodelling occur in the pulmonary vasculature and lead to the progressive rise in the pulmonary arterial pressure. However the main determinant of outcome in patients is the development of right ventricular failure. This was for many years felt to reflect a simple feature of the direct pressure effect and afterload on the right ventricle. Right ventricular failure is the leading cause of death and drives symptomology in PAH patients. As discussed above, current drugs do not however target right ventricular function.

More recent experimental evidence suggests that pulmonary pressure is not the sole determinant of RV failure [36, 37].

The PAB model in the rat described above is produced by constricting the pulmonary artery and is one in which there is a progressive increase in the afterload. Interestingly this model, although producing RV hypertrophy, depending on the tightness of the band, does not lead to RV failure as defined by RV dilatation, reduced cardiac output, and echocardiographic measurements (TAPSE) [38]. Contrast this with the other experimental animal models of pulmonary hypertension such as monocrotaline or chronic hypoxia + SU-5416 as described above. In these models there is very often clear evidence of RV dysfunction and failure. This would suggest that there is more to the process inducing RV failure than just pressure overload. Potential explanations would include that the lesions in the pulmonary vasculature are releasing additional factors which could lead to direct myocardial suppressant effects [39].

As the afterload increases that the RV is faced with, there is initially an adaptive hypertrophy, which allows the RV to function well as according to the physiological principles of the Starling curve. However with time and progressive increases in the pulmonary circulation pressures, the RV begins to show maladaptive compensatory mechanisms with dilatation and a fall in the RV function. Molecular pathways which are responsible for this abnormal maladaptive response are now being identified and include dysregulated reactive oxygen species, differential microRNA expression, and aberrant apoptosis.

From a molecular understanding, there are reductions in capillary density in the RV and associated increase in fibrosis observed in the SUGEN/hypoxia animal model. These changes are not seen in the LV and suggest a RV specific effect. Similar changes have been seen in the RV biopsies of some patients with pulmonary hypertension. The differences between the left and right ventricles are interesting and should not be entirely unexpected. For a start they are derived from different embryological sources, and recent research has shown different constitutive gene expressions between them when exposed to pressure afterload (e.g. the WNT pathway) [37].

Furthermore there is a distinct change in the energy metabolic pathways employed by the RV when exposed to pressure afterload. There is a switch to a more glycolytic pathway which initially is an attempt to try and conserve energy compared to the fatty acid metabolism it normally employs. However with time this can have effects on mitochondrial function and is felt to be one of the potential switches which leads to the maladaptive phenotype [40].

Conclusions and Clinical Perspectives

Although current treatment strategies have greatly improved the quality of life of pulmonary hypertension patients, survival remains poor. Further investigation into the molecular pathways involved in pulmonary hypertension that may lead to novel therapeutic strategies for patients. Furthermore, drugs that target components of the oestrogen pathway such as aromatase (i.e. anastrozole) and CYP1B1 (2, 4, 3′, 5′-tetramethoxystilbene) may prove to be beneficial for the treatment of PAH. As these drugs are already widely used in the treatment of cancer, this improves the translational potential of preclinical research.

Gaps in Knowledge

- Mechanisms underlying the sex differences in PAH onset and development
- Pathobiology of the RV and how it adapts and then fails in the face of an increased afterload

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Cerebral Small Vessel Disease and Vascular Cognitive Impairment

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_42

Key Concepts

- Cerebral small vessel disease is a term that can be applied to pathological changes, neuroimaging features or clinical presentations.
- Cerebral small vessel disease is a syndrome rather than a single disease entity.
- Various lesions associated with cerebral small vessel disease can be identified through neuroimaging; these should be considered together as reflecting a 'whole brain process' rather than taking each lesion type in isolation.
- Cerebral small vessel disease is a dynamic process (where lesions normally progress but can regress), and the classical features may represent the end stages of a process that has been progressing for years.
- Consensus, standardised definitions of the neuroimaging and pathological changes of cerebral small vessel disease have been important catalysts for progress and collaboration; consensus definitions around the clinical phenotype are now needed.

42.1 Introduction

Compared to the end organ damage seen in the heart, kidney and peripheral vasculature, chronic vascular changes in brain structure and function have traditionally received less clinical and research attention. The landscape is now changing, with recent, exciting progress in our understanding of pathophysiology and clinical manifestations. In this chapter we will primarily focus on the chronic, progressive syndrome of cerebral small vessel disease (cSVD). We will consider in turn the pathology, neuroimaging features and clinical phenotypes. Acute presentations of small vessel disease, such as lacunar stroke, are discussed in other chapters of this textbook.

42.2 Definitions

The term cSVD encompasses neuropathological, radiological, neuropsychological and clinical phenotypes (Fig. 42.1). The field of cSVD has been characterised by a confusing nosology with various definitions and terminologies. This inconsistency has complicated attempts at comparative or meta-analysis and ultimately has delayed research progress. Recent harmonisation efforts (focussed on pathological and neuroimaging phenotypes) have created a common language for describing many aspects of cSVD [1, 2]. In this chapter, where available, we will use these consensus terminology and definitions. Although we will consider cSVD as a distinct entity, separate from other neurodegenerative diseases such as Alzheimer's disease, increasing evidence suggests that this viewpoint is over-simplistic. The reality is that vascular and non-vascular processes frequently coexist and interact in a complex synergy that is not yet fully understood [3].

Contemporary thinking considers cSVD as a disease process that is manifest across the perforating cerebral arterioles, capillaries and venules. The vascular disease eventually causes damage to cerebral white and deep grey matter; this in turn causes characteristic neuroimaging appearances and eventually results in clinical symptoms and signs. Recent studies show that the cortex also is involved, although it is not clear yet whether this is a primary or a secondary effect.

An aetiopathogenic classification of cSVD has been proposed by Pantoni et al. [1]. Within this framework we can categorise cSVD by presumed causative factors of (1) arteriolosclerosis, by far the most common and best understood type of SVD; (2) amyloid angiopathy; (3) genetic, such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL); (4) inflammatory (auto-immune small vessel vasculitis, infectious vasculitis); (5) venous collagenosis; and (6) other aetiological processes such as post-radiation microvascular disease. This system reminds us that cSVD is a syndrome rather than a single disease and also highlights that under the rubric of 'small vessel', we need to consider arterial, capillary and venous circulations. These various pathologies are not mutually exclusive, and in particular arteriolosclerotic change may coexist with many of the other pathologies. In this review we will predominantly focus on the arteriolosclerotic subtype of cSVD, but we highlight recent reviews of some of the other subtypes for the interested reader [4, 5].

The most commonly recognised neuroimaging features of cSVD include recent small

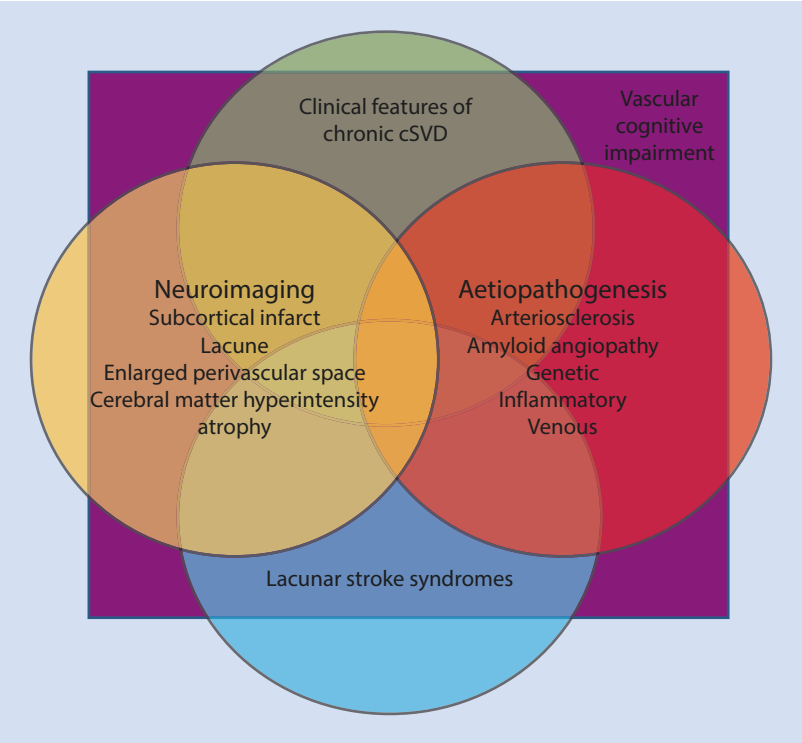

Fig. 42.1 The interplay between varying definitions of small vessel disease and vascular cognitive impairment

subcortical infarcts, lacunes, white matter hyperintensities, enlarged perivascular spaces, microbleeds and brain atrophy [2]. Again, recent attempts to harmonise the terminology used for describing neuroimaging cSVD changes have proven transformative in accelerating research and international collaboration. In contrast to the fields of cSVD pathology and radiology, to date there has been no consensus definition of the clinical cSVD syndrome. This represents an important area for future development.

42.3 Historical Perspectives

Descriptions in keeping with cSVD date back to the nineteenth century. Clinicopathological texts described small, deep infarcts on post-mortem brain examination, termed 'lacunae' from the Latin 'lacuna' meaning pool or cavity. An accompanying clinical presentation, 'the hemiplegia of old age', consisted of transitory hemiplegia, followed by a more prolonged period of gait disturbance, impaired memory, emotionalism and urinary incontinence. We would now recognise these features as lacunar stroke and chronic cSVD. In the early 1900s, Otto Binswanger described a clinical syndrome of progressive memory loss that had a vascular basis but was distinct from stroke. Alois Alzheimer differentiated these arteriosclerotic diseases from other diseases characterised by plaque deposition. However, Emil Kraepelin and contemporaries all described arteriosclerosis as the commonest cause of dementia. As the century progressed, attention moved to Alzheimer's eponymous disease, and interest in the vascular basis for dementia waned [6]. Major progress in our understanding of cSVD came from the seminal clinicopathological investigations of Charles Miller Fisher in Massachusetts General Hospital. Even today much of our understanding of cSVD, and the terminology we employ, can be traced back to this work. Clinical and research interest in cSVD gained momentum in the 1980s following the introduction of cross-sectional neuroimaging. Increasing availability and the use of neuroimaging such as computed tomography (CT) and more recently magnetic resonance imaging (MRI) have revealed the true prevalence of cSVD changes in middle-aged and older adults.

42.4 Epidemiology

The disease burden attributable to cSVD should not be underestimated. Directly, or in combination with other processes, cSVD accounts for almost half of all dementias and around one quarter of all ischaemic strokes. Haemorrhage is also seen, with up to 85% of these bleeds caused by cSVD. The proportion of disease attributable to cSVD may be even higher in certain ethnicities. Thus cSVD could be considered the most prevalent of all neurological disorders and one of the major determinants of physical and cognitive disability.

Although our understanding of the risk factors for cSVD remains incomplete, the strongest risk factors such as ageing and hypertension are increasing globally (with increasing life expectancy). Thus we are likely to see increases in prevalence of cSVD. One can speculate on reasons why such an important condition has received relatively little clinical, research, or health policy attention. Historically, research and practice have tended to focus on a single aspect of cSVD, such as lacunar stroke or white matter changes seen on neuroimaging. Studying these single features in isolation can give the impression of a relatively uncommon condition. It is only through considering cSVD at a whole brain level that we become aware of the true global importance of this condition [7].

42.5 Pathophysiology

Our understanding of the pathophysiology of SVD is improving, but there remain fundamental unanswered questions around the underlying biological mechanisms.

The cerebral 'small vessels' of interest are the smaller-arteries, arterioles, venules and capillaries. The pathology of hypertension-induced vascular change has been described in other chapters in this book. In the cerebral small vessels, we see many of these same changes are seen in renal and retinal small vessel disease, namely, fibrinoid necrosis, lipohyalinosis, vessel wall thickening and loss of smooth muscle cells. These changes cause narrowing of the vessel lumen and loss of elasticity that compromises blood flow autoregulation. The brain areas supplied by these small vessels, particularly those areas that are metabolically active and less able to cope with interruption to blood flow, are thus vulnerable to ischaemia. This partly explains why the disordered blood flow of cSVD causes a particular distribution of small vessel-induced brain pathologies [8].

While we recognise some of the vessel changes seen in cSVD, how these vascular lesions cause the various cerebral pathologies remains incompletely understood. It has been hypothesised that chronic or intermittent tissue hypoperfusion may result in degeneration of myelinated nerve fibres that is manifested as cerebral white matter disease, while acute occlusion of an end artery is the cause of focal lacunar type infarction. Both theories are biologically plausible but lack definitive empirical evidence. While narrowing and occlusion of an end artery is an attractive hypothesis, it seems likely that the process is more complex. Processes intrinsic to vessel flow seem to interact with other mechanisms such as increased blood-brain barrier permeability, disturbed lymphatics, local inflammation and endothelial dysfunction [9]. In fact the vessel wall changes or neuroimaging features seen may represent the late stage of a complex biological process that develops over many years. Although this complexity is a challenge, through understanding these processes, we may move closer to developing preventative or curative interventions.

42.6 Neuroimaging

Much of our understanding of cSVD has been driven by improvements in availability and resolution of non-invasive brain imaging. The small vessels of interest cannot usually be visualised with the imaging equipment available in clinical settings, and so we have been reliant on describing the more visible parenchymal lesions that accompany the small vessel pathology. CT can visualise certain features of cSVD and has been important for progressing our understanding of the condition and can play a role in assessment of cSVD on a population level [10]. However, when available, MRI is the preferred imaging modality. MRI offers higher sensitivity and specificity for the various manifestations of cSVD [2]. The use of high field strength MRI can offer greater definition of underlying structures such that we are now be able to image the small vessels themselves [11].

The lack of a consistent nomenclature for describing the imaging features of SVD has been

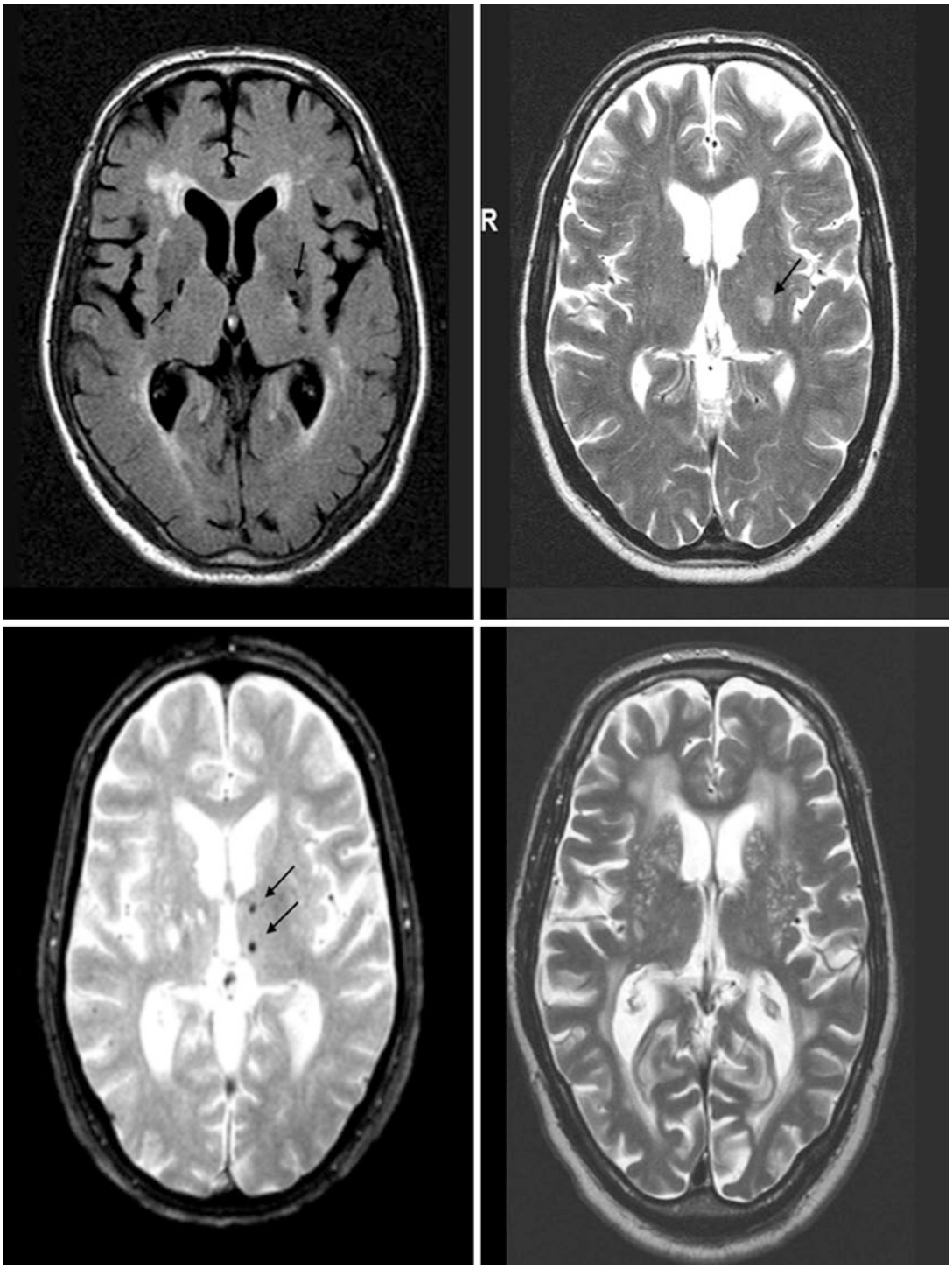

Fig. 42.2 Varying neuroimaging features of cerebral small vessel disease (clockwise from top right): acute small subcortical infarct (T2 MR image); enlarged perivascular spaces in basal ganglia and periventricular white matter hyperintensities (T2 MR image); microbleeds (GRE MR image); lacunes (FLAIR MR image)

previously described. Through the development of the STandards for ReportIng Vascular changes on nEuroimaging (STRIVE) materials, we now have a standard set of operationalised descriptors for all the common cSVD neuroimaging features [2] (Fig. 42.2). We will describe each of these below:

Recent Small Subcortical Infarct There is neuroimaging evidence of recent infarction in the territory of one of the perforating arterioles. To qualify as 'recent', there must be imaging features or clinical symptoms that are consistent with a lesion occurring in the previous few weeks. These lesions are no bigger than 20 mm and are best seen with diffusion-weighted MRI sequences. They often, but not always, present with clinical symptoms as a lacunar stroke syndrome.

White Matter Hyperintensities of Presumed Vascular Origin White matter hyperintensities (WMH) were the first lesions of cSVD to be described on imaging. In the CT era, they were called leukoaraiosis. They are the best recognized, commonest and most studied feature of cSVD [12]. The modifier '… of presumed vascular origin' recognises that white matter disease is a fairly non-specific process, and while cSVD is the commonest aetiology, these lesions can also be caused by a wide variety of metabolic, infectious and inflammatory conditions.

WMH are seen most clearly on fluid-attenuated inversion recovery (FLAIR) MRI sequences. They are usually symmetrically distributed in the hemispheres and brainstem with size varying from small punctate lesions to large confluent areas. While initially focal, they may coalesce in advanced states. The amount of WMH can be quantified either by an ordinal visual rating scale, of which the Fazekas scale is the most well-known, or by (semi-)automatic quantitative measurement of lesion volume.

WMH increase in prevalence with increasing age of the population studied; indeed in certain older adult cohorts, prevalence of WMH may reach 100%. WMH incidence and progression are also associated with vascular risk factors such as hypertension, smoking and diabetes. These epidemiological patterns give some clues as to pathogenesis but only explain a small proportion of the variance of WMH seen in older adults. Regardless of aetiology, the presence and volume of WMH are associated with adverse outcomes independent of other vascular risk factors. In a systematic review of 46 studies including 18,625 participants, WMH was associated with a threefold increased risk of stroke and doubling of risk of dementia and death [12].

Lacunes of Presumed Vascular Origin The consensus definition is of a round or ovoid, subcortical, fluid-filled cavity measuring between 3 and 15 mm in diameter. These lesions are thought to be consistent with sequelae of a previous acute small subcortical infarct or haemorrhage (whether symptomatic or not) in the territory of one of the perforating arterioles, and formerly they were called 'old lacunar infarcts'. Lacunes are common and prevalence increases with age. A systematic review described population prevalence of lacunes as around 10% of people aged 65 years old and 40% of people aged over 80 years [13]. Not all of those with radiological lacunes have corresponding clinical symptoms. A phenomenon of the clinically 'silent' infarct is described. In unselected populations, MRI studies have demonstrated silent subcortical infarcts in more than a quarter of subjects aged 70 years or over [13]. This makes 'silent' infarcts about five times more common than infarcts that present with a recognisable stroke syndrome.

Cerebral Microbleeds Microbleeds are small (generally 2–5 mm in diameter) perivascular foci of haemosiderin. They are thought to represent blood product leakage through small vessels, possibly within macrophages. The iron content allows them to be visualised as small, rounded, homogeneous and hypointense lesions, best seen on gradient echo MRI sequences where they exhibit 'blooming' artefact due to signal dropout from the haemosiderin. Microbleeds are common to both the arteriolosclerotic and amyloid angiopathic processes that underlie cSVD. In general, although not exclusively, microbleeds situated in the deep grey and white matter are seen in the context of systemic hypertension and are associated with lacunes and WMH. Cerebral microbleeds that are found in lobar structures are said to be more closely related to amyloid angiopathy. In both locations, the presence of microbleeds increases the risk of intracerebral bleeding. There seems to be a dose-dependent relationship with bleeding risk increasing with the number of microbleeds seen.

Enlarged Perivascular Spaces Perivascular spaces (previously Virchow-Robin spaces) are normal anatomical features representing interstitial cerebrospinal fluid-filled spaces that surround the small deep penetrating arterioles and venules. These spaces are not usually seen in health, but when they become enlarged, they may be seen, particularly on T2-weighted MRI, as multiple characteristically small, round or lineated, high-

signal areas in the basal ganglia and centrum semiovale. Enlarged perivascular spaces are associated with increasing age and in conditions including hypertension and inflammation. Originally thought to be a benign neuroimaging feature, enlarged perivascular spaces are now considered an early feature of cSVD and, thus not surprisingly, have been demonstrated to be associated with WMH and worse cognitive function. As with microbleeds, lesion location may point to differing pathological process. Some studies suggest that perivascular spaces in the centrum semiovale white matter are a sign of amyloid angiopathy, while perivascular spaces in the deep grey matter are a sign of arteriolosclerosis.

Brain Atrophy Loss of brain tissue is a non-specific feature of cSVD and can be generalised or focal. Some loss of brain tissue is seen with ageing, and differentiating 'normal' from abnormal can be a challenge. Age-stratified templates or quantitative imaging can help in this regard. The term atrophy implies progressive loss over time, which can only be inferred (rather than directly measured) from imaging taken at a single time point. This emphasises the need for serial imaging in studies of cSVD.

Other Neuroimaging Features The phenotypic expression of cSVD is heterogenous, and this variability is only partly explained by the neuroimaging features defined in STRIVE. There are certain other neuroimaging features that are commonly, but not exclusively, associated with cSVD. For example, imaging evidence of intracerebral haemorrhage can be a feature of cSVD. As with other lesions, location is important. It is said that haemorrhage deep within the brain parenchyma is a feature of arteriolosclerosis, while lobar bleeds are more common in amyloid angiopathy.

A focus of recent research activity has been assessing for potential cSVD changes not visible on routine imaging. These may represent early stages of cSVD processes. For example, in 'normal appearing white matter', advanced imaging techniques may reveal altered blood-brain barrier permeability, white matter integrity and myelination. These invisible or 'pre-visible' features offer the potential for intervention. The early, subtle changes in interstitial fluid mobility and water content may be reversible, while the better recognised imaging signatures of gross demyelination and axonal damage are less likely to be reversible and probably represent a late-stage phenomenon.

Examples of novel imaging features of cSVD include microinfarcts and diffusion abnormalities. Microinfarcts are ischaemic lesions previously only seen on light microscopy but now visible in vivo using high field MRI. Diffusion imaging is used to quantify the diffusion of water molecules along axons. Post image processing allows quantification of damage to white matter tracts that is not severe enough to cause a lesion on standard imaging. Values known as 'fractional anisotropy' (FA) and 'mean diffusivity' (MD) can be measured at the voxel level. FA/MD derangement is found both in WMH and in areas of normal appearing white matter prior to WMH development. Further early markers of cSVD are likely to be identified in the near future [11].

Quantifying Neuroimaging Features For many of the neuroimaging features described, there exist scales that allow quantification through visual assessment. Ordinal scales have been described for WMH, perivascular spaces and atrophy, while discrete lesions such as lacunes and cerebral microbleeds can be counted. Increasingly we recognise that the component features of cSVD should not be considered in isolation, because the clinical phenotype is not determined by isolated features but by the overall constellation and severity of the disease. A scale of total cSVD burden has been developed that collates all the features into one score. The scale runs from 0 to 4, with 1 point for each of one or more lacunes, moderate to severe white matter hyperintensities, one or more microbleeds, and moderate-severe basal ganglia perivascular spaces. In research settings voxel-based quantification of all the features of cSVD is also possible [14].

42.7 Clinical Features

The clinical manifestations of cerebral small vessel disease are varied and probably not yet fully recognised. A distinction can be made between acute symptoms such as stroke and more chronic, progressive symptoms. However, this classification may be an oversimplification as it is recognised that lesions with the same pathological or neuroimaging appearance may cause acute stroke in one person and be clinically 'silent' in another.

There is further heterogeneity within the classifications, for example, acute cSVD can represent both ischaemic and haemorrhagic strokes, while the clinical expression of more chronic cSVD can range from relatively asymptomatic only detectable on detailed neuropsychological testing through to severe dementia and disability.

For acute small vessel stroke, a series of common clinical presentations are recognised. As cSVD lesions tend to occur in the same brain regions (basal ganglia, centrum semiovale, thalamus, internal capsule and brainstem), several classical lacunar syndromes are recognised, such as pure motor stroke syndrome, pure sensory stroke syndrome and ataxic hemiparesis. Within the Oxford Community Stroke Project (OCSP) clinical classification, these presentations are labelled lacunar stroke (LACS). This system is useful for initial treatment and prognosis, but in about one fifth of cases, the clinical presentation is not matched by relevant neuroimaging features. Aetiological classification systems such as used in the Trial of Org 10172 in Acute Stroke Treatment (TOAST) combine investigations and risk factors to suggest a potential causative factor(s). In this system 'small vessel disease' is distinct from large vessel atherosclerosis and cardioembolic stroke. Acute stroke presentations are considered further in other chapters of this book.

The more chronic clinical features of cSVD include a variety of cognitive, mood and physical impairments. In contrast to the pathological and neuroimaging features of cSVD, there is no consensus, operationalised definition and no assessment scale for quantifying severity (Fig. 42.3).

Cognitive and Mood The best recognised and most feared clinical manifestation of cSVD is cognitive decline. Vascular cognitive impairment (VCI) indicates the entire range of cognitive deficits associated with cerebrovascular disease [15]. It ranges from mild cognitive impairment to vascular dementia, in which the cognitive dysfunction negatively affects daily functioning. Vascular dementia is the second commonest cause of dementia, after Alzheimer's disease, and cSVD is the most important contributor. It is increasingly recognised that cSVD may also contribute to cognitive decline in other neurodegenerative diseases, with the distinction between vascular and non-vascular dementia becoming increasingly blurred. Patients with an Alzheimer's disease dementia diagnosis in life often have evidence of cerebrovascular disease on post-mortem examination. In fact, there is considerable overlap in the risk factors for both Alzheimer's disease and vascular dementia, many of which are classical cardiovascular risk factors [3].

In contrast to Alzheimer's dementia with its typical clinical picture of early memory loss, a typical pattern of neuropsychological impairment in cSVD is less obvious. Impairments in all cognitive domains can be seen, and this is perhaps unsurprising due to the widespread locations of corresponding lesions. A core feature of executive function deficit is often seen, either in isolation or in combination with other impairments. Executive function includes cognitive aspects such as planning, initiation and problem-solving. Executive function is said to be dependent on frontal lobe and related subcortical structures, but it is likely that problems in cSVD arise from disruption of executive neural networks rather than direct structural damage. The importance of executive function in cSVD presentations needs to be considered when interpreting cognitive screening tests. Many of the commonly used instruments focus on language and memory with relatively little testing of executive function [16].

Depression and/or depressive symptoms are also common in cerebrovascular disease and demonstrate a bidirectional association with cSVD with depression representing both a risk factor for and a consequence of cerebrovascular disease. Many clinical features of depression such as apathy and psychomotor retardation may be direct consequences of cSVD. There is continuing debate as to whether incident later life depression in the context of cSVD is the same disease as midlife depression [17]. Other mood and emotional issues such as anxiety and emotional lability are also seen in cSVD and stroke. Observational studies suggest that other mood issues may be seen in as many as one third with cSVD-related stroke, but the nature of association and natural history remain poorly understood.

Physical Disordered gait and balance can be seen in cSVD. Instability, decreased mobility, reduced walking speed and decreased stride length have all been described. In some respects the clinical picture is similar to that seen in idiopathic Parkinson's disease but with features more prominent in lower

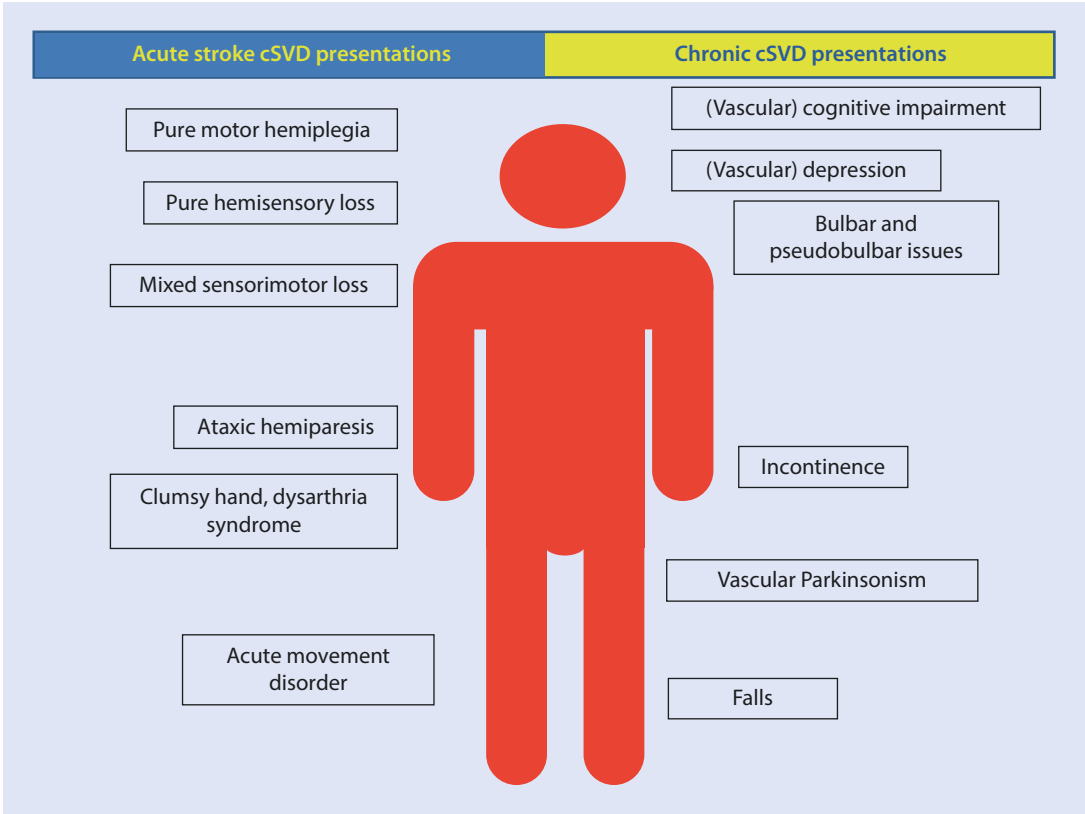

Fig. 42.3 Clinical features of cerebral small vessel disease, categorised as acute stroke presentations and more chronic features

limbs. Thus, labels vascular parkinsonism, lower body parkinsonism or 'marche a petit-pas' are often used. Perhaps unsurprisingly, cSVD is also associated with falls. CSVD may also be associated with urinary incontinence and in later stages, bulbar issues are described. In the context of all these features, cSVD is strongly associated with decreased functioning and independence. For example, the presence of WMH predicts decline in function, need for institutional care and death [10].

42.8 Risk Factors

An understanding of risk factors for cSVD is important as it may give clues as to underlying mechanism of disease or offer avenues for preventative treatment. Increasing age is obviously the most important risk factor, but age itself is not a modifiable factor. A substantial body of evidence suggests an association between cSVD and traditional vascular risk factors, such as hypertension, diabetes and smoking. However, these risk factors only explain a small percentage of the variation in expression of cSVD, and so the interplay of genetic, environmental or lifestyle factors must be important. It seems likely that lifetime exposures on a background of genetic susceptibility may be responsible for cSVD expression [18]. A further challenge to our understanding of risk factors is that the size and even direction of effect may change over the life course. For example, early and midlife hypertension seem to be associated with cSVD neuroimaging changes. However in older adults, particularly those with established cSVD, low blood pressure may be more harmful.

42.9 Treatment

Treatment of cSVD can include primary prevention and treatment of established disease. As cSVD seems to be associated with modifiable midlife risk factors such as hypertension

and smoking, these would seem a sensible target for preventative interventions. In fact, the evidence for a beneficial effect on cSVD is limited, but as benefit in other vascular beds is well established, few would dispute the importance of smoking cessation and treating blood pressure to target [19].

The treatment of the acute stroke manifestations of cSVD is explained in detail elsewhere in the textbook. Some have argued that cSVD-related stroke is a different entity and so should not be treated in the same fashion as other stroke aetiologies. Many trials of stroke have included patients with lacunar stroke (in fact, often disproportionately as there is often a bias towards patients with milder stroke), and there have also been trials with a specific cSVD stroke focus. Analysis of cSVD subgroups suggests that for standard treatments such as antiplatelet, antihypertensive and statin, beneficial effects are seen in both cSVD and other stroke subtypes. There may be differences in adverse events, for example, cSVD stroke seems to show an increased propensity to bleeding with antiplatelet, but net benefit still favours treatment [20].

Evidence-based treatments for established cSVD are lacking and represent an important area of current research activity. Curative treatment of advanced cSVD would require neurorestorative therapies, a prospect that seems unlikely in the near future. A more obvious target is treating early-stage cSVD, recognising that the appearance of the traditional imaging features probably represents a disease process that has been progressing for years. Given the important cognitive consequences of SVD, trials of drugs to treat cognitive symptoms are of particular interest, but results have been mixed. Trials of acetylcholinesterase inhibitors in vascular dementia suggested some cognitive benefit, but effect sizes were less than seen in Alzheimer's disease [20].

42.10 Cerebral Amyloid Angiopathy

Cerebral amyloid angiopathy (CAA) is the second most common type of cSVD, after arteriolosclerosis. It is a sporadic age-related disease, although a rare genetic variant exists. Pathologically, it is characterised by progressive accumulation of amyloid protein in the vessel walls. In contrast with the predilection for deep perforating vessels in arteriolosclerotic cSVD, cerebral amyloid angiopathy mainly affects the cortical and leptomeningeal small vessels. Neuroimaging markers are similar to arteriolosclerosis but with a distinct distribution. They include white matter hyperintensities, microbleeds in a lobar distribution, perivascular spaces in the white matter of the centrum semiovale, cortical superficial siderosis and cortical microinfarcts. CAA is frequently present in brains of patients with Alzheimer's disease but also has an independent contribution to vascular cognitive decline. Acute symptoms include haemorrhagic stroke from lobar haemorrhage and recurrent, often stereotyped, neurological symptoms which are called 'amyloid spells'. There are no specific preventive or disease-modifying treatments for CAA. Many recommend that antithrombotics should be avoided because of the haemorrhagic risk, but natural history of CAA remains poorly understood. This potential for differential treatment effects emphasises the importance of attempting to distinguish between arteriolosclerotic and CAA-related diseases of cerebral vessels [5].

Conclusions and Clinical Perspectives

The importance of harmonisation and creating common descriptors cannot be overemphasised in a field that has been characterised by confusing and inconsistent terminologies. The success of initiatives such as STRIVE should act as a template for similar work in clinical cSVD and preclinical models.

In clinical practice, cSVD may present to various clinical disciplines including psychiatry, neurology and geriatric medicine. Multidisciplinary, collaborative working will be required to advance our understanding of the disease.

Big data approaches, repurposing data from trials and other sources, may offer new insights or allow for hypothesis testing, but most previous studies simply did not collect sufficiently detailed neuroimaging or clinical data. There remains a role for focussed, prospective cohort studies with serial imaging, clinical and neuropsychological assessment.

Gaps in Knowledge

In the field of cSVD, it could be argued that we are still far from having all the answers, but we have a better awareness of the important questions and the challenges in answering them.

We recognise cSVD as a chronic, progressive condition, but fundamental questions remain around the epidemiology and natural history of the condition.

Increasing neuroimaging fidelity combined with improvements in informatics offers exciting potential to better characterise the pathology of cSVD in vivo. Increasingly attention will turn to areas of the brain that may appear 'normal' on conventional imaging to assess for early-stage changes of cSVD.

The ultimate goal is to establish evidence-based treatment strategies. This must be informed by the most important stakeholders, those living with or affected by cSVD. We need patients to tell us which aspects of the disease are of greatest importance to them to allow us to develop interventions that will have the greatest impact.

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Stroke

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_43

Key Concepts

- Stroke is a cerebrovascular incident resulting in neurological dysfunction; most commonly caused by an ischaemic stroke whereby a thrombus occludes a major vessel within the brain.
- Stroke is one of the leading causes of death and disability in the world, resulting in a large economic burden; however, treatment options are currently limited to the use of a tissue plasminogen activator (tPA) and/or a mechanical thrombectomy procedure to recanalize the blood vessel.
- The pathophysiology of ischaemic stroke is complex with numerous pathways converging to result in cell death and the development of an area of irreversibly damaged brain tissue called an infarct.
- Numerous elements of the pathophysiology of ischaemic stroke have been previously targeted as neuroprotective strategies in preclinical stroke models but have failed to translate clinically.
- The preclinical stroke research community has learned from previous failures and strives to improve the translational potential and quality of stroke research with improved study design, rigour and reproducibility which is the benchmark for other preclinical disease models.

43.1 Introduction: What Is Stroke?

Stroke was defined by the World Health Organisation in the 1970s as a clinical syndrome of "rapidly developing clinical signs of focal and at times global, loss of cerebral function, with symptoms lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin". However, due to the advances in modern medicine, this definition has been updated in recent years to include more specific definitions based on the cause or clinical presentation and also to distinguish transient ischaemic attack (TIA) from ischaemic stroke. Briefly, ischaemic stroke is defined as "an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction" with an infarction being defined by imaging or clinical symptoms persisting longer than 24 hours. Alternatively, TIA is defined as "a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia *without acute infarction*" [1]. Haemorrhagic stroke is defined as "rapidly developing clinical signs of neurological dysfunction attributable to a focal collection of blood within the brain parenchyma or ventricular system that is not caused by trauma," or in the case of subarachnoid haemorrhage, this may also include headache as a symptom and is caused by bleeding into the subarachnoid space (the space between the arachnoid membrane and the pia mater) [1]. Ischaemic stroke accounts for approximately 87% of stroke incidences with cerebral haemorrhage or subarachnoid haemorrhage accounting for around 10% and 3%, respectively [2].

43.1.1 Epidemiology

Stroke is the second leading cause of death worldwide accounting for around 10% of all deaths. It is also the third leading cause of loss of disability-adjusted life years (DALYs) accounting for nearly 5% of DALYs lost. The most recently available figures tell us that in 2013 there were an estimated 10.3 million new incidences of stroke worldwide with higher incidence in developed countries [3]. More specifically, in the United States (US), someone has a stroke every 40 seconds, 1 in 20 deaths are due to stroke, and the cost to the US economy is estimated at around $34 billion each year [2]. Meanwhile, in the United Kingdom (UK), the incidence is lower but still substantial, with a stroke occurring once every 5 minutes, accounting for around 1 in 14 deaths and costing the UK economy around £25.6 billion each year [4]. In both the United Kingdom and United States, stroke is one of the leading causes of disability contributing greatly to this economic burden. The worldwide occurrence of stroke is slightly higher in females than males with stroke affecting one in every five women ages 55–75 years and approximately one in six men [2]. Race is also a factor in stroke prevalence, with a higher prevalence of stroke in black individuals than white, Hispanic or Asian [2].

43.1.2 Classification and Causes of Stroke

As previously mentioned, the two major subtypes of stroke are ischaemic or haemorrhagic, a classification based on their pathology. The Bamford (or Oxford) classification system, however, uses the clinical presentation to divide stroke into four types: total anterior circulation, partial anterior circulation, lacunar circulation and posterior circulation strokes [5]. It provides important prognostic information, and the relationship with outcome reflects the link between the clinical syndrome and stroke topography on brain imaging, which in turn is indicative of potential aetiological mechanisms.

Ischaemic stroke is typically caused by either cardiac thromboembolism or atherothrombotic arterial occlusion, which can in turn be due to large artery atherosclerotic disease or small-vessel arteriosclerotic disease. Clinicians may use different classification systems to aetiologically categorise ischaemic stroke. The TOAST classification system for ischaemic stroke incorporates clinical, radiological, cardiac and laboratory investigations and assigns the cause of ischaemic stroke to one of five aetiological categories: large artery atherosclerosis (LAA), small artery occlusion (SVD), cardioembolic (CE), other determined pathology or undetermined pathology. Using this classification, LAA, SVD and CE account for 13.4–16.7%, 15.9–22.6% and 18.6–29.1% of cases, respectively [6]. Alternatively, the Causative Classification System (CCS-TOAST) allows for a more rapid, computer-assisted classification of the TOAST system. The ASCOD phenotyping system assigns a degree of likelihood that the stroke was caused by five categories of disease: A, atherosclerosis; S, small-vessel disease; C, cardiac pathology; O, other causes; and D, dissection. This allows for appreciation of the overlap between different aetiologies and the importance of dissection as a cause of ischaemic stroke in younger people [7].

Haemorrhagic stroke is classified based on its location and is usually caused by hypertension, amyloid angiopathy or structural brain disease such as arteriovenous malformation [5]. Haemorrhagic stroke, however, is not a focus of this chapter, and the following sections will focus on ischaemic stroke.

43.1.3 Symptoms of Ischaemic Stroke

The most common symptoms of stroke are sudden speech disturbances, sudden arm or leg weakness or numbness (in particular on just one side of the body), sudden facial weakness, sudden visual disturbance and sudden loss of balance/coordination [5]. In an effort to increase public awareness of stroke and increase hospital admissions early after stroke onset, the Department of Health in England introduced the "Stroke—Act FAST (Face, Arms, Speech: Time to call Emergency Medical Services)" campaign in 2009 which has been successful in increasing public education on stroke [8] and has since been introduced in various other countries.

Various scales are used in the hospital to grade patients' severity of stroke symptoms. The most commonly used is the National Institute of Health Stroke Scale (NIHSS) which gives patients a score between 0 and 42 (the greater the score, the more severe the stroke) based on various symptoms such as consciousness, facial weakness, speech disturbance, eye movement and vision and limb weakness or ataxia [9]. Similarly, various scales are used to grade patients' level of disability in the weeks or months following a stroke, the most common of which is the modified Rankin Scale (mRS). This gives patients a score between 0 and 6, where 0 means no symptoms present and 6 means dead. Scales like these are useful for clinicians to provide prognostic information or to monitor patient recovery, but they are also of particular use in clinical stroke studies. For example, the NIHSS can be used for trial inclusion criteria or for subgroup analysis, while the mRS is a common outcome measure to measure patients' level of disability at 90 days [10].

43.1.4 Risk Factors and Current Treatments for Ischaemic Stroke

Ischaemic stroke has a number of risk factors such as hypertension, atrial fibrillation, lack of regular physical activity, poor diet, diabetes and smoking [11]. As with many diseases, prevention is preferable to a cure, and therefore management of these risk factors is the first line of stroke prevention. For example, management of hypertension can

result in a 41% reduction of stroke risk with just a 10 mmHg reduction in systolic blood pressure [2], while the introduction of smoking ban legislation in numerous countries worldwide has been associated with a reduced incidence of stroke [12].

Immediately following an ischaemic stroke, the current most commonly available treatment is intravenous alteplase which is a tissue plasminogen activator (tPA), a clot-busting drug. This dissolves the occluding blood clot with the aim to restore blood flow and reduce the period of ischaemia in order to salvage non-infarcted brain tissue with the concept that "time is brain". This drug has a very narrow effective treatment window and is licensed for use only up to 4.5 hours from the onset of symptoms resulting in low numbers of patients being eligible for treatment. Additionally, alteplase can increase the risk of haemorrhagic stroke [11]. Tenecteplase is a genetically modified tPA which is commonly used in the treatment of myocardial infarction and may have some favourable properties in comparison to alteplase. A procedure known as thrombectomy, where the blood clot is mechanically removed from the a large blood vessel within in the brain, has also shown great promise in recent trials, with improvements in revascularization at 24 hours (76% vs 34%, thrombectomy vs standard care) and of 90-day functional outcome compared to standard medical treatment [13]. More recently, this procedure has proven effective with an extended treatment window up to 24 hours (since the onset of stroke symptoms) [14], and as a result, the American Heart Association Guidelines for the management of acute ischaemic stroke were updated to recommend the use of mechanical thrombectomy in patients between 6 and 24 hours, provided they meet appropriate selection criteria [15].

43.2 Pathophysiology of Ischaemic Stroke

This section will describe the molecular processes that take place following ischaemic stroke, rather than the processes that cause arterial occlusion, and is summarised in Fig. 43.1. For reviews providing further detail on the pathophysiology, see [16–19]. Although stroke refers to a collection of heterogeneous diseases, at the molecular level there is considerable consistency with regards to the cell signalling pathways involved.

43.2.1 Infarct Core and Penumbra

After the onset of ischaemic stroke, a core infarct (area of dead tissue due to lack of blood supply) develops locally to the obstructed vessel. While the blood supply in the surrounding brain tissue is significantly reduced, cell metabolism is maintained by collateral flow from other blood vessels within the brain. This region of the brain, known as the ischaemic penumbra, is potentially still viable and salvageable after a stroke [20]. Its viability, however, depends on the severity and duration of ischaemia, and, during the time lag to reperfusion, a physiological cascade occurs which places this penumbral tissue at further risk. If the time lag is too long, the penumbra may eventually be subsumed into the infarct core resulting in a larger infarct and thus more severe neurological symptoms [21].

43.2.2 Ischaemic Injury

The brain receives around 20% of the body's total oxygen despite accounting for only approximately 2% of body weight [22]. This high metabolic demand consequently makes the brain very susceptible to ischaemic injury.

43.2.3 Energy Failure

In the acute phase of ischaemic injury, the lack of oxygen and glucose results in a depletion of cellular stores of adenosine triphosphate (ATP) – the energy currency of the cell which is required for many key cellular processes. As a result of the lack of oxygen, cells switch to anaerobic respiration resulting in the production of lactate. This leads to an accumulation of lactic acid called metabolic acidosis and results in cell death through necrosis [17].

Mitochondria are responsible for aerobic respiration in cells, that is, they catalyse the conversion of adenosine diphosphate (ADP) to ATP using oxidative phosphorylation (OXPHOS). Pyruvate is produced through glycolysis of glucose in the cytosol but is then oxidised within the mitochondria generating NADH and $FADH_2$, which are then oxidised by the electron transport chain (ETC) in the mitochondrial membrane. OXPHOS produces a proton gradient across the mitochondrial membrane which then powers

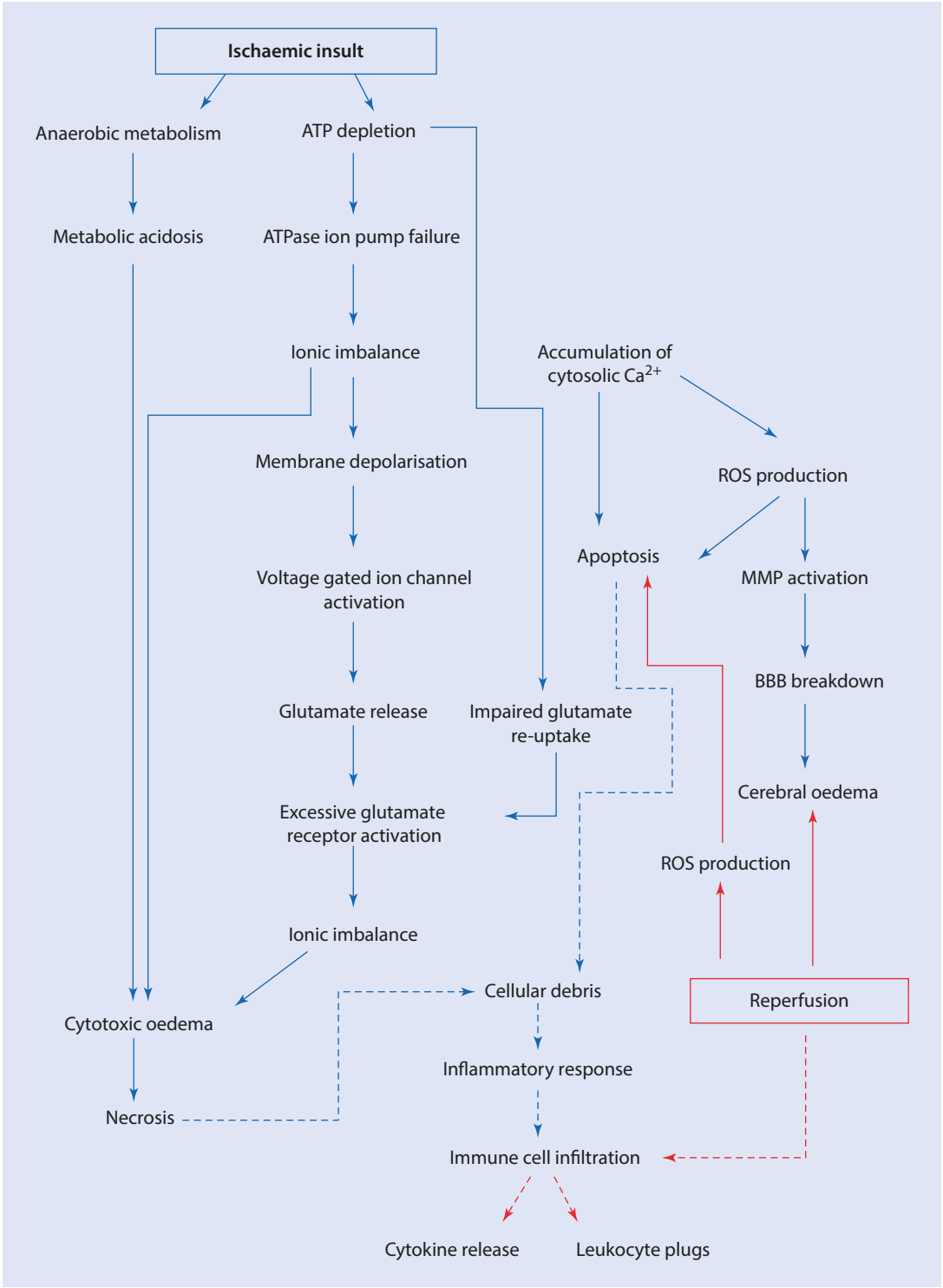

Fig. 43.1 The pathophysiology of ischaemic stroke. Schematic demonstrating the causes of cell death through necrosis or apoptosis resulting from ischaemia (blue arrows) and as a result of recanalization and reperfusion (red arrows); *ATP* adenosine triphosphate, *ROS* reactive oxygen species, *MMP* matrix metalloproteinases, *BBB* blood-brain barrier

ATP synthase. This proton gradient, however, is also important for the maintenance of low intracellular calcium ions (Ca^{2+}) by aiding Ca^{2+} uptake into the mitochondria, through the Ca^{2+} uniporter on the mitochondrial membrane. Therefore, reduction of respiration causes a rise in intracellular Ca^{2+} levels, and cells will utilise any remaining ATP stores to try to maintain Ca^{2+} homeostasis, resulting in more rapid ATP depletion.

43.2.4 Ionic Imbalance and Calcium Dysregulation

The depletion of ATP also affects the function of ATP-driven membrane ion pumps (ATPases), namely, sodium-potassium (Na^+-K^+) ATPase, Ca^{2+} ATPase and synaptic proton (H^+) ATPase. Low intracellular calcium levels are tightly controlled by Na^+-Ca^{2+} exchangers, on the mitochondrial and cell membrane, and Ca^{2+} ATPases, on the endoplasmic reticulum (ER), mitochondrial and cell membrane, in order to maintain a 10,000-fold gradient across the cell membrane. During ischaemia, failure of Na^+-K^+ ATPase results in K^+ efflux and Na^+ influx, which in turn causes reversal of the Na^+-Ca^{2+} exchanger. Coupled with the failure of Ca^{2+} ATPases, this leads to Ca^{2+} accumulation in the cytosol [17].

43.2.5 Excitotoxicity

Disruption in cellular ionic homeostasis causes the loss of membrane potential and membrane depolarisation. This in turn causes the activation of voltage-gated ion channels resulting in glutamate efflux. In normal neuronal electrical signalling, glutamate, a potent neurotransmitter, is released into the synapse and activates a family of receptors on the postsynaptic membrane (of the adjacent neuron): the NMDA, AMPA, kainite receptors or mGluRs. Activation of these receptors results in influx of Ca^{2+} and Na^+ and subsequent loss of K^+ which causes depolarisation and glutamate release so that this signal propagates from neuron to neuron. In the ischaemic cascade, ionic imbalance results in excess glutamate release, and the normal reuptake mechanisms for glutamate are impaired due to the energy failure state of the cell, and so this results in an extracellular accumulation of glutamate. This causes overactivation of the glutamate receptors and excess depolarisation in surrounding neurons, in an insult known as excitotoxicity. Severe excitotoxicity and ATPase failure ultimately lead to necrotic cell death due to cell ionic imbalance causing a passive influx of water into the cells known as cytotoxic oedema [17].

43.2.6 Apoptosis Initiation

Accumulation of intracellular Ca^{2+} also activates proteases, lipases and nucleases leading to degradation of key cellular proteins, the plasma membrane and nucleic acids. This causes irreversible cellular damage leading to the initiation of apoptosis (programmed cell death). Furthermore, calcium activates Ca^{2+}-dependent enzymes (calpains) which bind with pro-apoptotic proteins at the mitochondrial membrane. This results in the opening of mitochondrial transition pores (MTP) and the subsequent release of cytochrome C, from the mitochondria, leading to activation of caspase enzymes which are integral in the execution of apoptosis [23]. Opening of the MTP also further depletes cellular ATP by disrupting the mitochondrial membrane potential and therefore preventing the action of ATP synthase.

43.2.7 Reactive Oxygen Species (ROS)

Calcium dysregulation has further implications, in the ischaemic cascade, with the production of reactive oxygen species (ROS) (Fig. 43.2) [24]. As previously described, the ETC is responsible for the majority of cellular ATP; however, the ETC is also a major source of basal levels of cellular ROS: superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Increased mitochondrial Ca^{2+} promotes oxidation via the ETC and thus results in a greater production of ROS. Additionally, however, high mitochondrial levels of Ca^{2+} can inhibit enzymes of the ETC, for example, by causing disassociation of cytochrome C from the mitochondrial membrane and thus inhibiting complex III or by inhibiting complex I in combination with nitric oxide (NO). Ca^{2+} also activates additional enzymes contributing to ROS production, namely NADPH oxidases (NOX2, NOX3 and NOX4) and neuronal nitric oxide synthase

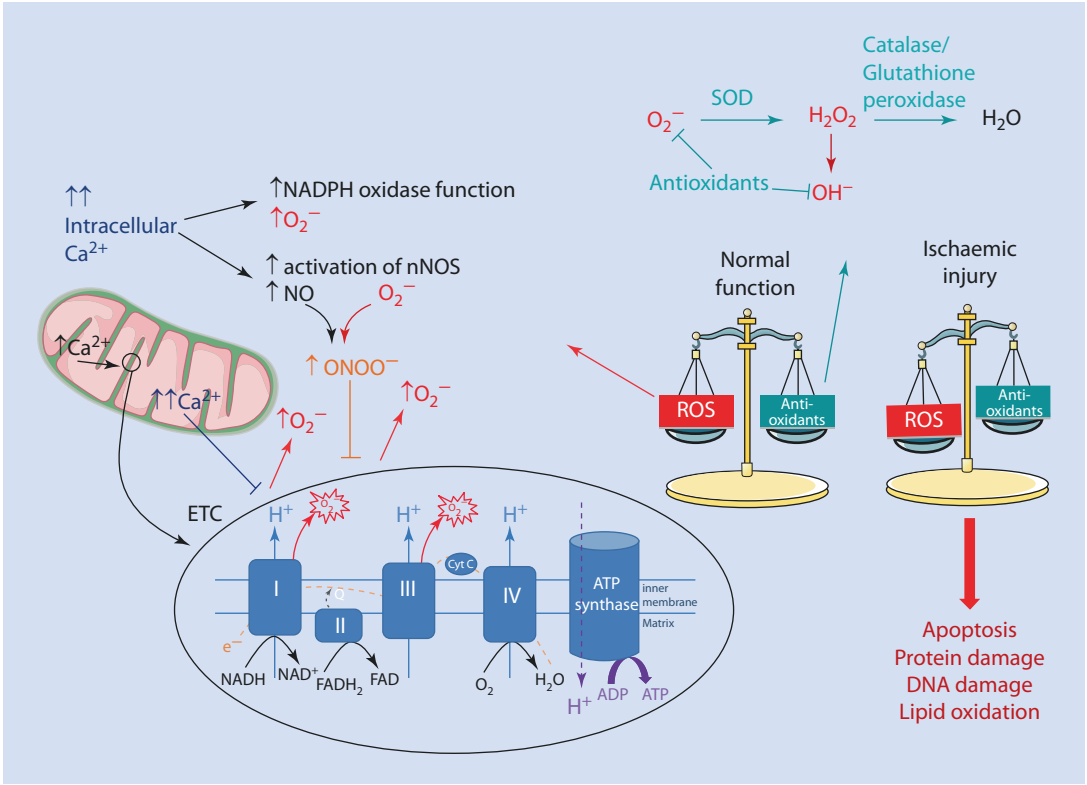

Fig. 43.2 ROS production in ischaemic stroke. Schematic representation of the sources of ROS resulting from increased calcium levels in ischaemic injury through NADPH oxidase activation or ETC stimulation/inhibition. Antioxidant strategies (top right, teal) exist within cells and manage basal levels of ROS under normal conditions. Ischaemic injury disrupts this balance leading to increased levels of ROS resulting in cellular damage and death. *ETC* electron transport chain, $O_{2\_}$ superoxide ion, *OH_* hydroxyl radical, *ONOO_* peroxynitrite, *nNOS* neuronal nitric oxide synthase, *SOD* superoxide dismutase, *ROS* reactive oxygen species

(nNOS). nNOS activation causes an increased production of NO, and although some studies have suggested a neuroprotective role of NO in the brain, through inhibition of NMDA receptors or increased vasodilation and therefore increased neuronal blood supply, this molecule is also considered neurotoxic. This is because, at high concentrations, NO combines with superoxide to form the highly reactive oxygen radical, peroxynitrite ($ONOO^-$). Cells have endogenous antioxidant enzymes and compounds, such as superoxide dismutase (SOD), catalase or α-tocopherol, to deal with excess ROS. If these antioxidant mechanisms are overloaded by too much ROS production, this leads to oxidative stress where ROS can damage proteins, lipids and nucleic acids leading to cellular damage which can result in the initiation of apoptosis. The brain is particularly vulnerable to oxidative stress because of its high respiration rate, its antioxidant defences are not high enough to deal with excess levels of ROS and also because of high levels of lipids, which are very vulnerable to ROS damage, and iron, which promotes free-radical reactions/damage [18].

43.2.8 Reperfusion Injury

Although ischaemia results in cell damage and death, recanalization of cerebral vasculature also causes further damage known as reperfusion injury. ROS generation, due to a combination of the reintroduction of oxygen and dysfunction of the ETC due to ischaemic damage, plays a key role in this injury. Moreover, an ischaemic accumulation of succinate within the mitochondria has been shown to drive reverse election transport (RET) which generates large amounts of ROS [25]. The damaging effects of ROS and oxidative stress

within the brain have already been discussed; however, coupling this with the physical restoration of blood flow and the brain is left open to further damage with inflammation and an immune response (discussed below). Matrix metalloproteinases (MMPs) are activated by ROS, pro-inflammatory cytokines and also by tPA (the clot-busting drug used for reperfusion). MMPs play a part in tissue remodelling (discussed later), but they are also involved in degradation of the blood-brain barrier (BBB). The BBB protects the brain by limiting the compounds which can pass from the bloodstream into the brain, and it is compromised following ischaemic injury due to MMP activity and damage to endothelial cells causing them to detach from the basal membrane. As a result, the BBB becomes more permeable, and increased BBB permeability causes cerebral oedema by allowing excess water, from the blood, into the brain. It can also increase the risk of intracerebral haemorrhage.

43.2.9 Inflammation and Immune Response

In the later stages of ischaemic injury and following reperfusion, an inflammatory response causes additional damage to the already compromised penumbra. This inflammatory response is considered sterile as it is not caused by infiltrating microorganisms.

Increased levels of intracellular Ca^{2+} and the cell stress response initiate activation of inflammatory transcription factors such as nuclear factor-kappaB (NF-κB) or mitogen-activated protein kinases (MAPK) signalling pathways. In addition, necrotic and apoptotic cell debris such as protein or nucleic acids, known as danger/damage-associated molecular pattern molecules (DAMPs), bind to and activate toll-like receptors (TLRs) on microglia, macrophages and endothelial cells inducing the release of pro-inflammatory signalling molecules. This inflammatory response induced during ischaemia results in the infiltration of leukocytes such as lymphocytes, neutrophils and monocytes, coupled with increased expression of endothelial adhesion molecules (such as p-selectin, ICAM-1 or VCAM-1). Increased BBB permeability following ischaemia assists this by allowing infiltrating immune cells' easier access to the brain parenchyma. The infiltrating leukocytes then accumulate within ischaemic tissue and adhere to endothelial cells, further exacerbating damage by releasing pro-inflammatory (e.g. IL-1β, TNFα) or cytotoxic (e.g. NO, ROS) molecules. Endothelial or platelet-derived p-selectin can also cause leukocytes to adhere to each other forming "leukocyte plugs" causing further obstruction within the blood vessel, worsening damage [19].

Although infiltrating neutrophils and monocytes play a role in the phagocytosis of damaged neurons or other cells, microglia are the resident macrophages of the brain. These cells are normally present in the brain in a resting or ramified state but are activated in response to insult or injury. Activated microglia contribute to the inflammatory response by releasing pro-inflammatory molecules; however, they also contribute to repair mechanisms by clearing dead cells and releasing anti-inflammatory cytokines [26].

43.2.10 Repair Mechanisms

Due to the damage induced by ischaemia and reperfusion injury, the brain responds with endogenous repair mechanisms in an effort to retain and restore function (Fig. 43.3).

As previously mentioned, collateral flow maintains the ischaemic penumbra to some extent during ischaemia; however, neovascularisation takes place following ischaemia to help improve blood flow compromised by ischaemic damage. This is via angiogenesis (the formation of new capillaries from existing blood vessels), vasculogenesis (the formation of new blood vessels) and arteriogenesis (the growth of new collateral arteries from existing arterioles) primarily driven by the upregulation of vascular endothelial growth factor (VEGF), induced by hypoxia and aided by production of NO, which dilates blood vessels improving flow and aids in the induction of vessel remodelling, for example, by increasing MMP9 activity. Protease enzymes such as MMPs are upregulated to aid in this remodelling process, whereby the MMP degrades the extracellular matrix proteins making space for new blood vessel growth.

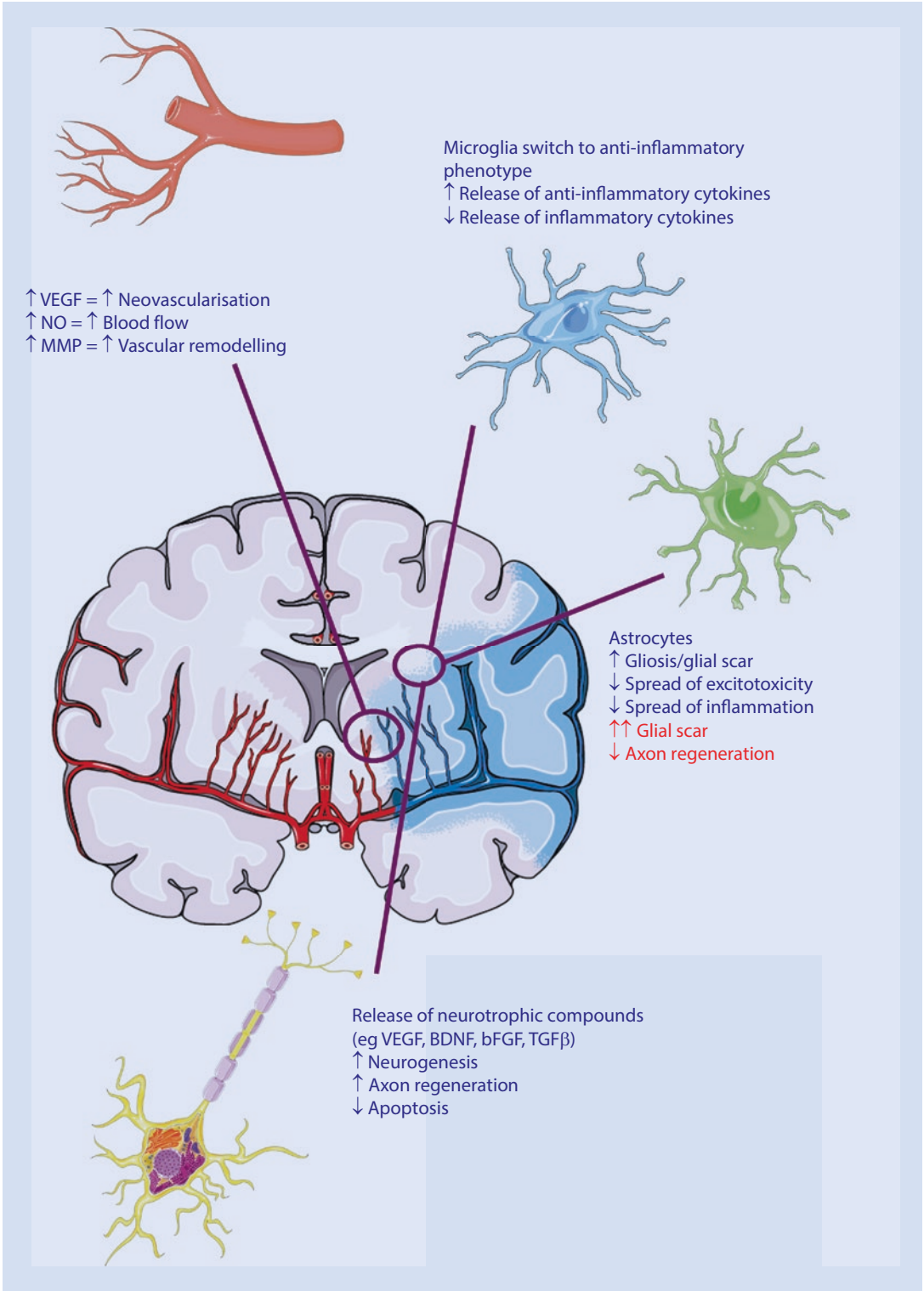

Fig. 43.3 Repair mechanisms in ischaemic stroke. Diagram summarising vascular, neuronal, microglial and astrocytic specific repair mechanisms following ischaemic injury

VEGF has also been shown to act upon neurons and promote neuronal repair. Production of this and other neurotrophic compounds, such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF), is upregulated following cerebral ischaemia to promote axon regeneration and neurogenesis. The subventricular zone (SVZ) of the brain contains neural stem cells which can proliferate and differentiate into new neurons (neurogenesis). This is stimulated by transforming growth factor β (TGFβ) superfamily signalling and activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. Neurotrophic compounds also promote repair and reduce injury following ischaemia by supressing apoptosis [27].

Ischaemic injury also stimulates astrocytes to initiate gliosis and lay down a glial scar. This creates a physical barrier and "cordons off" the damaged tissue to reduce its spread, for example, preventing the spread of inflammation or excitotoxicity. The glial scar also, however, prevents axon regeneration, and so if the scar is too large, it can become detrimental to brain repair.

Anti-inflammatory cytokines, such as interleukin-10 (IL-10) or TGF-β, are also upregulated, particularly in microglia, in response to ischaemic injury. IL-10 acts by inhibiting the actions of pro-inflammatory cytokines, while TGF-β induces the anti-inflammatory phenotype of microglia by preventing their release of pro-inflammatory molecules.

43.2.11 Pathophysiology of Haemorrhagic Stroke

Although not the focus of this chapter, an overview of stroke would not be complete without a comment on the pathophysiology of haemorrhagic stroke. Being caused by the rupture of a blood vessel rather than occlusion, haemorrhagic stroke does not suffer the ischaemic pathophysiology described above. It does, however, result in excitotoxicity, cytotoxicity, oxidative stress and inflammation. This is because the components of blood are directly exposed to the brain resulting in an inflammatory response and ROS production. In addition, the breakdown of red blood cells releases haemoglobin which is cytotoxic [28].

43.2.12 The Quest for Neuroprotective Therapies

As previously mentioned, the current treatments for ischaemic stroke are focussed on clot removal either pharmacologically, with the clot buster tPA, or mechanically, using the thrombectomy procedure.

Although reducing the period of ischaemia can improve patient prognosis, currently there are no neuroprotective drugs or strategies in use to minimise damage due to ischaemia or reperfusion. Over the years, many different aspects of ischaemia-reperfusion injury pathophysiology have been targeted as potential treatments for stroke but with little success in clinical trials. For example, excitotoxicity was targeted with NMDA receptor antagonists such as selfotel or dizocilpine [29]; calcium overload, which is integral to ischaemia-reperfusion-induced cell death, was targeted with calcium channel blockers (CCBs) such as nimidopine or verapamil [30]; immune response was targeted with anti-inflammatories such as minocycline or drugs to prevent leukocyte adhesion such as enlimomab [31]; and excess ROS production was targeted with ROS scavengers such as tirilazad mesylate or NXY-059 (also known as Cerovive) [18]. Similarly, other treatments targeting multiple pathways of ischaemic injury have been assessed, such as physical or pharmacological hypothermia. Hypothermia has been shown to have a neuroprotective effect through reducing cellular metabolic demand, preventing apoptosis, reducing ROS production and reducing inflammation, but so far, the beneficial effects seen in preclinical stroke models have not yet consistently translated clinically, and further work is required [32].

There have been various animal models of stroke, mostly rats or mice, developed in order to study stroke pathophysiology and search for neuroprotective strategies. These models involve the induction of focal cerebral ischaemia and may be permanent, to study the mechanism of and treat the ischaemic injury, or transient, to study and target reperfusion injury. Some examples of the most common models are the intraluminal filament model of middle cerebral artery occlusion (MCAO), where a filament is inserted into the internal carotid artery (ICA) at the neck and advanced into the circle of Willis to occlude the origin of the middle cerebral artery (MCA) and

either left in place (permanent, pMCAO) or removed after a period of time (transient, tMCAO); the electrocoagulation model of pMCAO, where electrocoagulation forceps are used to permanently occlude the MCA via a craniectomy; the embolic model, where a blood clot is formed outside the body and injected via a catheter into the ICA to occlude the origin of the MCA which can then be dissolved with tPA; and the endothelin-1 (ET-1) model, where ET-1 is injected via stereotactic injection into the tissue surrounding the MCA; ET-1 is a potent vasoconstrictor and causes temporary occlusion of the MCA which then gradually relaxes allowing for reperfusion [20].

The failure of many neuroprotective strategies to translate clinically is thought to be due to the poor quality of preclinical trials. For this reason, the stroke community came together in 1999 to produce the stroke therapy academic industry roundtable (STAIR) recommendations, for advancing preclinical research, which have since been updated in the 2009 STAIR report and the newer RIGOR [33] and IMPROVE [34] guidelines. These guidelines focus on improving the quality and validity of preclinical studies with emphasis on the use of randomisation and investigator blinding to minimise bias, the use of power calculations to define group sizes, the use of functional outcome measures rather than infarct volume alone, the use of comorbidity rather than healthy animal models and the replication of trials in different animal models and/or laboratories to demonstrate reproducibility and robustness [33]. These guidelines aim to improve the quality of preclinical studies so that drugs or therapies are more likely to succeed in clinical trials where the population is more heterogeneous and displays multi-morbidities. The stroke research community is committed to resolving the issues relating to translating preclinical research to successful clinical treatments for stroke. This is evidenced by the continuously improving guidelines for stroke research but also by the creation of the MULTIPART (Multicentre Animal Research Team) network (► http://www.dcn.ed.ac.uk/multipart/default.htm). This international network was established with a vision to perform large multicentre preclinical trials, similar to phase III clinical human trials, to combat the translational stroke research crisis.

Other potential confounders limiting efficacy in clinical trials are unsuccessful recanalization with tPA and the variety of stroke subtypes included in trials. The recent successes of numerous mechanical thrombectomy trials, in improving patient outcome following stroke and extending the length of the stroke treatment window, mark the beginning of an exciting new era of stroke research. This is because the imaging techniques, used prior to thrombectomy, allow for reduction in patient heterogeneity, by the inclusion of only specific types of vessel occlusion in clinical trials, and allow treatments to be given when salvageable brain tissue is apparent. Meanwhile, the procedure itself improves recanalization success and also offers a local, intraarterial treatment opportunity which may be considered too invasive otherwise [35].

Conclusions and Clinical Perspectives

- Stroke remains a substantial cardiovascular and neurological problem with complex aetiology and pathology.
- Recent research advances have provided a new treatment option for some patients, with the introduction of mechanical thrombectomy procedures which can be utilised as late as 24 hours after stroke onset.
- Although no neuroprotective therapies have yet been successful, advances in brain imaging techniques may provide better opportunities for successful clinical trials by reducing patient heterogeneity, while improvements in the quality of preclinical research may result in more promising drug targets.

Gaps in Knowledge

- Further research is required to better understand the failures of previous neuroprotective trials to produce successful neuroprotective therapies.
- Although the basic pathophysiology of ischaemia-reperfusion injury in stroke is well understood, the roles and interactions of specific cell types in the neurovascular unit are less well understood and may provide insight for the direct targeting of drugs.
- The use of large multicentre preclinical trials in comorbid or multi-morbid animal models may help to improve the success of translation of stroke therapeutics.

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Peripheral Vascular Disease

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_44

44

Key Concepts

- Peripheral vascular disease (PVD) is common and is usually asymptomatic but can progress to critical limb ischaemia and eventually limb loss.
- PVD is associated with disease in other vascular territories and consequent increased cardiovascular and overall mortality. Secondary preventative measures, such as lifestyle and risk factor modification, are an important part of management.
- Diagnosis is based on a careful and detailed history and simple clinical examination of the arterial supply of the lower limb, occasionally supplemented by non-invasive imaging.
- For most, conservative management is recommended, with endovascular or open surgery being offered depending on clinical severity, disease pattern and patient factors.
- New therapeutic options such as gene and cell therapy under clinical testing may provide future management options in patients not suitable for conventional therapy.

44.1 Introduction

Peripheral vascular disease (PVD) is the name given to atherosclerosis affecting the arterial vasculature predominantly of the lower limb. Given the systemic nature of atherosclerosis, patients with PVD commonly have concomitant disease affecting the coronary, cerebral and carotid arteries. Thus PVD is associated with poor outcomes with increased overall and cardiovascular mortality [1]. In early disease, patients are usually asymptomatic, but a minority can gradually progress to symptomatic disease and even fewer progressing to critical limb ischaemia (CLI) with rest pain and eventual tissue loss. This is usually a reflection of the arterial disease pattern affecting the three segments responsible for perfusion of the lower limb – aortoiliac, femoral popliteal and crural – below the knee disease. This chapter discusses epidemiology, risk factor modification, assessment and the evidence for the management options of the patient presenting with symptoms of PVD. Finally, newer modalities for treatment of PVD are considered.

44.2 Epidemiology and Natural History

It is challenging to determine an accurate figure for the prevalence of PVD in the general population. Historically, screening studies focused on selected cohorts of patients and used self-administered questionnaires that could have led to under-reporting. The ankle-brachial pressure index (ABPI) is the most useful non-invasive method of assessing and screening for asymptomatic disease in the general population with values <0.9 indicative of arterial disease.

The Edinburgh Artery Study reported the prevalence of asymptomatic PVD to be 7–15% among the middle-aged and elderly population [2]. As age is a known risk factor for atherosclerosis and therefore PVD, it is not surprising that the prevalence of PVD increases with age. Further research in the USA found the prevalence of PVD among those over the age of 40 years was 4.3%, increasing to 14.5% in those over 70 years [3]. In the general population, the prevalence of intermittent claudication has been quoted in the literature as 1.1–6.1% [3–5], with the incidence increasing sharply with age.

Patients with PVD are at increased risk of disease progression as well as cardiovascular mortality. About 7–15% of asymptomatic patients progress to intermittent claudication over a 5–7-year period [2, 6]. Risk factors found for declining ABPI and subsequent progression of disease include increasing age, smoking, uncontrolled hypertension, diabetes and raised LDL cholesterol [7]. Interestingly, the Edinburgh Artery Study reported that disease severity did not correlate with cardiovascular risk: both asymptomatic patients with PVD and those with intermittent claudication – a seemingly more severe disease – had the same increased risk of cardiovascular events and death [2].

The majority of patients with intermittent claudication will remain stable with no deterioration or improvement in their walking distance without any intervention apart from risk factor modification and structured exercise. Twenty-five percent of patients experience clinical deterioration with only a minority of these requiring revascularisation or major amputation [8].

44.3 Risk Factors and Disease Modification

The pathogenesis of cardiovascular disease and PVD is similar, and many of the modifiable risk factors for heart disease are applicable to arterial disease of the lower limb. The process of atherosclerosis in vasculature of the lower limb is less understood compared to the coronary vessels. It is thought the development of plaques in the artery in the lower limb leads to narrowing or stenosis of the vessels. These plaques, covered by a fibrin layer, are fragile and susceptible to rupture which leads to thrombosis within the vessel, thereby further obliterating it [9, 10]. The increased demand for oxygen and nutrients accompanied by an inadequate supply due to occlusion of the arteries leads to the symptoms typical of PVD – pain and eventually tissue loss.

Risk factors for the development of PVD can be categorised into modifiable and non-modifiable or the presence of existing disease. Logistic regression analyses adjusted for age and gender have shown that black race/ethnicity, current smoking, diabetes, hypertension, hypercholesterolemia and poor kidney function are significantly associated with PVD [3].

Evidence from several studies have shown that there is a clear and strong association between age and increased risk of PVD [2–4]. Gender differences are more equivocal with studies showing no difference, while others show a preference towards one gender [2, 6, 11]. Smoking remains the key modifiable risk factor: in addition to the established increased risk of cardiovascular death, cancer-related death and obstructive pulmonary disease, smoking is strongly associated with and one of the most significant risk factors for progression of PVD. Risk factor modification in PVD heavily focuses on smoking cessation with all forms of nicotine replacement therapy helpful [12].

Diabetes and renal failure present two groups of patients in whom complex and varying multi-organ disease present require greater consideration. In addition to macro- and microvascular disease, the individuals with diabetes suffer from a differing pattern of PVD with more diffuse and distal disease of the lower limb compared to the nondiabetic. As a result, individuals with diabetes are more likely to require revascularisation and are at an increased risk of amputation. Often traumatic ulcers initiated by neuropathy destabilise what previously was a stable perfused foot to a limb that requires greater perfusion to allow ulcer healing but whose PVD prevents the increase required. Glycaemic control helps prevent progression and development of PVD in diabetics: the UK Prospective Diabetes Study reported a 28% increased risk of PVD for every 1% increase in HbA1c [13]. Additionally, individuals with diabetes should have adequate blood pressure control if hypertensive, as this not only reduces the cardiovascular risk but also the risk of amputation [14].

Patients with end-stage kidney disease (ESKD) are also at risk of developing PVD with the prevalence among haemodialysis patients reported between 12% and 38% [15]. Additionally, PVD has been shown to be an independent predictor of all-cause and cardiovascular mortality in patients on haemodialysis [15]. Patients also have concomitant disease such as diabetes, which produces a disease pattern similar to the non-renal failure and diabetic vasculopath – diffuse, distal disease with neuropathy and proclivity to infection. Investigation in the patients with ESKD is also challenging as the use of contrast-enhanced magnetic resonance angiography (CE-MRA) with gadolinium is often contraindicated due to the risk of developing nephrogenic systemic fibrosis (NSF), and the use of iodinated contrast agents in computer tomography angiography (CTA) risks further injury to the kidney. Management in this select cohort of patients includes risk factor modification and secondary preventative measures such as cessation of smoking and antiplatelet therapy. Further options include angioplasty, bypass or amputation depending on disease severity and patient factors.

Hypertension is a significant risk factor for cardiovascular and peripheral vascular disease. Optimum blood pressure (BP) control not only reduces the risk of cardiovascular disease but delays the progression of arterial disease with NICE recommending a target BP less than 140/85 mmHg with either an angiotensin-converting enzyme (ACE) inhibitor or calcium channel blocker depending on age and ethnicity with multimodality treatment reserved for hypertensives who are uncontrolled with one agent [16].

Ethnicity has also been shown to correlate with the risk of developing PVD. In a large study in the USA, it was reported that African Americans were 2.8 times more likely to screen for PVD than non-Hispanic whites [3]. This can be explained by

the higher prevalence of hypertension and diabetes (risk factors for PVD) among the African American population [17].

There is an association between elevated cholesterol and PVD with elevated cholesterol levels increasing the risk of developing PVD [18]. HMG-CoA reductase inhibitors are the main therapeutic option for patients with PVD and elevated cholesterol, with clinical trials reporting that treatment with statins improves walking distance and quality of life [19–21].

The use of antiplatelet therapy has become the standard of care for secondary prevention in patients with both cardiovascular and peripheral vascular diseases [22].

44.4 Chronic Lower Limb Ischaemia

PVD can be described as a clinical spectrum of chronic vascular insufficiency. Patients can often have disease that is asymptomatic, with occasional progression to symptomatic disease (intermittent claudication) and very occasionally to limb threat (critical limb ischaemia – Table 44.1). Diagnosis is usually entirely clinical, based on the history and supportive examination of the lower limbs. The ABPI is typically reduced below 0.9 and, with increasing severity of disease, gradually declines. However the ABPI is most usefully employed as a supportive measurement that reenforces the clinical diagnosis as investigation is only merited where intervention is proposed and this is a clinical judgement. The following outlines key features associated with clinical stages of PVD, examination and assessment of the chronically ischaemic limb.

Table 44.1 Severity of peripheral vascular disease (Fontaine classification)

Stage		Description
I	Asymptomatic	Disease present but no symptoms
II	Intermittent	Cramping pain in leg/thigh muscles precipitated by walking and relieved by rest
	Claudication	Mild claudication
	IIa	Moderate to severe claudication
	IIb	
III	Rest pain	Constant pain (worse at night)
IV	Tissue loss	Presence of ulceration or gangrene

44.4.1 Intermittent Claudication

Intermittent claudication (IC) can be defined as cramp-like pain that develops in the muscle groups after walking that occurs in a characteristic pattern, is eased by a short period of rest and recurs in the same pattern at the same distance. The muscle groups affected are usually distal to the atherosclerotic lesion – buttock and thigh claudication signals disease of the aortoiliac segments, whereas calf claudication indicates disease of femoral-popliteal segments. Pain, due to muscle ischaemia, is absent at rest and develops progressively while walking. This is because of a failure of the exercise-induced vasodilation to supply the muscle groups: at rest there is adequate supply for the tissues but not for increasingly metabolically active muscles. It is easily differentiated from other conditions such as osteoarthritis and spinal stenosis by the clinical picture: in spinal stenosis, the pain is usually relieved by flexing the spine, walking uphill or lying flat, whereas osteoarthritis affects the joints and is usually present on standing and tends to ease with walking. Examination often reveals diminished or absent pulses and reduced ABPI (<0.9).

44.4.2 Critical Limb Ischaemia (CLI)

CLI is a very different disease state that, without treatment, can result in tissue and eventually limb loss. Although it has a textbook definition (rest pain of more than 2 weeks of duration or ulceration/gangrene with an ankle pressure of <50 mmHg or toe pressures <30 mmHg), it should be easily recognised with appropriate history and examination [23]. Perfusion that is inadequate for the tissues at rest is very typical: is severe, worse on elevation and only relieved by dependency. Thus pain is often described as worse at night while asleep (due to the loss of the beneficial effects of gravity on blood

flow), and the patient often has to hang his foot off the bed or stand up and walk in order to relieve the pain, which may lead to dependent oedema. The pain is severe requiring opioid analgesia. Rest pain may be accompanied by tissue loss in the form of ulceration between the toes, painful heel hacks or frank necrosis of the digits. Clinical examination may reveal a pale foot when it is raised above the level of the heart (Buerger's test positive). Infection may rapidly supervene in ischaemic tissue, leading to misdiagnosis due to the redness, heat and swelling. A key in recognition is the clinical picture of pain on elevation. Critical limb ischaemia requires immediate assessment and intervention to prevent limb loss.

44.5 Assessment of the Ischaemic Lower Limb

An adequate knowledge of anatomy is important before assessing the lower limb (Fig. 44.1). The aorta bifurcates at the level of the umbilicus to form the left and right common iliac arteries. The common iliac vessels then bifurcate to form the internal and external iliac arteries. The external iliac crosses the groin crease to become the femoral artery which descends down the leg before becoming the popliteal artery just above the knee joint. Below the knee joint, the popliteal artery divides into the anterior tibial and posterior tibial arteries. The anterior tibial artery continues into the dorsum of the foot as the dorsalis pedis artery and the posterior tibial artery pass behind the ankle to supply the foot.

A thorough history and clinical examination is the basis in evaluating the chronically ischaemic leg. Details of the duration, quality and character of the pain as well as the effect on the quality of life of the patient should be documented. Symptoms of disease affecting other vascular territories should be sought. These include symptoms of cerebrovascular, renovascular and cardiovascular disease. Risk factors for atherosclerosis and a history of smoking should be obtained, as should any prior vascular interventions.

A general systemic exam should be performed before a detailed assessment of the lower limb vasculature that should include blood pressure monitoring, random blood glucose measurement and palpation of central pulses. Pulse assessment entails palpation of the abdominal aorta (which may be difficult in the obese abdomen), femoral, popliteal, posterior tibialis and dorsalis pedis pulses. Peripheral pulses are generally poorly examined. Capillary refill is invariably reported but often influenced by environmental conditions. It is important that the patient is positioned appropriately and correct anatomical landmarks are used in locating the pulses. Additionally, the examination room should be warm and the patient properly exposed. The absence of pulses is not always indicative of the degree of arterial insufficiency, and it is therefore important to assess for overall perfusion and also for tissue loss. This is particularly important in the neuropathic limb.

ABPI measurements can be assessed in the vasculopath, but the optimal use of these is for cases of diagnostic doubt to separate symptoms of PVD from coexistent disease such as joint pain. Ankle pressures are compared to the blood pressure in the arm (higher pressure), with values of 1.0–1.2 considered normal. Patients with diabetes and chronic kidney disease often have heavily calcified vessels which may result in abnormally high ankle pressures, and therefore one should be suspicious of an abnormally high ABPI. Ideally these patients should have their toe pressures or the arterial waveform assessed to detect the degree of stenosis or occlusion. The normal elasticity of arteries gives a unique triphasic waveform on Doppler, which in turn can be used to give valuable information of the degree of stenosis. This is reflected by the change in waveforms with lesions of moderate stenosis (50% reduction) giving a biphasic waveform with monophasic waveforms indicating >70% stenosis.

Duplex USS may be employed to locate the anatomical lesion and degree of stenosis in patients with PVD. It allows both the visualisation of vessels and detailed assessment of waveforms and blood flow. Changes in the peak systolic velocity (PSV) can also give an indication of the degree of stenosis. The PSV is measured in the normal artery proximal to a stenosis and then within the stenosis. A 50% stenosis will be reflected by a doubling of the PSV with greater increases indicating more severe stenosis. The combination of PSV, waveform assessment and visualisation of the artery provides useful information on the severity of the stenosis.

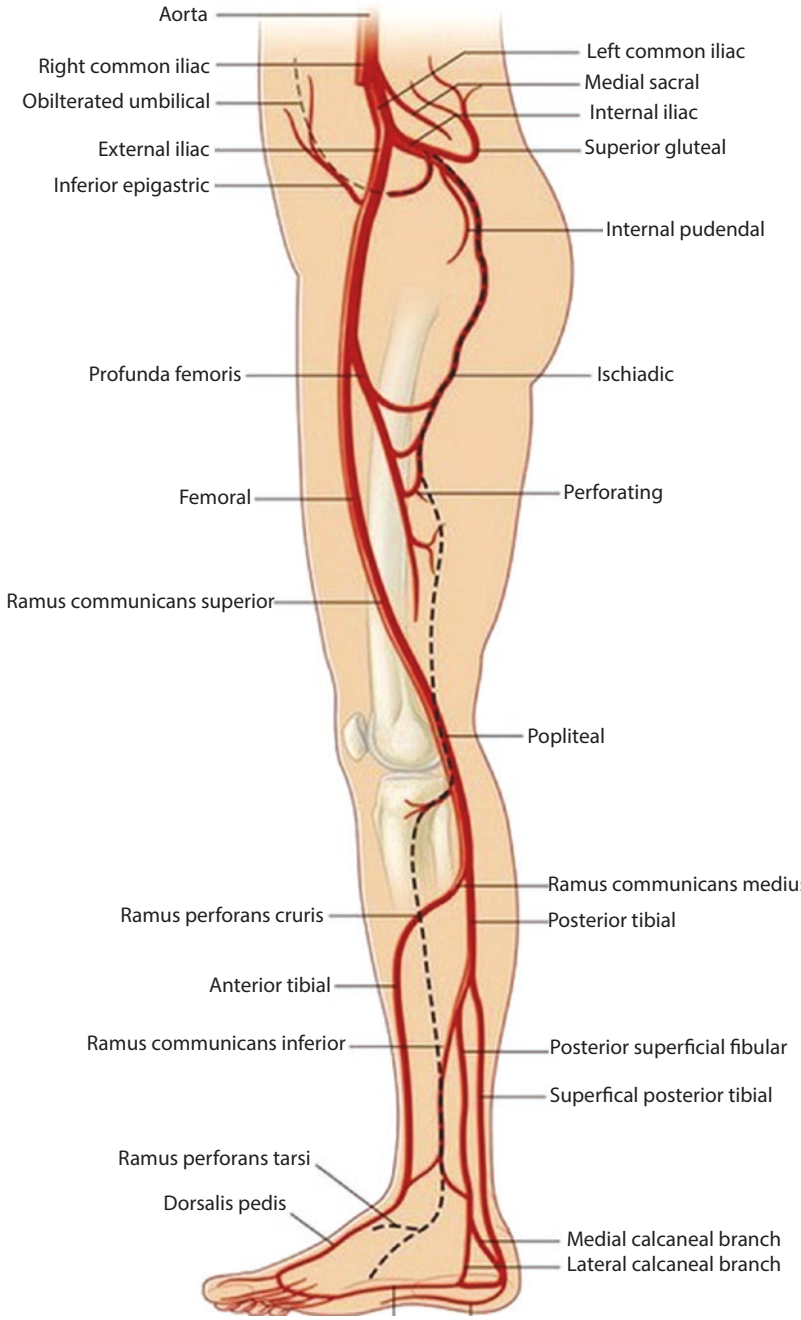

Fig. 44.1 The arterial supply to the lower limb

44.6 Imaging Modalities

44.6.1 Catheter Angiography

The use of digital subtraction angiography (DSA) in the investigation of PVD as a first-line modality is no longer recommended. Its invasive nature, the use of iodinated contrast material, exposure to ionising radiation and complications (embolisation, pseudoaneurysms, AV fistulas, haematomas) have all led to a reduction in its use except for selected patients in whom other imaging modalities have been inconclusive – most typically due to severe calcification. However, it does

offer excellent views of distal vessels and allows both diagnostic and therapeutic procedures to be performed simultaneously.

44.6.2 Magnetic Resonance Angiography (MRA)

The use of MRA has been shown to have high accuracy for detecting and localising occlusive disease in patients with PVD [24]. The non-invasive nature, use of non-iodinated contrast and lack of radiation exposure make it an attractive option, and it is now the first-line investigation for PVD. Various imaging protocols and techniques have evolved that provide more accurate detail of distal vessels, reducing venous contamination which previously limited interpretation of crural vessels. It tends to underestimate the degree of calcification in vessels and overestimate the degree of stenosis. Contraindications include patients with metallic heart valves, cardiac pacemakers, cochlear implants or metallic ocular foreign bodies. Patients with impaired renal function are also at increased risk of nephrogenic systemic fibrosis (NSF) which is related to the use of the contrast agent gadolinium, and these patients may benefit from time-of-flight MRA. Prosthetic joints may impair the direct visualisation of the overlying arteries.

44.6.3 Computed Topographic Angiography (CTA)

CTA is generally used when imaging is required urgently or MRA is contraindicated [25]. The advantage of CTA is that it allows accurate vessel measurement, and 3D images can be obtained which allows for accurate planning for endovascular procedures. Limitations of its use include the use of iodinated contrast agents (and the possibility of developing contrast-induced nephropathy (CIN)) and use of ionising radiation.

44.7 Management of PVD

44.7.1 Medical

As noted previously, modifiable risk factors for PVD should be addressed in the initial consultation. Although the role of the vascular specialist has been advocated by some, secondary preventative measures are best prescribed and monitored by the general practitioner. This will include optimal management of comorbidities and cessation of smoking. Patients with intermittent claudication should be encouraged to exercise with supervised, structured programmes offering a significant improvement in walking distance equivalent to angioplasty over 2 years [26]. Additionally, given that PVD has similar risk factors as cardiovascular disease, it is recommended that patients are managed with antiplatelet therapy (aspirin or clopidogrel) and statins. The role of antiplatelet therapy is mainly to reduce cardiovascular disease progression with several studies showing a beneficial reduction in risk [27, 28]. Furthermore, a large review found that statins reduced disease progression and improved symptoms and walking distances in patients with symptomatic PVD and hyperlipidaemia [29]. Vasoactive drugs may be prescribed for patients with intermittent claudication as they have been proven to have an effect on walking distance. Although randomised trials have shown no difference among different vasoactive drugs, NICE recommends that naftidrofuryl be used as first line since it is the most cost-effective [30].

Prostanoids are powerful vasodilators and have an effect on platelet function and improve endothelial function. The method of administration is usually intravenous with side effects ranging from mild to life threatening, and therefore its use is limited to the hospital setting. Iloprost has been evaluated in several trials with the most benefit seen in patients with critical limb ischaemia and Buerger's disease [31, 32].

44.7.2 Endovascular and Surgical

Intervention in patients with intermittent claudication depends on several factors. Most importantly, the patients' claudication distance will determine how limiting their disease is and the effect on their quality of life. Young patients with very short claudication distances will be severely handicapped if it limits their ability to work or enjoy other activities and therefore will benefit from intervention. However, if the distance at which they claudicate has no effect on quality of life, then a conservative approach may be more suitable. Before intervening in patients with intermittent claudication, a trial of best medical

therapy, smoking cessation and risk factor modification should be implemented. Failure to improve with these measures may warrant intervention. This clearly differs from patients with critical limb ischaemia who invariably will require some form of intervention. The type of intervention will largely depend on the overall health of the patient as well as the pattern and severity of disease.

Once chronic limb ischaemia is confirmed, a decision is made on whether the patient will need or will benefit from revascularisation. If they are candidates for revascularisation, they should be imaged with Duplex USS or more commonly by contrast-enhanced MRA or CTA. The decision for endovascular versus open revascularisation will depend on the severity of disease (focal versus multisegmental), anatomical location (supra-inguinal versus infrainguinal) and overall health of the patient.

The Trans-Atlantic Inter-Society Consensus (TASC) working group published guidelines (TASC I and II) on which lesions should be treated with endovascular versus open surgery. These guidelines are based on anatomical lesions and do not take into account individual patient factors. No advice is offered on the management of infra-popliteal vessel disease (TASC II). Further guidelines (TASC III) are currently being developed.

44.7.2.1 Suprainguinal Disease

The options for suprainguinal disease (aortoiliac) are either endovascular or open. TASC recommends that simple aorto-iliac disease (types A and B) is best treated with angioplasty (PTA) with more severe disease – longer, multi-level stenotic lesions (types C and D) requiring open surgical repair. The decision for PTA alone versus PTA and stenting will depend on whether the lesion is stenosed or occluded and the recoil following angioplasty.

Common iliac lesions within close proximity of the aortic lumen are at risk of occluding the contralateral vessel if stented, and occasionally, bilateral or kissing stents are required to maintain contralateral patency. Complications with angioplasty and stenting occur in 2.6–11.6% and include bleeding, pseudoaneurysm formation, arterial rupture, embolisation, CIN and stent-related problems.

Open surgical intervention in patients with aorto-iliac disease is a significant undertaking, and this option is based on general fitness and the presence/absence of severe cardiopulmonary disease. Patients undergoing aortic surgery should undergo cardiopulmonary testing usually with an echo and pulmonary function tests or cardiopulmonary exercise testing.

In those deemed fit, the option for occlusive disease of the infrarenal aorta is a graft that originates in the aorta proximal to the disease that lands in the most proximal patent distal artery – usually an aorto-bifemoral graft (ABG). This is an extensive operation with an intraoperative mortality of 5–10%. Long-term outcomes are usually good with patient survival of 73–88% and a primary patency rate of 85% at 5 years [31]. In patients who are unfit or those with a hostile abdomen, the operation of choice is an axillo-bifemoral bypass. For all prosthetic grafts, the greatest fear for the vascular surgeon is graft infection.

For extensive disease affecting the external iliac extending into the common femoral artery, the options include an ileo-femoral bypass, femoro-femoral crossover or ileo-femoral crossover. Principles related to successful bypass procedures include good inflow, adequate runoff and a suitable conduit for bypass. The options for conduit are autologous vein or prosthetic graft. Generally, the vein has better patency rates and lower infection rates than synthetic grafts, but newer biological grafts may be better.

When disease is limited to the common femoral artery or extends into the deep profunda artery/superficial femoral artery, the treatment of choice is a femoral endarterectomy and repair using a vein or bovine pericardial patch. Due to the location near to a joint that is subjected to flexion/extension, it is thought that these lesions are not suitable for angioplasty; however, there are no randomised trials comparing both interventions.

44.7.2.2 Infrainguinal Disease

There has been a change in the management of infrainguinal disease with open surgical bypass being replaced by a more selective policy to intervention and better angioplasty options. This increasingly selective policy is in response to the recognition of the drastic impact of abrupt occlusion or loss of prosthetic bypasses that had been performed more readily in the past. Success with infrainguinal bypass will depend on good inflow and runoff vessels as well as a suitable conduit.

The options for disease affecting the femoral-popliteal segments include angioplasty or open surgical bypass. As recommended by TASC, type A and B lesions are managed by endovascular techniques, but the re-intervention rate is higher. Patients with more severe disease are more likely to require open surgical bypass either femoral-popliteal bypass (above or below knee) or femoral-distal bypasses. Long saphenous vein is ideal as it provides better 5-year patency rates compared to synthetic grafts for bypasses both above and below the knee [32, 33].

Angioplasty options for infrainguinal disease are variable and can range from routine angioplasty +/− stenting or subintimal angioplasty. Femoral-popliteal lesions (types A and B) are best treated with angioplasty with 1-year patency rates of 70%. The routine use of stenting in these segments is less clear. Meta-analyses have not supported the routine use of stenting in the femoral-popliteal segment [34]. Subintimal angioplasty is a well-described technique where a guidewire is used to access the subintimal space above an occlusion. The wire then re-enters the true lumen of the vessel, following which the subintimal space is angioplastied to create a new lumen. Through remodelling, the new lumen created acts as the new "conduit" for blood flow. Success of this procedure depends on adequate inflow into the subintimal space as well as adequate runoff. Complications of this procedure include failure to regain entry into the true lumen due to severe calcification, perforation of the artery and distal embolisation. This procedure is suitable for very long and multifocal occlusions.

The BASIL (Bypass versus Angioplasty in Severe Ischemia of the Leg) trial randomised 452 patients with infrainguinal disease to either bypass or angioplasty. This trial found no significant differences in amputation-free survival, quality of life or all-cause mortality up to 2 years. Thirty-six percent of patients died by 5 years [35]. The conclusions drawn from this trial were (1) fit patients likely to survive more than 2 years with useable vein should be offered an operation and (2) angioplasty should be reserved for those not suitable or unfit for bypass.

Few patients may be unsuitable for both angioplasty and surgical bypass. Often these patients with intractable rest pain are offered an amputation to alleviate their symptoms. Several medical options are available; however, the long-term results of these treatments are often poor. Iloprost, in a meta-analysis of 700 patients with critical limb ischaemia, was reported to significantly reduce death and amputation [25]. However the long-term benefits remain unclear, and therefore its use is declining. In patients with non-reconstructible disease with rest pain, chemical lumbar sympathectomy may offer some early short-term benefits, but in the majority, the long-term effects are poor and inevitably lead to an amputation.

44.7.3 Gene Therapy

The use of angiogenic growth factors has great appeal in patients with occlusive disease not suitable for intervention. Genes coding for growth factors, such as vascular endothelial factor, fibroblast growth factor and platelet-derived growth factor, can be injected into ischaemic tissue to stimulate angiogenesis and lead to improved blood flow into the limb. The limited clinical trials investigating the effectiveness of gene therapy have shown improvements in ABPI, rest pain and increased flow detected by radiography [36]. However these trials often have a degree of heterogeneity (growth factor used, endpoints, severity of PVD, method of gene delivery) making comparisons difficult. A meta-analysis of trials investigating the effectiveness of gene therapy has reported no significant differences in main outcomes [37]. Further clinical trials are still needed to investigate the effectiveness and benefit of these therapeutic options.

44.7.4 Cell-Based Therapy

Endothelial progenitor cells are necessary for vasculogenesis. They can be found in the peripheral circulation, but the major source of these cells is the bone marrow. Small clinical trials have reported some benefit with cell-based therapies, with improvements in ABPI, rest pain and collateral vessel formation. There is some benefit to be gained from treatment with granulocyte-colony stimulating factor (G-CSF) which has been reported to also increase collateral flow and improvement in symptoms. The combination of G-CSF with progenitor cells seems to offer the most benefit with significant improvements in lower limb pain and ulcer healing [38].

These new modalities offer great potential in the treatment of patients with peripheral arterial disease. Potential complications include unwanted neovascularisation such as in the eye and malignant lesions, as well as the theoretical risk of bleeding. However, further clinical trials are needed to assess the long-term benefits and potential risks, before they are offered as an alternative treatment option for PVD.

Conclusions and Clinical Perspective

- Among the UK population, peripheral vascular disease is common. Risk factors for peripheral vascular disease such as diabetes, hypertension, end-stage renal disease and high cholesterol are similar to those for cardiovascular disease, and therefore PVD can be a surrogate marker for cardiovascular risk.
- Patients can often be asymptomatic or can present with severe critical limb ischaemia with evidence of tissue loss. Patients with less severe symptoms can often be managed conservatively by modifying risk factors and supervised exercise programmes. Patients with severe, debilitating disease often require surgical or endovascular intervention depending on the pattern of disease.
- New gene and cell-based therapies are novel techniques that may be of use in patients with chronic limb ischaemia; however clinical trials are needed to assess the effectiveness of these newer therapeutic modalities.

Gaps in Knowledge

- PVD is a preventable disease process. Ensuring patients at risk are identified and treated appropriately may help in reducing those patients requiring intervention. The role of screening in the general population is disputed. Further studies looking at the cost effectiveness of screening in those at risk are needed to determine whether the number of surgical interventions would be reduced.

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Vascular Malformations and Tumours

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R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2_45

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Key Concepts

- Nomenclature and classification are critical in understanding the different pathologies and communicating with colleagues.
- The pathogenesis of the majority of the vascular tumours and malformations is poorly understood.
- As a result of the poor understanding of the basic pathogenesis of these conditions, the management of the majority of these lesions is relatively crude with non-specific endovascular procedures (embolization, sclerotherapy) or surgical (excision, debulking) forming the mainstay of management options.

45.1 Introduction

When considering the pathogenesis, presentation, investigation and management of vascular tumours and anomalies, the use of accurate terminology and of a classification system is critical. Any classification system should be continually reviewed and renewed to ensure that it remains appropriate and fit for purpose. An ideal classification takes into account the pathogenesis and clinical presentation and then acts as a guide to the clinical management of conditions. Historically the classification and nomenclature used for these conditions have caused significant confusion, with different specialists using different names for the same condition and conversely the same name for different conditions [1]. This proliferation of names also gives the erroneous impression that the conditions they describe are fully understood and that there is a vast knowledge base underpinning the nomenclature. In 1998 a more rational and clinical approach was taken by the International Society for the Study of Vascular Anomalies [2]. This classification system divides vascular malformations into tumours and vascular malformations – the key pathophysiological feature differentiation between the two categories being that the tumours have abnormal endothelial cell turnover and the malformations have normal rates of cell turnover (in the undisturbed state).

The most common vascular tumour is the infantile haemangioma – in fact it is the commonest tumour of childhood. All other vascular tumours are rare by comparison.

Vascular malformations are subclassified by their primary tissue of origin (capillary, venous, arterial, lymphatic or mixed) and by the rate of blood flow through the lesion into low flow or high flow [3].

45.2 Infantile Haemangiomas

Infantile haemangiomas are the most common childhood tumours with an incidence reported to range from 1% to 10% [4–6]. There is an association between low birth weight and haemangioma [5].

Most studies show that head and neck lesions predominate; however an excellent well-powered study of a Dutch population suggests that truncal lesions are more common [6].

45.2.1 Natural History

Whilst the majority of tumours are cutaneous (Fig. 45.1), they can occur at any site or depth of tissue. The deeply placed lesions may remain occult or only be discovered incidentally but may present (particularly if they are large) with complications associated with or caused by the tumour [7]. It is unusual for an infant to present with visceral lesions in the absence of cutaneous lesions, and likewise the presence of five or more cutaneous lesions should prompt examination and investigation for deep lesions [8].

With regard to cutaneous infantile haemangiomas, around 30–40% are obvious at birth [9]. A small herald lesion – a blanched area – or other precursor lesions may be apparent at birth or soon after, and the majority become apparent by the 3rd and 4th week [6]. After its initial appearance, the lesion enters a proliferative phase of rapid neonatal growth reaching 80% of its maximum size by 5 months with growth levelling off between 10 and 12 months [10].

It is during the proliferative phase that most complications occur. Depending on size and site, ulceration, bleeding, obstruction (airway, eye, ear canal), skeletal disproportion and high-output cardiac failure can all occur. Once the proliferative phase has reached its maximum, the lesion enters a second, slower, involuting phase. Whilst they are considered as two distinct phases, there

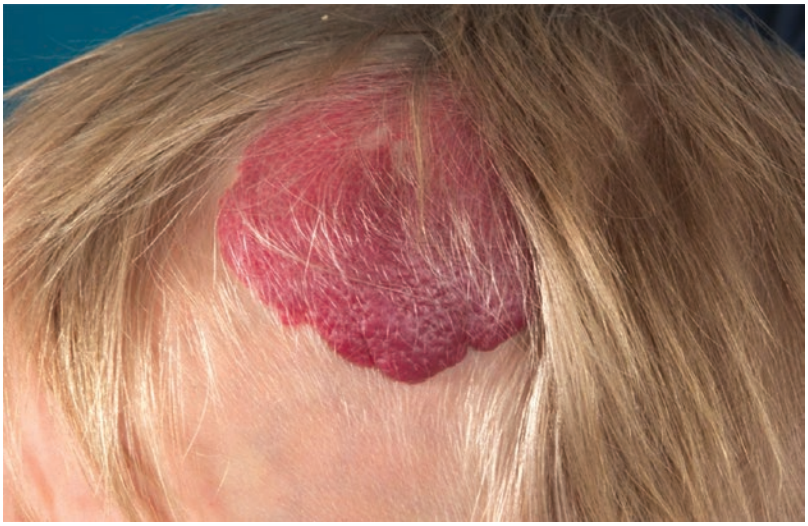

Fig. 45.1 Typical haemangioma just beginning to involute with grey flecks visible in the centre of lesion

is, in actuality, a gradual change from one to the other with a degree of overlap. This second phase lasts, on average, until around the age of 7 years with 70% having completely resolved by this point [11]. There is no correlation between the size of the original lesion or the age of onset and the degree of resolution [10]. Classification: The straightforward infantile haemangiomas are probably best classified as solitary (focal), multiple (multifocal) or territorial (regional).

Management: Diagnosis is usually made on clinical grounds, but if there is doubt, biopsy can be useful (though excessive bleeding is a risk). Histopathologically, infantile haemangiomas are characterised during the proliferative and involuting phases by the presence of the GLUT1 marker (erythrocyte-type glucose transporter enzyme 1) [12].

Active intervention is rarely indicated but may be required in cases presenting with (1) high-output cardiac failure; (2) airway obstruction in cases of tracheal, laryngeal tongue base, oral or nasal tumours and obstruction of vision (to prevent the development of amblyopia); and (3) ulcerated, painful and bleeding lesions and in some cases for cosmetic reasons [13].

Interestingly, it has been found that haemangiomas produce an enzyme (3 iodothyronine deiodinase) that breaks down normal thyroxine and can therefore lead to hypothyroidism. This is only reaches significance in large lesions, and for this reason routine measurement of TSH and thyroxine levels is suggested for patients with large lesions [13].

If active treatment is indicated, primarily for functional problems, there are a number of systemic and local interventions that are helpful. Systemic treatments with corticosteroids, beta blockers, interferon or vincristine have been used. Generally different centres will use either steroids or propranolol (with propranolol being most efficacious if administered in the 1st year of life) as a first-line treatment reserving interferon and interferon for nonresponders [14].

Local interventions include topical beta blockers (Timolol) and high-dose topical corticosteroids; intralesional treatments include steroids and bleomycin.

In refractory cases with limited response, embolisation and surgery may be considered [15].

Subtypes: There are two forms of congenital haemangioma – rapidly involuting congenital haemangioma (RICH) and non-involuting congenital haemangioma (NICH). There are also additional rarer lesions that form part of a syndrome [3].

45.3 Vascular Tumours

Other than infantile haemangiomas, vascular tumours are rare, may be benign or malignant and may also have systemic effects such as a consumptive coagulopathy (Kasabach-Merritt phenomenon [16]). Examples include pyogenic granuloma, Kaposiform haemangioendothelioma, Tufted angioma and angiosarcoma. Management: Pyogenic granulomas can usually be treated with limited surgical intervention with either shaving or local

excision although satellite lesions may form following excision (rarely). The more aggressive tumours are usually treated with a combination of surgery and chemotherapy.

45.4 Vascular Malformations

45.4.1 Capillary Malformations (and Other Superficial Lesions)

This collection of conditions, variously known previously as port wine stains, capillary haemangiomas and/or naevus flammeus, are relatively common. For practical purposes they can be divided into:

1. Common (cutaneous) types, Sturge-Weber syndrome, megalencephaly-capillary malformation
2. Hyperkeratotic lesions (e.g. verrucous haemangioma, angiokeratoma)
3. Telangiectasias (spider angioma, Campbell De Morgan spots, hereditary haemorrhagic telangiectasia (HHT)) [3]

45.4.1.1 Epidemiology

The published incidence rates for common cutaneous capillary malformations vary from 0.1% to 2% of newborns. Sex distribution is equal [17].

45.4.1.2 Natural History

The common cutaneous lesions are usually seen at birth though they may be less obvious because of anaemia or skin pallor. Whilst some lightening may occur during the 1st year of life, the majority do not significantly change in colour with development. In adulthood they often darken, and the overlying skin may thicken and take on a cobblestone appearance. This can lead to significant progressive disfigurement [17].

45.4.1.3 Classification

The common cutaneous lesions can occur at any site. When they occur in the head and neck, they may follow one or more of the dermatomes supplied by the three sensory divisions of the trigeminal nerve and can be associated with overgrowth of the underlying skeleton and soft tissues [18].

Sturge-Weber syndrome is probably the best known syndromic form of a capillary malformation. This is a triad of a capillary malformation affecting the upper face (usually unilateral), vascular anomalies of the meninges and ocular choroid. Two of these features are necessary to make the diagnosis. As the lesions progress with age, bony overgrowth, coupled with the darkening and thickening of the cutaneous lesions, can result in significant facial disfigurement. The meningeal vessels are enlarged and tortious, whilst the cortical vessels are hypoplastic. This leads to cerebral atrophy with subcortical calcifications. The calvarium can become thickened as part of the overall overgrowth phenomenon. Seizure activity is common, and whilst neurological impairment is not an inevitable result of the syndrome, where seizures are severe or intractable, impairment can occur [19]. The ocular lesions include ectatic vessels in the sclera, conjunctiva, retina or choroid, and glaucoma can ensue in around 60% of cases [20].

Megalencephaly-capillary malformation is a condition with capillary malformations typically affecting the midline of the face (forehead or upper lip) in association with megalencephaly. Hemihypertrophy may also occur and there is an associated increase in risk of Wilms tumour [21].

A proportion of capillary lesions are thickened and are thought to be of a different subtype – this subtype has not been fully studied or classified, and they can be termed verrucous haemangiomas. They often start as dark capillary stains but begin to thicken and roughen in early childhood with a well demarcated but irregular margin [22].

Telangiectasias are common lesions of the mucous membranes or skin. They are linear, punctate or stellate dilated small vessels.

Spider Telangiectasias: Characterised by a central arteriole with radiating small vessel in a starburst akin to the legs of a spider (hence spider naevus). These lesions usually appear in infants and the majority disappear after puberty. Acquired lesions are seen in pregnancy and hepatic failure. This points to the role of oestrogen in the genesis of these lesions – liver failure leads to the reduced breakdown of oestrogen and thus its increase in the systemic circulation as happens naturally in pregnancy. Diagnosis can be confirmed by central compression and then

release – this leads to initial blanching followed by radial blushing [23].

Campbell De Morgan spots are common lesions seen in the ageing population and are thought to be harmless. Whilst there are reports of their association with systemic disease, there is no convincing supporting evidence for this. The lesions themselves are 2–5 mm in diameter, smooth and elevated from the underlying skin, and they may become pedunculated. They are non-pulsatile and there is typically a surrounding halo of pallor [23].

Hereditary haemorrhagic telangiectasia (HHT) is a more serious condition with the potential for significant, life-threatening bleeding. It is inherited in an autosomal dominant pattern and a number of mutations have been identified. The telangiectasia may occur at any site; commonly in the mucous membranes, GI tract, cutaneous, visceral or cerebrally. The lesions are arteriovenous malformations, and the condition is also known as Rendu-Osler-Weber syndrome. The presentation and symptomatology are highly variable, and the diagnosis is confirmed clinically with three out of four features – multiple telangiectasias, spontaneous recurrent epistaxis, visceral lesions and an affected first-degree relative [24].

45.4.1.4 Management

The management of this diverse group of conditions is highly dependent on the condition, and its effects and an accurate diagnosis are necessary to minimise the adverse effects of the condition.

Simple, non-syndromic capillary malformations are managed to minimise the resultant deformity. The available treatment options include laser treatment and/or surgery. Surgical excision is reserved for small lesions where primary closure would be achievable or in more severe cases where there is a necessity the lesion can be excised and the skin resurfaced utilising skin grafts or free tissue transfer techniques [25]. Laser treatment is the most common form of treatment, and a pulsed dye laser is the first-line choice of device. The principle of treatment is to cause photocoagulation whilst minimising surrounding tissue damage and hence reducing scarring. Laser treatment necessitates a trial patch to evaluate the response; multiple treatments are usually necessary, and whilst lightening of the lesion is common, complete elimination is not the norm, and other types of laser are often tried to improve the result [26]. Where overgrowth occurs, particularly of the facial skeleton, corrective orthognathic surgery can be performed in a conventional way; however, if the cutaneous lesion remains, it is probable that the skeletal overgrowth will recur, even in adulthood after the completion of normal growth [27].

45.4.2 Lymphatic Malformations

The commonest form of lymphatic malformation is a disorder of lymphatic vessels or nodes leading to the accumulation of extravascular (extralymphatic) fluid known as lymphoedema. Abnormalities of the central lymphatic system (primarily the thoracic duct and its tributaries) are rare but can occur [28]. The formation of mass lesions of lymphatic vessels coalescing into larger fluid-filled cavities (macrocysts) or smaller microcysts leads to the lesions variously known as lymphatic malformations, lymphangioma and cystic hygroma (seen in the neck). The most apt term for these lesions is lymphatic malformation with division into macrocystic, microcystic or mixed, where both microcysts and macrocysts can occur together. The division between the two types is rather arbitrary, but cysts that can be aspirated are termed macrocystic – those too small for this are termed microcystic [3]. This pragmatic approach is useful as the macrocystic lesions are more amenable to treatment with sclerotherapy.

Lymphatic malformations can occur anywhere with the exception of the central nervous system where there is no lymphatic tissue. They occur most commonly in the neck and other sites where there are major lymphatic vessels such as the groin and retroperitoneal region.

Epidemiology: The exact incidence of lymphatic malformations is not known, but the literature estimates it to be around 1–5/10,000 live births [29].

45.4.2.1 Natural History

Larger lesions are often diagnosed antenatally; however others may not manifest until later on in life. Rarely the lesions are so large as to present airway or other obstructive symptoms, but

the majority are asymptomatic [30]. Gradual growth in step with the child's growth is the norm; however superimposed on this are relatively rapid fluctuations in size that can be caused by posture, constriction and concomitant infections.

45.4.2.2 Management

Lymphatic malformations causing airway embarrassment need early intervention, and emergency tracheostomy may be necessary (for instance, in large cervical lesions). Once the diagnosis has been made, usually on MRI scanning [31], the options are for surgical debulking and/or sclerotherapy. The macrocystic lesions respond better to sclerotherapy than the microcystic lesions, but sclerotherapy can be tried for both types. Sclerotherapy involves the direct puncture of the lesion, aspiration of the lymphatic fluid and then instillation of the sclerosant agent usually under ultrasound or x-ray control. The sclerosant agent then causes an inflammatory response leading to fibrosis and scarring, hence reducing the size of the lesion. Surgery is reserved for debulking large lesions or those causing other adverse effects [28]. The timing of surgery is determined by the problems that the lesion is causing and the risks of intervention must be balanced against the potential benefits. The use of surgical coblation techniques is useful in reducing the complications of surgery [32].

In later life as the child grows, lymphatic malformations often become swollen and tense with concomitant infections, and this enlargement may lead to complications; these acute inflammatory episodes may require urgent treatment that can include respiratory support, antibiotics and/or steroids. In the longer term, it is thought that these infective episodes create an inflammatory response and can act as a form of sclerotherapy, shrinking the lesion [33].

In the vast majority of cases, cure is not possible and would only be accomplished with a complete surgical resection which often necessitates extensive damage to surrounding tissues. This collateral damage, particularly in the head and neck, would be debilitating or disfiguring. Furthermore, the margins between normal and affected tissues are not clearly defined increasing the risk of incomplete excision [34].

For these reasons the management of all but the smallest lymphatic malformations is in the main, supportive with active interventions targeted at specific symptoms with clear and well-defined objectives. A staging system proposed by de Serres et al. suggests a guide to when intervention is indicated [30]. Sclerotherapy with bleomycin, sodium tetradecyl sulphate or OK432 (Picibanil, lyophilized mixture of Group A *streptococcus pyogenes*) as well as other agents have been utilised [28].

45.4.3 Venous Malformations

Classification: Venous malformations may be either (i) anomalous anatomic veins or (ii) venous anomalies that are separate from named venous branches [3]. This chapter will only discuss the second category.

45.4.3.1 Epidemiology

These lesions are relatively common with 1–4% of the population. There is equal sex distribution [29].

45.4.3.2 Natural History

Slow-flow venous malformations are present at birth, but not all of them are clinically apparent. They tend to grow in proportion as the child grows; however they can respond to hormonal changes such as puberty or pregnancy, and growth may be accelerated during these periods [35]. In addition the lesions expand when venous pressure is increased such as during a Valsalva manoeuvre or when the lesion is dependent. Recurrent episodes of increased venous pressure can lead to stretching of the walls of the venous cavities, and this can also result in enlargement [36]. Calcification of stagnant blood within the venous cavities results in the formation of phleboliths; these can vary in size and are often palpable and visible on radiological investigations [37].

Symptoms can vary – most are painless – but some discomfort or pain can occur particularly after interventions that may cause a consumptive coagulopathy. If pain is a significant symptom, low-dose aspirin may be of use [38]. Troublesome bleeding is not usually an issue; even after significant traumatic injuries, haemorrhage can be

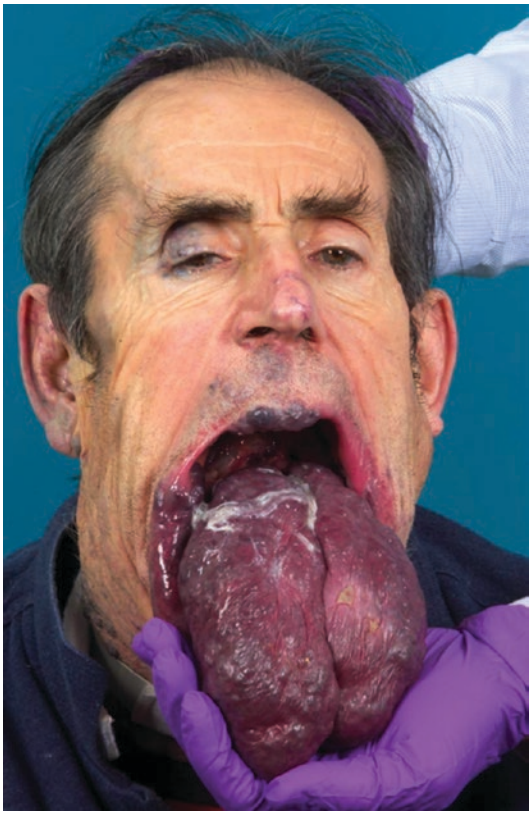

Fig. 45.2 Large multiple venous slow-flow vascular malformations affecting the tongue, lips and right orbit

controlled easily with pressure, reflecting the slow-flow nature of the lesions. The lesions can occur in any organ or location, but cutaneous sites are the most common (Figs. 45.1 and 45.2). These lesions, similar to capillary malformations, can cause overgrowth of the underlying tissues, including the bone. This can result in limb and extremity size discrepancies as well as skeletal and occlusal abnormalities in the head and neck region [39].

Central nervous system lesions can also occur, and their effect at these critical sites can range from being entirely asymptomatic to devastating should haemorrhage occur [40].

45.4.3.3 Management

The management of each particular lesion depends on its site and effects, but in general if the lesion is small and accessible, complete excision will result in cure. This approach is rarely possible as most lesions are too large, with indistinct margins to allow for reliable excision without extensive disfigurement or debilitating damage to surrounding structures. The main objectives in treatment are to mitigate the effects of the lesion and achieve this with as little collateral damage as possible. The mainstay of treatment is sclerotherapy with one or more of a variety of different agents including bleomycin, sodium tetradecyl sulphate and absolute alcohol. These interventional radiology techniques may be augmented with surgical manoeuvres to maximise the effect of the irritant agent by compressing or compartmentalising the lesion; this also has the effect of minimising the rapid systemic distribution of the sclerosant agent [41]. Surgical resection of anatomically amenable lesions has a role; however these procedures often are debulking procedures rather than truly curative resections. Nonetheless, debulking can often lead to significant improvement in symptoms, deformity and quality of life and should not be discounted completely.

45.4.4 Arterial Malformations

In these lesions an abnormal communication forms between arteries and veins. These may be congenital or acquired, either in accidental trauma or intentionally as a surgical procedure (for venous access in dialysis patients). In congenital lesions, there are often multiple connections between the arterial and venous system. Whatever the cause of the abnormal connection(s), the effects are similar. The flow through the arteriovenous malformation (AVM) increases. This shunting from high pressure to low pressure has several effects – the blood flow distant to the lesion reduces as more blood is diverted to the low resistance system, a proximal collateral arterial supply develops, and then this can lead to reverse flow in the artery immediately distant to the AVM. This in turn reduces the flow to the distal portion and can lead to ischaemia. The veins are thus subjected to abnormal high pressures and respond by becoming thickened and "arterialised". The abnormal flow results in

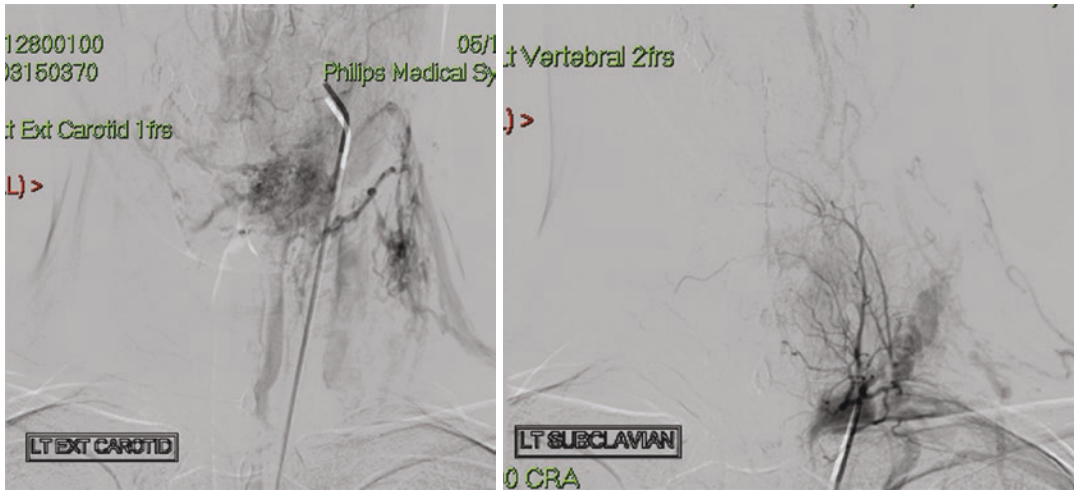

Fig. 45.3 Angiogram showing extensive fast-flow arteriovenous malformation in the left neck and upper thorax primarily supplied by left external carotid artery and left subclavian arteries

high turbulence, and hence a bruit is often heard and thrill palpated [42]. In very large AVMs, the increased blood flow can result in high-output cardiac failure [43].

45.4.4.1 Natural History

The AVMs usually inextricably enlarge with time, and many seem very sensitive to hormonal changes. Schobinger has staged the progression of AVMs (though it was Mulliken who published this classification): Stage I, Stage II, Stage III and Stage IV.

Stage I: Quiescent – cutaneous warmth and blush, Stage II: Expansion – bruit, pain, Stage III: Destruction – ulceration, bleeding, infection, Stage IV: Decompensation – hight output cardiac failure [43].

Bleeding from AVMs due to ulceration, infection or trauma can be torrential and life threatening. As the lesions enlarge, the recruitment of additional feeding vessels and draining veins increases the local damage they incur, and their adverse systemic effects progress [43].

45.4.4.2 Epidemiology

There is very little reliable data on the incidence or prevalence of extracranial AVMs. But in case series of all vascular malformations, they represent a small proportion (approximately 5%) of cases seen [44].

45.4.4.3 Classification

AVMs may be solitary, multiple and/or part of a syndrome. Intracranial AVMs are discussed below.

45.4.4.4 Management

As a result of the potentially serious consequences of progressive growth and worsening of AVMs, active intervention is recommended for virtually all lesions. The most successful treatments usually follow complete resection of the lesion; this is often difficult without the use of preoperative embolization techniques and intraoperative bleeding and in their absence can often be catastrophic. Embolisation techniques can be utilised in isolation, but this approach, without excision of the nidus, risks the development of additional supplying arteries that may not be amenable to further embolisation [45]. Some lesions because of their location or size are not resectable, and treatment with repeated embolisation procedures may be necessary to palliate the effects of inoperable lesions (Fig. 45.3). In terms of surgery, it is often helpful to consider their resection akin to the resection of a malignant tumour. In many cases resection leads to both functional and cosmetic deformities that present significant ongoing challenges.

Embolisation can be achieved with a variety of intravascular techniques and materials. These

techniques include intravascular access either to the arterial or venous side of the lesion (or both) and or direct puncture of the lesion. The materials used for embolization can be aimed at occluding the vessel by causing the blood to clot or by mechanically obstructing the vessel, and the effect may be temporary (used pre-surgically) or permanent. Alternatives include absorbable materials such as Gelfoam, nonabsorbable materials such as gelatin-acrylic spheres, liquid agents that solidify such as cyanoacrylate (Superglue), Onyx (ethylene vinyl copolymer dissolved in dimethyl sulfoxide) and PHIL (precipitating hydrophobic injectable liquid). A variety of mechanical devices are also used, coils, detachable balloons and coated metal coils (to induce thrombosis), and the use of combinations of devices and agents is common [46]. Ethanol can also be used to act as a sclerosant in AVMs.

45.5 Intracranial Vascular Malformations

Vascular malformations of the central nervous system are a heterogenous group of disorders and occur from morphogenetic areas affecting arteries veins or a combination of vessels. There may be classified histopathologically into arteriovenous malformations venous angioma, capillary telangiectasia and cavernous malformation. They may also be classified functionally into those with AV shunting (AVM, dural AV fistula and vein of Galen VOG malformation) and those without AV shunting (venous angioma, capillary telangiectasia, cavernous malformation and sinus pericranii). They may occur in isolation or be associated with syndromes.

45.5.1 Arteriovenous Malformation

Most arteriovenous malformations are parenchymal lesions (pial AVM); they are usually congenital and supratentorial in 85%. It is unusual for them to be multiple (<2%), but this may be the case when they are associated with syndromes such as HHT, Sturge-Weber or Woburn-Mason [47, 48].

Pathologically the surrounding brain parenchyma shows signs of haemorrhage with gliosis and ischaemic changes. Typically they present in the second to fourth decade of life and may present with haemorrhage, seizures or focal neurological deficit due to steal from adjacent areas or mass affect. They are associated with a cumulative lifelong risk of haemorrhage of 2–4% every year. Spontaneous regression of these lesions does occur but is exceptionally rare [49]. As in the management of cutaneous avms in order to effect a cure, complete obliteration of the nidus is required. Treatment options include endovascular embolisation followed by surgery or stereotactic radiosurgery. All but the smallest AVMs require multiple modality treatment, and the more complex may be incurable [50, 51].

45.5.2 Dural AV Fistula

Thought to occur after sinus thrombosis due to increased angiogenesis, dAVFs may vary vastly in size although multiple lesions are uncommon. They mostly affect adults (4060), and their presenting complaint depends on the sinus affected, e.g. transverse sinus/sigmoid sinus will result in a bruit and tinnitus and cavernous sinus (carotid cavernous fistula) results in pulsatile proptosis, chemosis and orbital pain. Lesions which have cortical venous drainage result in seizures dementia and progressive neurological deficit. The majority (>90%) follow a benign course; however malignant dAVFs have an aggressive clinical course with haemorrhage and neurological deficit as do multiple dAVFs. If the patient is not at risk of immediate haemorrhage, treatment may be conservative with observation with or without carotid compression. If they are at risk of haemorrhage, treatment is with embolisation of the arterial components or surgical resection of the involved dural venous sinus. In addition stereotactic radiosurgery can be used for these lesions [52].

45.5.3 Vein of Galen Aneurysmal Malformation

The embryonic precursor of the vein of Galen is a single transient midline vein known as the median prosencephalic vein and if this persists can result

in a direct AV fistula between the deep choroidal arteries and its remnant. It is rare in adults; however in symptomatic children, it may present in the neonatal period with high-output cardiac failure and a cranial bruit. In older children with macrocrania, hydrocephalus, developmental delay and seizures to headaches. If untreated, it may result in progressive brain damage, intractable cardiac failure and death. Treatment is aimed at control of the lesion in order to allow normal brain development; this is achieved with staged arterial embolisation at 4–5 months [53].

45.5.4 Cavernous Malformation

These lesions may be inherited or acquired and are formed of angiogenically immature blood-filled locules called caverns. They do not contain brain parenchyma; the adjacent parenchyma shows reactive changes. 2/3 are solitary, and they typically present at 40–60 years with seizure headache and focal neurological deficit. They have a haemorrhage risk of around 0.5% per year. If symptomatic, they may be resected microsurgically or steriotactically if surgically inaccessible [54].

45.5.5 Capillary Telangiectasia

These lesions are usually asymptomatic and discovered incidentally on brain imaging. They represent a collection of engorged thin-walled vessels surrounded by normal brain parenchyma; they are most likely congenital and do not generally require treatment [55].

45.5.6 Sinus Pericranii

Transcalvarial communication between the intra and extracranial venous drainage systems is mostly congenital. These are rare and typically present in children and young adults with a non-tender, non-pulsatile, blue, compressible scalp mass which increases in size on Valsalva manoeuvre and reduce in size on standing up. The extracranial component may be removed for cosmesis [56].

45.6 Genetics of Vascular Malformations

As the understanding of genetics increases, the number of vascular lesions that have their genetic causes grows. When considering Mendelian inheritance, there are examples of each type – sporadic, X-linked, autosomal dominant, recessive – and as the relevant molecular pathway is identified, the opportunity for targeted therapeutic interventions becomes possible. This would allow a move away from mechanical and surgical interventions to targeted drug therapies. Taking hereditary haemorrhagic telangiectasia (HTT) as an example, this is an autosomal dominant condition, the locus of the mutation is 9q33–34, and the transforming growth factor β (TGFβ) receptor is the abnormal protein. This opens up the possibility of understanding the abnormality and testing candidate therapeutic interventions in an animal model [57].

Conclusions and Clinical Perspectives

Vascular tumours and malformations are a diverse and complex group of conditions that can have major life limiting or altering effects on a wide group of patients and their families. These patients present to specialists of many disciplines and management often involves multidisciplinary care. At present surgery still has a significant role in the treatment, but its role is diminishing, and interventional radiology techniques and novel pharmacological interventions are increasing in importance. ◘ Table 45.1 outlines some of the more recent developments in the understanding of the biochemical basis of many of the conditions, their significance and potential therapeutic interventions.

Table 45.1 Table showing some recent developments in the understanding of infatile haemangioma, and vascular malformations linking the findings to explanations and possible therapeuric interventions.

	Finding	Importance	Explanation	Extrapolation
Infantile haemangioma	Tissue-specific markers (Lewis Y, merosin, FcyRII and GLUT-1) [58, 59]	Coexpression by placental microvessels	1. Embolism of placental endothelial cells via right to left shunt	High levels of VEGF produced by the placenta (and also IH). sFlt-1 produced in maternal serum and amniotic fluid binds to VEGF preventing uncontrolled growth – post-party's lack of sFlt-1 results in uncontrolled response to VEGF by IH – explaining the possible role for VEGF inhibitors
			2. Abnormal (endothelial phenotype) angioblastic colonisation of mesenchyme	
	Mesodermal-like stem cells present in IH [60]	Regulated by RAS	1. Inherent response to systemic RAS	1. Explains action of β-blockers (decrease renin levels) and ACEI/AT2RB
			2. Independent production of angiotensin 2	2. Explains incompleteness of effect and variability of response
	Hypoxia-induced mediators of progenitor cell trafficking present in IH (VEGF-A HIF-1a, MMP-9, oestrogen) [61]	Tissue hypoxia leading to angiogenesis (via hypoxia-induced mediators)	Results in endothelial progenitor cell (blood vessel precursors) mobilisation and thus neovascularisation	Explains precursor white patch
				Potential further targets for treatment (role of oestrogen esp.)
Capillary malformation	Decreased density of perivascular nervous tissue [62, 63]	Decreased vascular tone leading to progressive dilatation	Inverse correlation between nerve density and blood vessel diameter	Poorer response to laser therapy in low-density nerve high-density blood vessel lesions
				Supports a neural role in progression of CM
	Increased VEGF-A and VEGF-R2 [64]	Known to be involved in vascular tissue proliferation	Could be involved in proliferation or vast dilatation	Possible role for VEGF blockers
	Specific mutation	Found in lesional skin of both syndromic and non-syndromic CMs and also in affected brain tissue of patients with Sturge-Weber	Timing of mutation possibly explains severity and defines the tissues involved	Potential target for gene therapy
	Somatic activating mutation encoding a p.Arg183Gln amino acid substitution in GNAQ) [65]			

(continued)

Table 45.1 (continued)

	Finding	Importance	Explanation	Extrapolation
Lymphatic malformations	VEGF-C [66]	Present in high levels in LMs esp. in proliferative phase	Rapamycin reduces cell proliferation via mTOR in VEGF-C driven growth in the proliferative phase	Rapamycin could be used for some cases (either in the proliferative phase or in cases where cell proliferation continues)
				VEGF-C could potentially be used as a marker for proliferation
	Genetic abnormalities in thePI3K/AKT/mTOR pathway [67]	Present in CLOVES, proteus, Klippel–Trénaunay syndrome	PI3K inhibitors reduce proliferation in individuals with these genetic abnormalities	PI3K inhibitors reduce proliferation in individuals with these genetic abnormalities
Venous malformations	Endothelial receptor on ch9 [68]	Autosomal dominant inheritance of a gene in families with VMs	The EC-specific receptor tyrosine kinase TIE2 gene had been mapped previously to 9p21 by in situ hybridization	Explains familial VM
				Genetic screening for affected individuals
				Extrapolation of mechanism leading to new targets for therapy
	Tyrosine kinase receptor deficiency [69]	Thought to cause abnormal interaction between smooth muscle and endothelial cells leading to fewer muscle cells surrounding dilated venous channels	Tie-2 receptor TK has a specific kinase-activating mutation which leads to an increase in receptor autophosphorylation and altered signalling thereafter	Specific points on the signalling pathway could provide targets for therapy
Arteriovenous malformations	TGFβ signalling pathway	A number of targets on the TGF signalling pathway are implicated in the formation of AVMs	Alk-1, Eng, SMAD 4-deficient mouse models all exhibit increased AVM formation	These could represent important targets in themselves for future therapies or be used a study models for future therapies as root cause an effect become clear with further research
	Alk-1			
	Eng			
	SMAD 4 [70]			
	NOTCH receptor [71, 72]	NOTCH 4 activity is increased in AVMs unknown role	Notch receptors are transmembrane proteins that promote arterial endothelial cell (EC) specification by enhancing expression of arterial molecular markers and suppression of venous marker expression. Abnormal signalling induces enlarged AV connections and shunting (venolisation of arteries)	A potential target for future therapies
		NOTCH 1- and 3-deficient mouse models exhibit increased rates of AVMs		

Gaps in Knowledge

There are still huge chasms in our understanding of how the identified genetic changes, receptor differences, protein expression and environmental factors interact to form and allow these conditions to progress; however this is an extremely exciting time as the surface is beginning to be scratched in unravelling some of the complexities involved.

- Pathogenesis of vascular tumours
- Pathogenesis of vascular malformations
- Full mechanism of action of propranolol in the management of infantile haemangiomas
- The interactions and relationships between the genotype and phenotypes

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Supplementary Information

R. M. Touyz, C. Delles (eds.), *Textbook of Vascular Medicine*,
https://doi.org/10.1007/978-3-030-16481-2

Glossary

Association studies Association mapping is based on the idea that genetic variants underlying complex traits occur with a relatively high frequency (>1%), have undergone little or no selection in earlier populations and are likely to date back to >100,000 years ago (common disease/common variant hypothesis). Association analysis has potentially far greater power than linkage analysis for detecting variants with modest effect on disease risk, provided that the genetic marker is close enough to exhibit strong linkage disequilibrium (LD) with the functional variant. Unless targeting a specific, known polymorphism, all genetic association studies utilize one important population-genomic feature in their design: linkage disequilibrium (LD). LD is an extremely useful feature, as it means investigators do not need to genotype all polymorphisms in a region of interest. Instead, they can select a subset of SNPs that are proxies for the majority of all common genetic variation nearby (so-called tagSNPs).

Genetic architecture The genetic architecture of a trait refers to the number of distinct alleles that impact on the trait variation, their frequencies and penetrances.

Genome-wide association (GWA) studies Genome-wide association studies (GWAS) offer a hypothesis-free approach that systematically tests hundreds of thousands or more variants in the genome without prior knowledge of the location of the causal variants. GWAS were made possible after assembling human genetic variants in large human genome reference projects such as the International HapMap Project, the Human Genome Project and the 1000 Genomes Project. For GWAS, large sample sizes are required to generate sufficient statistical power to overcome the multiple hypotheses that are tested. The high number of false-positive results is addressed by stringent multiple testing correction and seeking evidence from multiple replication and validation studies of the top signals. GWAS are also blind to rare and structural variants.

Haplotype Haplotype is a linear arrangement of closely linked alleles on the same chromosome that is inherited as a unit. Individual's genotypes at multiple tightly linked SNPs have two haplotypes, each containing alleles from one parent.

Hardy-Weinberg equilibrium (HWE) The Hardy-Weinberg equation is a mathematical equation that can be used to calculate the genetic variation in a population at equilibrium.HWE is based on five assumptions:

1. Random selection: When individuals with certain genotypes survive better than others, allele frequencies may change from one generation to the next.
2. No mutation: If new alleles are produced by mutation or alleles mutate at different rates, allele frequencies may change from one generation to the next.
3. No migration: Movement of individuals in or out of a population will alter allele and genotype frequencies.
4. No chance events: Luck plays no role. Eggs and sperm collide at same frequencies. When assumption violated and by chance some individuals contribute more alleles than others to next generation, allele frequencies may change. This mechanism of allele change is called genetic drift.
5. Individuals select mates at random.

Heritability Heritability is the proportion of phenotypic variation due to the genetic differences between individuals within a population. Estimates of heritability can be used to describe the relative components of variance attributable to genetic factors and environmental factors. The heritability of a continuous trait is defined as the proportion of its total variance that is attributable to genetic factors in a particular population.

Linkage disequilibrium (LD) LD can be simply defined as a non-random association between alleles at adjacent loci. That is, the presence of combination of alleles or markers in a population more often or less often than expected if the loci were segregating independently in the population. When a variant is first introduced into a population by mutation, it will be perfectly correlated with nearby variants, but over successive generations meiotic recombination will break up the correlations, and LD will decay. The LD between two markers within the same genomic region is commonly measured by the absolute value of D' and r^2. The higher the value of D', the lower the possibility that a recombination event occurred between these two loci ($D' = 1$ means that the two markers have not been separated by a recombination event). The absolute value of r^2 is more commonly used to quantify and compare LD in the context of mapping. When $r^2 = 1$, the two markers have not been separated by recombination and have the same allele frequency. In this case of perfect LD, the two markers are completely linked, and observation at one marker provides complete prediction about the other.

Linkage studies The concept of linkage analysis is to search for alleles or chromosomal segments that are shared by affected relatives more than expected by random Mendelian segregation. These segments are passed entirely from the parents to the offspring without recombination at meiosis. Linkage analysis is only carried out in families with affected relatives and involves genotyping of several markers that spread over the entire genome. Markers that flank the disease gene or mutation tend to highly segregate with disease status in families. Identifying markers within such a segment that consistently accompanies the disease may indicate presence of susceptibility genetic factors nearby these markers. However, presences of such factors are neither necessary nor sufficient for the disease to develop.

Penetrance Penetrance is the likelihood, or probability, that a particular genotype will be expressed in the phenotype. A penetrance of 100% means that the associated phenotype

always occurs when the corresponding genotype is present, while a penetrance of 30% indicates that only 30% of those carrying a particular allele exhibit a phenotype.

Phenotype Phenotype or trait is the observable or measurable characteristic that is the target of genetic dissection.

The International HapMap Project The International HapMap Project (► http://www.hapmap.org) has constructed genome-wide maps of LD patterns in multiple populations. One of the main objectives of the project is to identify set of SNPs that are in LD blocks to allow more efficient genotyping.