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Gérard Lizard *Editor*

Implication of Oxysterols and Phytosterols in Aging and Human Diseases

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Editor
Gérard Lizard
Department of Biochemistry (Biochemstry of the
peroxisome, inflammation and lipid metabolism)
University of Bourgogne & Inserm
Dijon, France

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This book is dedicated to my family: Sarab, Diane, Denis, and "Miss" Garance.

I also thank my parents and family for having instilled in me the sense of work, effort, and perseverance.

I also greatly acknowledge some collaborators, who have been my Ph.D. students: Dr Amira Zarrouk, Dr Imen Ghzaiel, Dr Anne Vejux, and Dr Thomas Nury (pioneering works) as well as Dr Khouloud Sassi, Dr Meryam Debbabi, Dr Maryem Bezine, Dr Wiem Meddeb, and Dr Asmaa Badreddine. These collaborators have greatly contributed to a better understanding of the biological activities of oxysterols in several age-related diseases (cardiovascular, neurodegenerative, ocular and muscular diseases, and some cancers) and of their impact on oxidative stress, inflammation, organelle activity (mitochondria, peroxisome), and cell death induction.

Many thanks, Prof Mohammad Samadi, dear friend, for the synthesis of many oxysterols and phytosterols.

Louis Pasteur (Born in 1822 in Dole (Jura, France), not far from Dijon) said "Le hasard ne favorise que les esprits préparés."

Preface

Oxysterols and phytosterols are strongly bio-active molecules deriving from cholesterol (Massaad et al. 2017; Lizard et al. 2021). These molecules have lot of biological and physiological activities, and depending on their structures and concentrations, they can exhibit deleterious or beneficial activities.

Briefly, cholesterol, initially called cholestérine, was discovered by Michel Eugène Chevreul in 1815 during his work on fats related to the soap and candle industry. In 1884, Emile Littré has dubbed cholestérine "cholestérol". The name of cholesterol originates from the ancient Greek *chole* (bile) and *stereos* (solid), because it was discovered in its solid form in gallstones in 1758 by François Poulletier de La Salle. Subsequently, during about a century, several studies were carried out on the cholesterol structure and biosynthesis leading to four Nobel Prizes. In 1928, Adolf Windaus was awarded Nobel Prize in Chemistry for his research on sterols; in 1964, Konrad Bloch and Feodor Lynen were awarded Nobel Prize in Physiology and Medicine for discovering the mechanism of the cholesterol and fatty acid metabolism; and in 1975, John Cornforth was awarded Nobel Prize in Chemistry for his studies on enzymes allowing him to detail the biosynthesis of cholesterol. Progressively epidemiological and clinical evidences support the hypothesis of an implication of hypercholesterolemia in the initiation and progression of human atherosclerosis and cardiovascular diseases. In 1985, the Nobel Prize of Medicine was discerned to Michael S. Brown and Joseph L. Goldstein for their work on cholesterol metabolism and Low Density Lipoprotein receptor which leads to the discovery of statins, a new generation of hypocholesterolemic drugs, which are now widely used for the treatment of hypercholesterolemia.

It was progressively established that oxidized cholesterol derivatives, namely oxysterols, obtained either from cholesterol by auto-oxidation or by enzymatic catalysis could be involved in many diseases such as cardiovascular diseases but also other age-related diseases, ocular diseases (age-related macular degeneration, cataract), neurodegenerative diseases (Alzheimer's diseases), sarcopenia, osteoporosis, and some cancers, mainly prostate cancer, some types of breast cancer, and glioblastoma. It is increasingly possible that some oxysterols are implicated in chronic inflammatory pathologies (inflammatory bowel disease). Several oxysterols modulate specific metabolic pathways. These molecules have multiple biological activities and can regulate oxidative stress, inflammation, cell death, as well as cell differentiation

and cholesterol homeostasis. At the cellular level, depending on their structures, oxysterols can act at the level of the plasma membrane, endoplasmic reticulum, organelles (mitochondria, peroxisome, and lysosome), and/or at the nuclear level. Several of these oxysterols, in particular those resulting from the oxidation of cholesterol on its side chain, can be ligands or activators of the following receptors: (i) nuclear receptors, such as liver X receptors (LXRs) α or β and retinoic acid receptor-related orphan receptor α and γ (RORα [NR1F1] and RORγ [NR1F3]), but also (ii) cytoplasmic receptors such as SREBP (sterol regulatory element binding transcription protein), NPC1 (NPC intracellular cholesterol transporter 1/Nieman-Pick type C1), FXR (NR1H4, farnesoid X receptor alpha), oxysterols binding proteins (OSBPs), OSBPs-related proteins (ORPs), and cholesterol epoxide hydrolase (ChEH) (also named anti-estrogen binding site (AEBS); ChEH is an hetero-oligomeric complex comprising 3beta-hydroxysterol-delta(8)-delta(7)-isomerase (D8D7I) and 3beta-hydroxysterol-delta (7)-reductase (DHCR7)) as well as (iii) membrane receptors such as receptor tyrosine kinases and the Epstein–Barr virus-induced gene 2 receptor (EBI2, also known as GPR183). Some of these receptors are involved in the control of cholesterol trafficking, cell proliferation, and cell death. For the receptors (RORs, FXR, LXRs, EBI2), there are several lines of evidence for their involvement in inflammation. Other oxysterols oxidized at C7, such as 7-ketocholesterol (7KC) and 7-β-hydroxycholesterol, which either minimally or do not interact with these receptors, are potent inducers of inflammation and are known to have an important role in the pathophysiology of many age-related diseases (cardiovascular, ocular, and neurodegenerative diseases). These C7-oxidized oxysterols trigger both the production of inflammatory cytokines and prostaglandins. Prostacyclin (PGI2) production, which promotes platelet aggregation, has also been described in 7KC-treated endothelial cells. The ability of 7KC to induce inflammation is likely to occur mainly through the TLR4 receptor both in vitro and in vivo. To date, the pro-inflammatory activities of oxysterols are thought to be involved in chronic inflammatory diseases (cardiovascular diseases, inflammatory bowel disease), as well as in common (multiple sclerosis, Alzheimer's disease) and rare neurodegenerative diseases, such as X-linked adrenoleukodystrophy (X-ALD). Certain oxysterols can also act on bacteria, viruses, and parasites. Thus, several oxysterols are involved in the immune response and can act on infectious agents; their involvement in the immune response and cytokine storm is very likely, because some of their receptors are associated with immune activities and signaling pathways by which oxysterols promote cytokine production.

Phytosterols are structurally related to cholesterol and are mainly C28 and C29 carbon steroid alcohol (Lizard 2011; Rezig et al. 2022). Plant sterols, also named phytosterols, are integral components of the membrane lipid bilayer of plant cells. Unlike animal systems in which cholesterol is most often the single final product of sterol synthesis, each plant species has its own characteristic distribution of phytosterols, with the three most common phytosterols in nature being β-sitosterol, campesterol, and stigmasterol. In addition to the free sterol form, phytosterols are also found in the form of conjugates,

particularly fatty acyl sterol esters. In humans, phytosterol absorption is considerably less than that of cholesterol. Some investigations support that phytosterols decrease cholesterol absorption, and thus reduce circulating concentrations of cholesterol. Indeed, in the intestine, phytosterols compete with cholesterol, leading to reduced cholesterol absorption and, as a consequence, to a lower plasma LDL-cholesterol concentration.

Today, it is well admitted that oxysterols and phytosterols are strongly bio-active lipids which act on several signaling pathways. There are several lines of evidence of their involvement in many diseases especially age-related diseases.

This book has been written with the contribution of members of the European Network for oxysterol research (ENOR; https://www.oxysterols.net/) from Australia, Austria, Brazil, Finland, France, Germany, India, Ireland, Italia, Lebanon, Morocco, Norway, Poland, Russia, Spain, Tunisia, Turkey, United Kingdom, United States of America, and Switzerland.

This book provides information intended for a wide biomedical public as well as for researchers wishing to acquire broad and recent knowledge on oxysterols and phytosterols.

Dijon, France Gérard Lizard

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About the Editor

Gérard Lizard is an INSA-Lyon biochemist engineer, PhD in Molecular and Cellular Biology, and PhD in Human Biology. After a post-doc in pharmaceutical industry, he was recruited to INSERM in 1991 and since 2012 has been director of the Biochemistry Laboratory "Peroxisome, Inflammation and Lipid Metabolism" at the University of Burgundy. He was one of the first researchers to work on cell death by apoptosis associated with oxidative stress in the field of atherosclerosis, which led him to demonstrate that certain oxidized derivatives of cholesterol (oxysterols) induce a mode of cell death by oxiapoptophagy characterized by oxidative stress, apoptosis, and autophagy. Gérard Lizard has gradually oriented his research on aging (biogerontology) and the pathophysiology of age-related diseases by focusing on the lipotoxicity of oxysterols and the impact on organelles (mitochondria, peroxisome). Gérard Lizard has been the director of 20 PhD theses and is the co-founding member of the European Network for Oxysterol Research (ENOR).

Part I

Chemical Characteristics

1 The Structure of Oxysterols Determines Their Behavior at Phase Boundaries: Implications for Model Membranes and Structure–Activity Relationships

Anita Wnętrzak, Anna Chachaj-Brekiesz, Jan Kobierski, and Patrycja Dynarowicz-Latka

Abstract

The presence of an additional polar group in the cholesterol backbone increases the hydrophilicity of resulting compounds (oxysterols), determines their arrangement at the phase boundary, and interactions with other lipids and proteins. As a result, physicochemical properties of biomembranes (i.e., elasticity, permeability, and ability to bind proteins) are modified, which in turn may affect their functioning. The observed effect depends on the type of oxysterol and its concentration and can be both positive (e.g., antiviral activity) or negative (disturbance of cholesterol homeostasis, signal transduction, and protein segregation). The membrane activity of oxysterols has been successfully studied using membrane models (vesicles, monolayers, and solid supported films). Membrane models, in contrast to the natural systems, provide the possibility to selectively examine the specific aspect of biomolecule-membrane interactions. Moreover, the gradual increase in the complexity of the used model allows to understand the molecular phenomena occurring at the membrane level. The interest in research on artificial membranes has increased significantly in recent years, mainly due to the development of modern and sophisticated physicochemical methods (static and dynamic) in both the micro- and nanoscale, which are applied with the assistance of powerful theoretical calculations. This review provides an overview of the most important findings on this topic in the current literature.

Keywords

Cholesterol · Cholesterol oxidation products · Oxysterols · Model membranes · Langmuir monolayers · Interface · Structure–activity relationship

1.1 Model Techniques for Investigation of Oxysterols' Activity on Membrane Level

It has been proven that oxysterols, due to their structural similarity to cholesterol, have the ability to insert into biomembranes. Interestingly, in many cases, the activity of oxysterols on this level differs from that observed for the parent molecule. The effect of biomolecules (including oxysterols) on the structure and function of natural membranes can be studied directly in vivo;

A. Wnętrzak (✉) · A. Chachaj-Brekiesz · P. Dynarowicz-Latka
Faculty of Chemistry, Jagiellonian University, Kraków, Poland
e-mail: anita.wnetrzak@uj.edu.pl

J. Kobierski
Department of Pharmaceutical Biophysics, Faculty of Pharmacy, Jagiellonian University Medical College, Kraków, Poland

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however, the results of such experiments are difficult and sometimes impossible to interpret due to the high complexity and variability of living systems. At the same time, artificial systems provide a highly useful, simplified, but well-defined platform to conduct systematic studies under controlled conditions with a wide range of physicochemical characterization techniques. Importantly, these models not only mimic the cell membrane in vitro but also provide insight into the basic biophysical processes, giving access to molecular details of interactions between membrane components. This allows to verify hypotheses about the mode of action of the biomolecule of interest, its toxicity, or to identify molecular targets. So far, many membrane models have been applied (for reviews see (Eeman and Deleu 2010; Rosilio 2018) and references therein); however, the most commonly used for studies on lipids (including oxysterols) are monolayers, vesicles, and solid-supported bilayers (see Fig. 1.1). Therefore, these models are briefly described below.

Let us start with Langmuir monolayers, which are floating films formed by spreading a solution of lipid(s) on the water surface using the so-called Langmuir trough (for details regarding the methodology and applications see (Dynarowicz-Latka et al. 2001; Stefaniu et al. 2014; Oliveira et al. 2022). The obtained film represents half of the membrane bilayer; however, at certain conditions (surface pressure of 30–35 mN/m), its packing is identical to that in bilayers, which is referred to as monolayer–bilayer correspondence (Marsh 1996; Feng 1999; Brockman 1999). The use of Langmuir monolayers as models of biological membranes is supported by their simplicity of preparation, easy modification of experimental parameters (such as pH, ionic strength, and temperature), and membrane composition to reproduce the conditions close to those in cells (Nobre et al. 2015). Langmuir films can be characterized by a variety of experimental methods (for examples and references see Table 1.1), which provide information about membrane organization in microscale as well as general parameters describing the overall system. The latter parameters are calculated based on the experimental dependencies of surface pressure (π) and molecular area (A): (i) in-plane elasticity, expressed quantitatively by values of compressional modulus (C_s^{-1}) (Behroozi 1996; Davies and Rideal 1963; Miller and Liggieri 2009) and (ii) excess thermodynamic functions of mixing with the excess Gibbs free energy (ΔG^{exc}) being most frequently used (Dynarowicz-Latka and Kita 1999). ΔG^{exc} is applied to characterize both the kind (attraction/repulsion) and strength of interactions of chosen molecule with the other constituents of the surface layer. The main limitation of Langmuir monolayers is their reduced stability during measurements at physiological temperatures (due to increased subphase evaporation). Moreover, obtaining information on molecular organization at the nanoscale (with AFM and coupled spectroscopical methods) requires the transfer of films to a solid support, which may modify interactions with respect to the air/water interphase.

The second applied laboratory membrane model is vesicles, which are bubbles (size 0.01–10 μm) filled with water (or aqueous solution) having at least one lipid bilayer. When vesicles are formed by phospholipids, they are often called liposomes (Jones 1995; Deleu et al. 2014). The preparation of vesicles involves the following steps: (i) formation of a dry lipid film (by solvent evaporation) at temperatures above the lipid phase transition, (ii) rehydration in aqueous phase, and (iii) sonication. Depending on the experimental conditions, multilamellar (MLVs) or unilamellar (ULVs) vesicles can be formed. The latter can be classified according to their size as small (SUVs), large (LUVs), or giant unilamellar vesicles (GUVs). Vesicles, contrary to monolayers representing one leaflet, are composed of two leaflets reflecting the curved, bilayer structure similar to that of natural membranes. Therefore, in the case of this model, the most frequently studied aspects involve monitoring vesicle shape and size changes with the scattering techniques as well as transport across the membrane and pore formation (with fluorescence microscopy and partition coefficient). The use of lipid vesicles as membrane models suffers from some disadvantages resulting mainly from

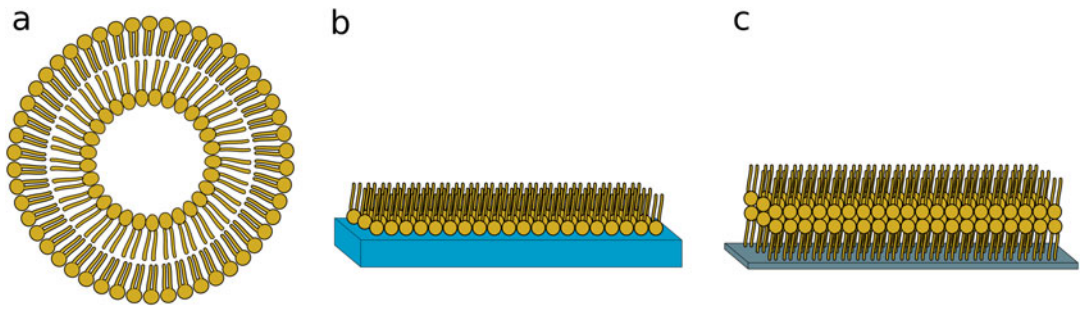

Fig. 1.1 Artificial laboratory models of membrane: (**a**) vesicle, (**b**) Langmuir monolayer, (**c**) solid-supported bilayer

preparation techniques and limited composition range. Namely, there are difficulties in obtaining vesicles that are homogeneous in size and layer number because of their ability to undergo spontaneous fusion. Moreover, it is impossible to independently regulate the lipid composition and molecular packing density. The range over which the lipid composition can be varied without changing the surface curvature and physical state of the vesicles is limited.

Another frequently used membrane model is supported bilayers, which can be obtained by the deposition of monolayers, vesicles, or micelles onto a solid support (most often mica sheets, silica wafers, or gold). It should be noted that the deposition of monolayers with Langmuir–Schaefer technique allows to obtain surface layers mimicking lipid asymmetry between inner and outer membrane leaflets. Moreover, the transfer onto solid support enables to study artificial membranes with the experimental techniques characterized by the spatial resolution decreased to the nanoscale. As a result, the application of AFM as well as coupled nanospectroscopic methods enables tracking of local changes in membranes (Dufrêne and Lee 2000; Sofińska et al. 2022). The main disadvantage of this model is the possible modification of crucial interactions between molecules in the studied systems, which results from the change of the interface (from air/water to air/solid).

As mentioned above, each of the discussed membrane models offers certain advantages and disadvantages, and none of them is universal. Therefore, upon studying a certain biomolecule and a particular aspect of its activity, both the appropriate membrane model and the most optimal characterization technique should be applied individually.

The growing computing power of microprocessors and the easier access to cluster calculating make computational chemistry a tool increasingly used on a par with experimental methods. Among computational studies of biomembranes, molecular dynamics (MD) simulations are the most commonly used. These simulations show the trajectory of the molecules in a defined system for a specified period of time and temperature. The information is obtained by numerically solving Newton's equations of motion, which include the potentials of covalent bonds and electrostatic and van der Waals interactions. Molecules with kinetic energy are therefore able to overcome potential barriers as long as these are lower than the total energy, parameterized by a given temperature. When the system is simulated for a sufficiently long time, the solutions will cover the entire available space.

The undeniable advantage of MD is that there is no restriction in terms of the molecules that build the system or their configuration. Due to this, information unavailable from experiments can be obtained practically for any system at a reasonable cost and time. Computer programs used for this type of calculation have more often a user-friendly interface, making them almost a

Table 1.1 Artificial membrane models together with most frequently used methods of their analysis and obtained information

Membrane model	Analysis method	Obtained information	References
Langmuir monolayers	π–A isotherms measurement	• Lift-off area $A_{lift\text{-}off}$ (the onset area of the surface pressure rise) and collapse pressure values • In-plane elasticity of monolayer reflected by C_s^{-1} (compressibility modulus) • Type and strength of interactions between components based on thermodynamical parameters A_{12} (mean molecular area) and ΔG^{exc} (excess Gibbs free energy of mixing) • Affinity of biomolecule to lipids/membrane • Mutual miscibility (or immiscibility)	(Dynarowicz-Latka and Kita 1999; Dynarowicz-Latka et al. 2001; Oliveira et al. 2022)
	ΔV–A curves measurement	• Apparent dipole moment of molecules in film • Orientation of molecular dipoles • Surface dissociation • Interactions between film components • Interactions between monolayer components and substances in the aqueous phase	(Oliveira, and Bonardi 1997; Nakahara et al. 2005; Chachaj-Brekiesz et al. 2022)
	BAM and fluorescence microscopy	• Film texture and thickness • Phases and their coexistence • Aggregation and formation of domains • Collapse mechanism	(Hönig and Möbius 1991; McConnell 1991; Daear et al. 2017)
	PM–IRRAS, SHG and SFG	• Orientation of molecules at surfaces • Molecular conformation • Functional groups involved in interactions with subphase and other film components	(Eisenthal 1996; Volpati et al. 2014; Sofińska et al. 2022)
	GIXD	• Surface packing, ordering, and orientation of molecules • Film thickness	(Bera et al. 2020)
Vesicles	Fluorescence spectroscopy and imaging	• Single vesicle size and lamellarity • Membrane integrity, fusion, or aggregation • Insertion of bioactive compounds into membrane • Domain formation and phase separation	(Domenech et al. 2009)
	FFF, NTA, flow cytometry, SEC	• Vesicles size distribution • Polydispersity	(Kanásová and Nesměrák 2017)
	TEM methods	• Single vesicle size and lamellarity	(Liu et al. 2010)
	DLS	• Vesicles size distribution • Zeta potential • Electrical surface charge • Isoelectric point of vesicles	(Carvalho et al. 2018)
	SAXS	• Vesicle lamellarity • Molecular packing • Domain formation and phase separation	(Bouwstra et al. 1993)
Solid-supported films	AFM	• Topography • Aggregation and formation of domains in nanoscale	(Dufrêne and Lee 2000; Sofińska et al. 2022)

(continued)

Table 1.1 (continued)

Membrane model	Analysis method	Obtained information	References
		• Phases and their coexistence • Mechanical properties of films	
	AFM-IR and TERS	• Mapping of molecular distribution on surface • Orientation of molecules in films • Molecular conformation	(Sofińska et al. 2022)
	Electroanalytical methods	• Membrane permeability to ions and water • Surface ordering and defects in molecular assembly	(Dziubak and Sek 2021)

Abbreviations: AFM (atomic force microscopy), AFM-IR (atomic force microscopy infrared spectroscopy), BAM (Brewster angle microscopy); DLS (dynamic light-scattering); FFF (field flow fractionation); GIXD (grazing incidence X-ray diffraction); NTA (nanoparticle tracking analysis); PM–IRRAS (polarization modulation-infrared reflection-adsorption spectroscopy); SAXS (small-angle X-ray scattering), SEC (size-exclusion chromatography); SFG (sum frequency generation spectroscopy), SHG (second harmonic generation spectroscopy); TEM (transmission electron microscopy); TERS (tip-enhanced Raman spectroscopy)

"black box". Because of this, the user does not need to know all the theoretical chemistry behind the calculations to get reliable results. However, the disadvantages of MD include the limited evolution time of the simulated system. For currently available computing power, system evolutions lasting nanoseconds are typically obtained, while experiments cover evolution time many orders of magnitude longer. Another disadvantage of computer simulations is that they are only implementations of theoretical models which, due to the finite computational resources, take into account only selected elements of the physical model.

Model studies (theoretical simulations as well as laboratory experiments) are usually carried out in the following order: starting with the study of the properties of pure biomolecule of interest, through simplified two-component systems (biomolecule-membrane component) that enable to identify particular membrane components important in the mode of action of a biomolecule, and ending with biomolecule interaction with multi-component systems that mimic the composition of a selected biomembrane. The following sections describe the research on oxysterols in that order.

1.2 Characteristics of Pure Oxysterols at Phase Boundary

Most processes in living organisms take place at phase boundaries rather than in bulk phases; therefore, it is reasonable to study properties of bioactive compounds at interfaces. The phase boundary typical for living organism is the border of the lipid cell membrane (oil, with relative electric permittivity $\varepsilon = 2$) and the aqueous environment surrounding the cell (water, $\varepsilon = 80$). What is interesting, its properties are similar to the other common interface—the border between water ($\varepsilon = 80$) and air ($\varepsilon = 1$). In this section we present the characteristics of most important oxysterols at the air/water interface in comparison with their non-oxidized parent molecule, cholesterol.

Cholesterol is an important membrane sterol in mammals. At the air/water interface, cholesterol molecules are arranged in a well-ordered layer, where molecules are oriented vertically to the membrane plane. The sterane ring system together with the attached isooctyl chain line up toward the phase with lower permeability (air), while the hydroxyl group at C(3) is oriented toward the higher permittivity hydrophilic region (water) (Fidalgo Rodriguez et al. 2019). In contrast, the surface behavior of oxidized cholesterol

derivatives (oxysterols) in many aspects is different. From a physicochemical point of view, this is due to the presence of an additional polar group in cholesterol structure, which makes such a molecule bipolar. This influences hydrophilicity, molecular alignment, and both interactions (polar and hydrophobic) with other membrane constituents.

A simple method to examine differences in the organization of cholesterol and oxysterol molecules in membranes is the critical packing parameter (CPP). In a thermodynamically stable system, amphiphiles can be geometrically described by parameters such as optimal interfacial surface area a_0, the volume of the hydrocarbon chain V, and its critical length l_c. These parameters can be related by the formula (Israelachvili et al. 1976):

$$\mathrm{CPP} = \frac{V}{a_0 l_c} \tag{1}$$

Knowing the CPP value, one can determine a solid figure that circumscribes the geometric representation of the molecule as well as select the suitable model for experimental studies (for details see Table 1.2).

Unfortunately, determining the CPP value is not always easy. Calculating the critical length of the hydrocarbon chain and its volume for fully saturated chains is possible thanks to semi-empirical formulas. Determining the interfacial surface area per molecule is much more difficult because of many factors that affect this parameter. In addition to the steric interactions, its value can be modified by the ionic interactions. Furthermore, the presence of other components in the system or the level of hydration can affect a_0. For this reason, a_0 is determined mostly empirically, using diffraction experiments. For cholesterol, the a_0 value was determined at approximately 19 Å^2 (de Bernard 1958), which gives CPP 1.22 and means that cholesterol molecule is circumscribed by inverted truncated cone. However, there are no literature reports in which the CPP for oxysterols would be determined using this technique.

A different theoretical approach was used in (Kobierski et al. 2021b). First, molecular dynamics calculations were used to obtain thermodynamically stable systems made of amphiphilic molecules. Then the mean values of parameters a_0, l_c, and V were determined for these systems. This allowed to determine the CPP parameter, which for cholesterol was equal to 3, what translates into an inverted cone circumscribing the molecule. This value deviates from the one determined by (de Bernard 1958), which is probably most influenced by the fact that in diffraction experiments cholesterol in a mixture with phosphatidylcholine was investigated. For sterols oxidized at C(7), the CPP values were determined as: 1.40, 1.59, and 1.43, for 7-ketocholesterol, 7-K; 7α-hydroxycholesterol, 7α-OH; and 7β-hydroxycholesterol, 7β-OH, respectively. Thus, these molecules are described by inverted truncated cones, since an additional functional group at C(7) increases the surface area a_0. The CPP values for 25-hydroxycholesterol, 25-OH, were also determined in (Kobierski et al. 2021b), for the C(3)-OH exposed to water (CPP = 2.86) and for the C(25)-OH turned toward water (CPP = 2.95). The obtained values were similar to the one for cholesterol, which is due to the fact that the additional hydroxyl group is distant from the interfacial surface and does not significantly affect a_0.

An experimental method enabling further comparison of differences in the behavior of cholesterol and oxysterol in membranes is the use of the Langmuir monolayer technique based on analysis of the surface pressure—area per molecule isotherms complemented with microscopic and spectroscopic characterization methods. Figure 1.2 shows representative π-A isotherms for cholesterol and its derivatives oxidized at C(7) in sterane system (7-K, 7α-OH, 7β-OH), which are commonly synthesized in human organisms via nonenzymatic path or delivered with highly processed foods (Brzeska et al. 2016).

In comparison to cholesterol, all experimental isotherms measured for oxysterols bearing additional polar group attached in the B-ring of

Table 1.2 Values of critical packing parameter with the corresponding molecular shapes and the self-assembled structures

CPP	Molecular shape	Self-assembled structure
≤1/3	Cone	Micelle
1/3-1	Truncated cone	Vesicle/flexible layer
≈1	Cylinder	Planal layer
1-3	Inverted truncated cones	Inverse vesicle/flexible layer
≥3	Inverted cone	Inverse micelle

sterane skeleton have the lift-off points shifted toward larger area per molecule values. Among them, the monolayer of 7β-OH is the most expanded (as evidenced by the lift-off area as well as the smallest slope of experimental curve). This isotherm is characterized by the continuous rise in surface pressure upon compression without any visible discontinuities until the collapse (at 47 mN/m). This course of the isotherm indicates that 7β-OH molecules remain anchored in the polar phase with both hydroxyl groups and do not change orientation being tilted in respect to the interphase throughout the entire compression range until collapse (Fig. 1.2c). This results in

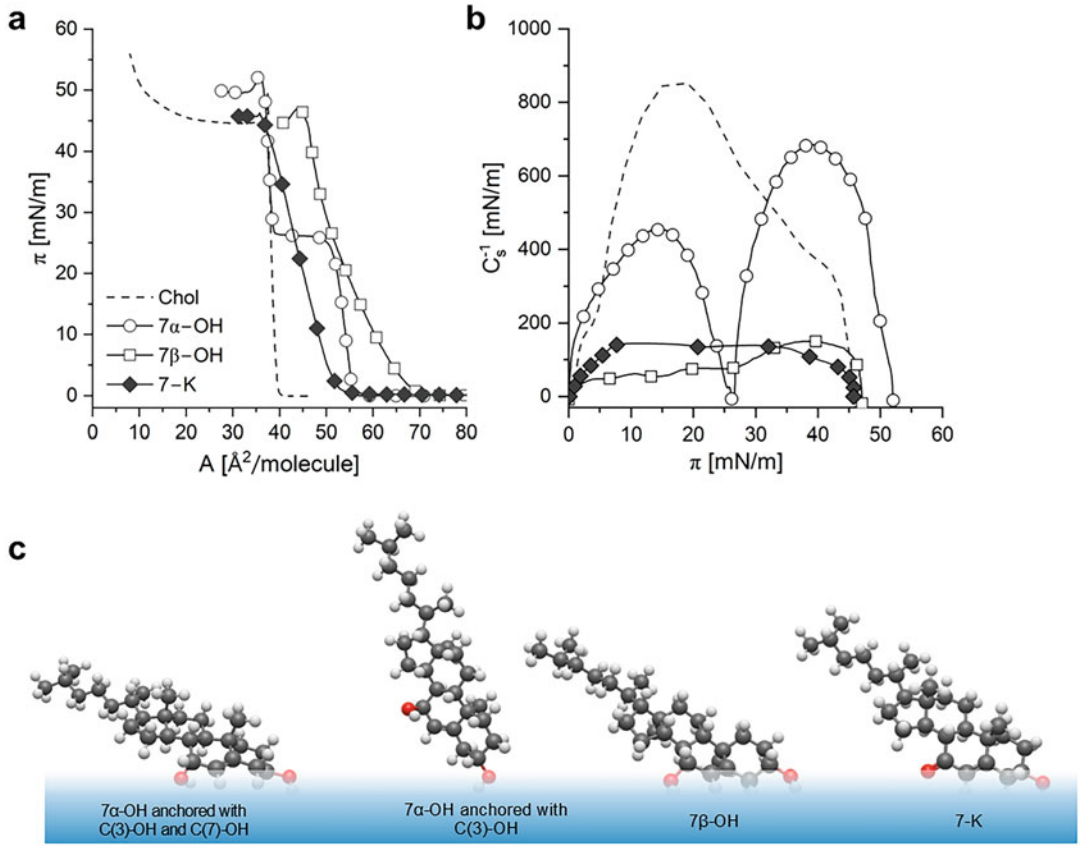

Fig. 1.2 Comparison of (**a**) representative surface pressure—molecular area (π-A) isotherms measured at 20 °C and (**b**) compressibility modulus—surface pressure (C_s^{-1}-π) plots for cholesterol and selected sterols oxidized at C(7) in the sterane ring system together with (**c**) surface anchoring schemes of these oxysterols (explanation regarding two different orientations of 7α-OH is included in in the text)

fluidization of 7β-OH film in comparison to cholesterol, as evidenced by lower compressibility moduli values (Fig. 1.2b) (Wnętrzak et al. 2019a). Analogical surface behavior is observed for 7-K; however, the lower hydration degree of carbonyl group at C(7) vs. hydroxyl group at C (3) causes that 7-K molecules are less inclined as compared to 7β-OH (Simon et al. 1992; Wnętrzak et al. 2017). The inclination of molecules in the film, reflected in film expansion and fluidization, was also observed for oxysterols bearing a polar group attached to the A-ring of the sterol system: 6-ketocholesterol, 6-K (Simon et al. 1992; Smondyrev and Berkowitz 2001; Li et al. 2003); 5,6β-epoxycholesterol, 5,6β-epoxy (Telesford et al. 2015); 4β-hydroxycholesterol, 4β-OH (Kulig et al. 2015). In contrast, 7α-OH shows surface behavior which is quite different from that described above for 7β-OH and 7-K (Wnętrzak et al. 2019a). Namely, although the 7α-OH isotherm lifts off at molecular area comparable with the value for 7-K, the curve shows a broad plateau region at a surface pressure approximately 25 mN/m (at 20 °C). This reflects the phase transition connected with the change in the tilt of 7α-OH molecules and simultaneous emerging of the C(7)-OH group out from the polar phase. Above the plateau, 7α-OH isotherm overlaps with the curve for pure cholesterol. The surface pressure of the transition decreases with the increasing temperature and remains noticeable even at a temperature of 36 °C. This suggests that under physiological conditions 7α-OH molecules surface anchoring and inclination is similar to that of cholesterol. The striking difference in surface activity between two 7-hydroxycholesterol epimers (7α-OH and 7β-OH) was evidenced by differences in the hydration degree of hydroxyl groups at C(3) and C(7) obtained from molecular dynamics simulations (Wnętrzak et al. 2019a). The explanation of these differences is delivered from geometric considerations of DFT optimized structures for 7α-OH and 7β-OH. Namely, the spatial orientation of C(7)-OH in 7α-OH molecule makes it unfavorable to being permanently anchored with both polar groups in polar phase as the area per molecule decreases. Therefore, for surface pressure values below the plateau 7α-OH molecules exist in two surface arrangements (anchored with both or one hydroxyl group), while below plateau molecules are anchored only with C(3)-OH. In contrast 7β-OH, is constantly anchored with both hydroxyl groups in water surface (Fig. 1.2c).

Oxysterols possessing an additional polar group in the isooctyl chain were initially perceived as typical bipolar amphiphiles, having the ability to anchor in the hydrophilic region with both polar moieties (Kauffman et al. 2000; Olsen et al. 2009). Further results from Langmuir monolayer studies (Wnętrzak et al. 2020; Chachaj-Brekiesz et al. 2023) and MD simulations (Galiano and Villalaín 2020) did not support this point of view. Figure 1.3 shows the comparison of representative π-A isotherms for cholesterol and its derivatives oxidized in the isooctyl chain (24(S)-hydroxycholesterol, 24-OH; 25-hydroxycholesterol, 25-OH; 27-hydroxycholesterol, 27-OH), which are synthesized in human organisms in the enzymatic path.

At first glance, the surface behavior of side chain oxidized sterols is analogous to that of cholesterol. This is reflected in similar lift-off area values and almost the same slope of experimental curves in comparison to cholesterol isotherm. Therefore, all physiologically crucial chain-oxidized oxysterols, similar to cholesterol, are initially anchored in the polar phase with only one polar group. MD simulations have shown that in the case of Langmuir monolayers from 25-OH, the population of molecules anchored with the C (3)-OH moiety is in a slight predominance compared to those anchored with C(25)-OH (Wnętrzak et al. 2020). It should be mentioned that other calculations revealed that it is also possible that a very small population of molecules can be anchored with both hydrophilic groups; however, these results were obtained for models containing phospholipids, which may stabilize such an arrangement (Nåbo et al. 2018). Further compression of films shows differences in surface behavior between particular sterols oxidized in the isooctyl chain and cholesterol. Namely, the experimental curves for 25-OH and 27-OH show

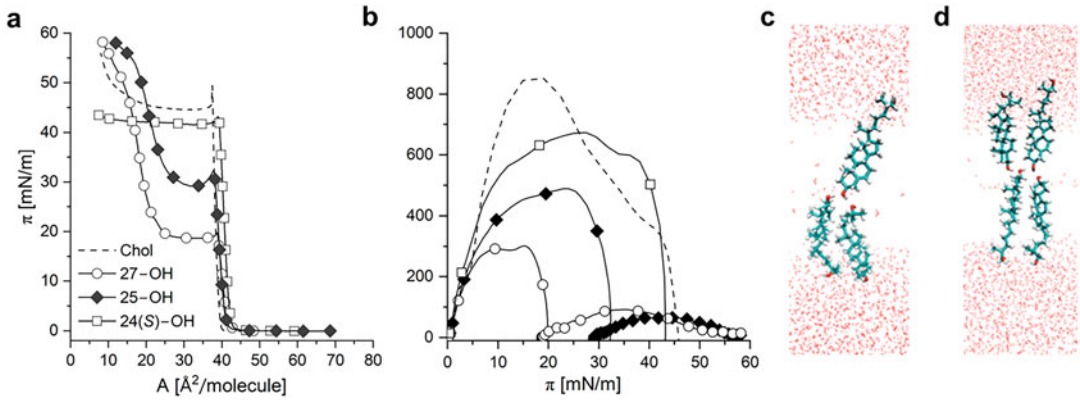

Fig. 1.3 The comparison of (**a**) the representative surface pressure—molecular area (π-A) isotherms measured at 20 °C and (**b**) compressibility modulus—surface pressure (C_s^{-1}-π) plots for cholesterol and selected sterols oxidized in the isooctyl chain; together with snapshots of molecular arrangements stabilizing bilayers of (**c**) 25-OH and (**d**) 27-OH existing above isotherms plateau. Snapshots of molecular arrangements were adapted from (Chachaj-Brekiesz et al. 2023) under CC BY 4.0 license

broad plateau regions that appear at approximately 32 mN/m and 20 mN/m (at 20 °C), respectively. At surface pressure values that are above the plateau, 25-OH and 27-OH molecules have the ability to spontaneously organise into bilayers, which was proved by optical thickness measurements, PM-IRRAS spectroscopy, and electric surface potential change. Bilayer structures are stabilized by the hydrogen bonding between hydroxyl groups of molecules from neighboring monolayers. MD simulations revealed that in the case of 25-OH, the stabilization is due to the dimers and trimers formation (Galiano and Villalaín 2020; Chachaj-Brekiesz et al. 2023), while 27-OH has the ability to form tetramers (Chachaj-Brekiesz et al. 2023). In contrast, 24-OH—similar to cholesterol is not able to spontaneously organize into bilayers. This may be explained by the fact that the hydroxyl moiety at C(24) is less exposed to hydrogen bonding due to the strong shielding effect of the isopropyl group.

All of the above prove that the surface properties of oxysterols and their ability to self-organize at the phase boundary depend on the type of additional polar group introduced, its position (in the sterane ring system or in the iso-octyl chain), and configuration. In the next section, the influence of oxysterols structure on the interactions with the main membrane constituents is analyzed.

1.3 Interactions of Oxysterols with Selected Components of Cell Membranes

The simplest method that allows for looking at the influence of the studied biomolecules on membrane is to select one of the main membrane lipids (e.g., DPPC and cholesterol) and study the mutual interactions in two-component system using the membrane model (e.g., monolayers, vesicles). This approach is certainly an oversimplification since natural membrane is a complex, multicomponent system; however, it can be useful in many cases; e.g., it allows to easily verify which of the membrane components is important in targeting the biomolecule to the cell and/or causes a toxic effect.

Let us focus in this section, most of all on the results from the Langmuir monolayers technique. An application of abovementioned methodology allows to track the following effects of the studied oxysterol introduction into the membrane lipid monolayer*:* (i) film in-plane elasticity (reflected

by the compressibility modulus, C_s^{-1}) and (ii) extent and character of mutual interactions (reflected by the mean molecular area, A_{12} and excess Gibbs free energy of mixing, ΔG^{exc}). Below we show the selected results of investigations on the influence of a representative ring-oxidized sterol (7-K) on a typical membrane phospholipid, POPC. For comparison purpose, the analogical experiments were conducted on the reference systems containing cholesterol, instead of 7-K (Fig. 1.4).

The starting points for the analysis of the influence of a biomolecule (in this case, cholesterol or 7-K) on the lipid film are π-A curves. Comparing the results presented in Fig. 1.4 a, up and down it can be deduced that the addition of cholesterol or 7-K condenses the monolayer formed by POPC (Mintzer et al. 2010; Wnętrzak et al. 2017), which is reflected in a shift of $A_{lift\text{-}off}$ toward smaller surface area and the increase in slope of the curves. A more accurate view is provided by the extent of deviations from the linear dependence of the mean molecular area (A_{12}) vs. the film composition (Fig. 1.4c). Negative deviations observed for both analysed systems indicate the contraction of the molecular area upon the introduction of sterols and suggest the existence of attractive POPC–sterol interactions. The direction and strength of POPC–sterol interactions was also evidenced by excess Gibbs free energy of mixing, ΔG^{exc} (Fig. 1.5), which is discussed later. Figure 1.4b shows the influence of sterols on the compressibility of the POPC film. The obtained results indicate the increase in rigidity and packing density of the POPC monolayer with the addition of sterol. The so-called condensing effect is well described for cholesterol (Hung et al. 2007; Alwarawrah et al. 2010) and can result from strong attractive interactions between Chol and phospholipids (surface complex formation) as well as the so-called "umbrella effect". In the "umbrella effect", the polar groups of phospholipids shield the hydrophobic core of cholesterol emerging it from the water, allowing for closer packing. The condensing effect was also described for oxysterols; however, its magnitude depends on the structure of oxidized sterol derivative and the particular phospholipid analyzed. Sterols oxidized in the sterane system typically introduce less pronounced condensation compared to cholesterol, which has been demonstrated for the following mixtures: 7-K/DPPC (Telesford et al. 2015), 7-K/POPC (Mintzer et al. 2010), 5,6β-epoxy/DPPC (Telesford et al. 2015), 7-K/SM (Mintzer et al. 2010; Wnętrzak et al. 2022b), 7β-OH/SM (Wnętrzak et al. 2022b), and 6-K/SM (Li et al. 2003). This is quite understandable since sterols oxidized in a sterane system typically are more inclined to the interface compared to cholesterol (see Sect. 1.2), and therefore require more space between neighboring phospholipids, which results in less condensation. 7α-OH is an exception to this rule, as in some conditions it adopts a horizontal orientation in phospholipid environment due to being anchored with only C(3)-OH in water. The effect of 7α-OH on bovine brain SM was shown to be comparable to this of cholesterol (Mintzer et al. 2010; Wnętrzak et al. 2019b). On the contrary, the effect of sterols oxidized in isooctyl chain on the surface rheology of phospholipid films is more complicated. The authors report both smaller (in the case of 25-OH/POPC (Mintzer et al. 2010), 25-OH/SM from the bovine brain (Mintzer et al. 2010), and 25-OH/GM_1 (Kobierski et al. 2021a)) or greater (in the case of 25-OH/SM from egg (Kobierski et al. 2021a; Wnętrzak et al. 2022b), and 25-OH/DPPC (Wnętrzak et al. 2021a)) condensation induced by oxysterol in comparison to the corresponding mixtures with cholesterol.

The most informative parameter that provides insight into the character and strength of mutual interactions, as well as film stability, is the excess Gibbs free energy of mixing, ΔG^{exc}. Thus, the negative or positive values of this function suggest attractive or repulsive interactions between the selected film components. More negative values also indicate greater stability of the floating monolayer. Here, we compare the values of ΔG^{exc} as a function of the sterol (Chol, 7β-OH, 7α-OH, 7-K, and 25-OH) content in the film of a selected membrane lipid (DPPC, POPC, DPPE, DPPS, SM, and GM_1). Figures 1.5, 1.6, and 1.7 summarize the ΔG^{exc} values for the surface pressure of 30 mN/m, which have been adapted from

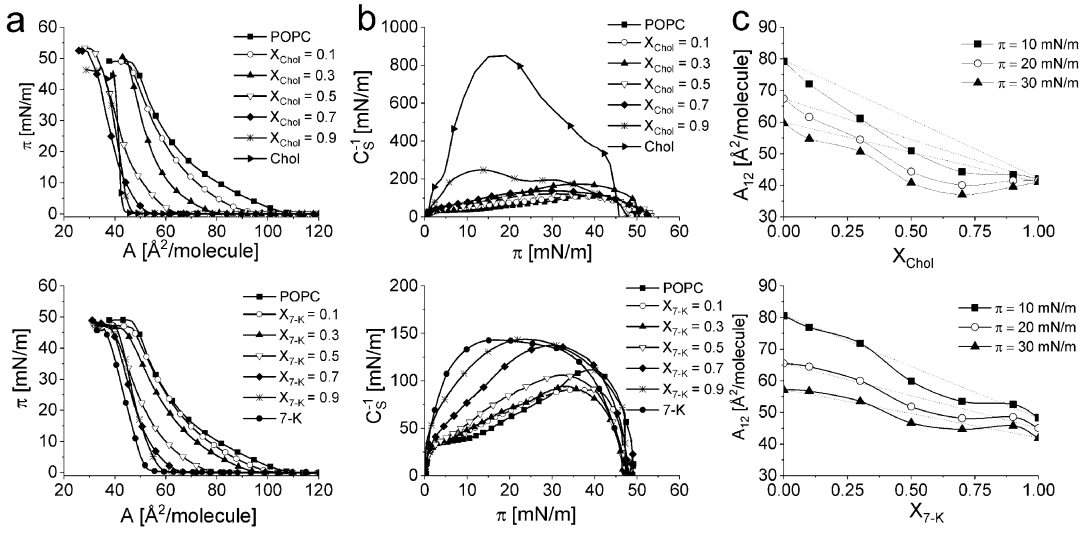

Fig. 1.4 Mixtures of cholesterol (up) and 7-K (down) with POPC: (**a**) surface pressure—molecular area (π-A) isotherms; (**b**) mean molecular area—molar fraction of sterol (A_{12}-X) plots; (**c**) compressibility modulus—surface pressure (C_s^{-1}-π) plots

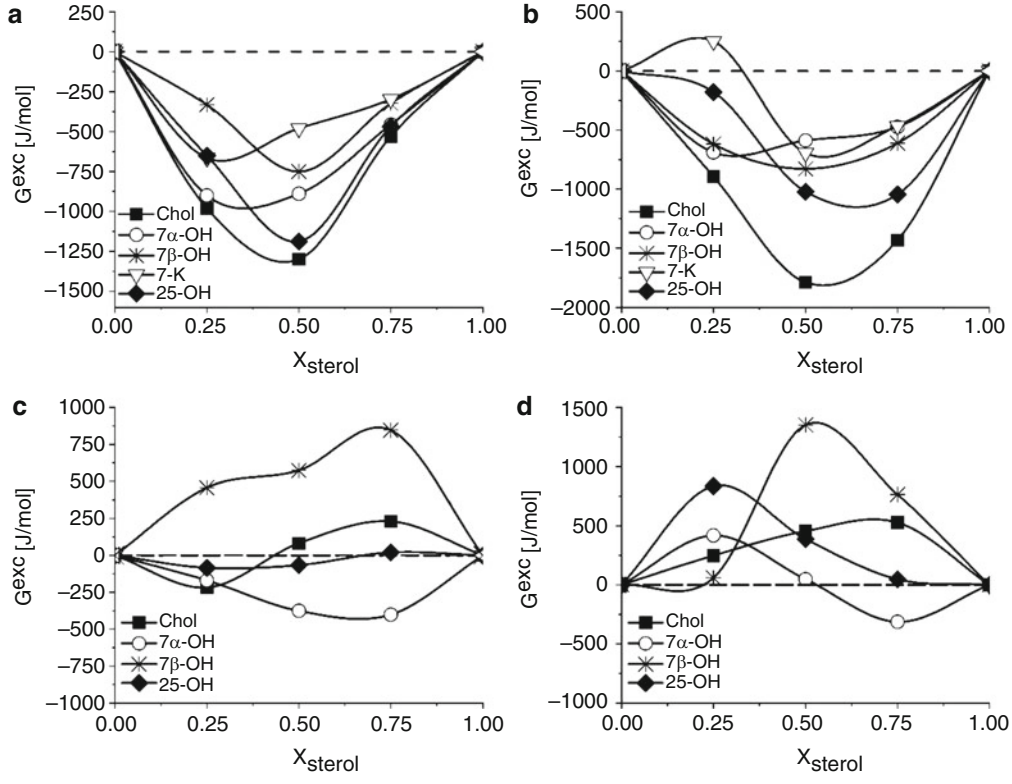

Fig. 1.5 Excess Gibbs free energy of mixing, ΔG^{exc} as a function of film composition (expressed as molar fraction of sterol, X_{sterol}) at 30 mN/m for mixed systems: (**a**) DPPC/sterol; (**b**) POPC/sterol; (**c**) DPPE/sterol; (**d**) DPPS/sterol

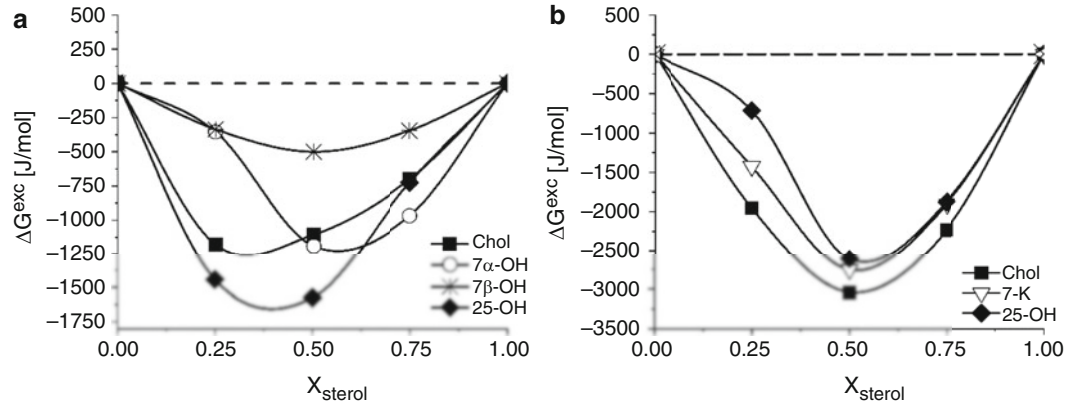

Fig. 1.6 Excess Gibbs free energy of mixing, ΔG^{exc} as a function of film composition (expressed as molar fraction of sterol, X_{sterol}) at 30 mN/m for mixed systems: (**a**) SM/sterol; (**b**) GM_1/sterol

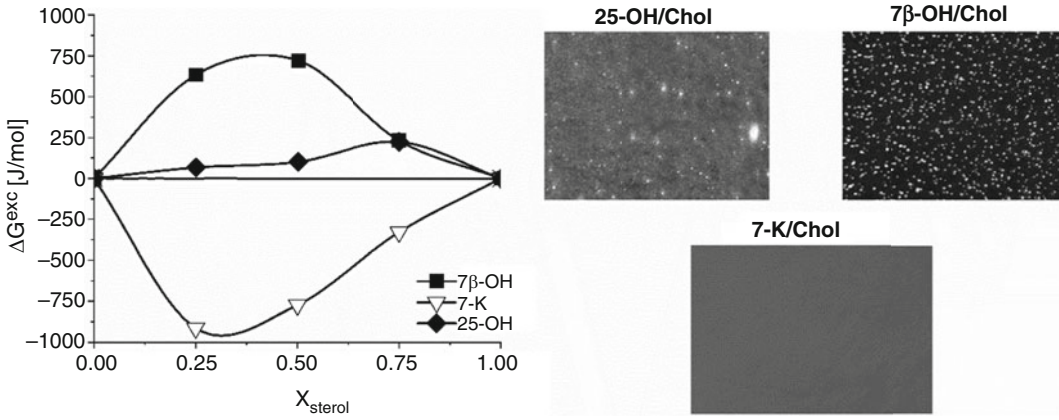

Fig. 1.7 Excess Gibbs free energy of mixing, ΔG^{exc} as a function of film composition (expressed as molar fraction of sterol, X_{sterol}) for mixed Chol/oxysterol systems together with BAM images for 1:1 mixtures at 30 mN/m. Adapted and modified with permission from (Wnętrzak et al. 2022b)

published papers (Telesford et al. 2015; Chachaj-Brekiesz et al. 2019; Kobierski et al. 2021a; Wnętrzak et al. 2021a, b).

Let us start our considerations with mixtures of sterols with the most common lipids in membranes, phosphatidylcholines (PC), represented by DPPC and POPC (Fig. 1.5). Generally, negative ΔG^{exc} values in all investigated systems show that the character of sterol interactions with PCs (POPC and DPPC) is attractive. The only exception from this rule occurs in the 7-K/POPC system with the smallest content of 7-K. It is worth to notice that the strongest interactions occur for PCs mixed with cholesterol (minimum at ca. −1800 J/mol) (Makyła and Paluch 2009; Jurak 2013; Makyła-Juzak et al. 2018). Furthermore, the observed minima in ΔG^{exc}–X_{chol} dependencies suggest the formation of surface complexes between molecules (Seoane et al. 1998; Keller et al. 2000; Gong et al. 2002). The introduction of an additional polar group into the sterane system in the case of 7β-OH, 7α-OH, and 7-K causes weakening of the interactions (ΔG^{exc} values oscillate around −1000 ± 200 J/mol). This phenomenon is the most pronounced for systems containing 7-K, suggesting a more

destabilizing effect of the carbonyl group compared to the hydroxyl group (Telesford et al. 2015; Chachaj-Brekiesz et al. 2019). Interestingly, clear minima in the ΔG^{exc}–X_{sterol} dependencies (as in the case of mixtures with cholesterol) are no longer observed. On the other hand, for the 25-OH molecule (with an additional hydroxyl group present in the isooctyl chain), the effect of reducing the stability and the strength of interactions caused by the introduction of an additional polar group is weaker. The minima in the course of the analyzed functions ΔG^{exc}-$X_{25\text{-}OH}$ are also visible. As we have shown in (Wnętrzak et al. 2021a), DPPC in the mixed monolayers interacts mainly through phosphate groups with the hydroxyl moieties at C(3) and C (25) of the 25-OH molecule. These functional groups are probably involved in the formation of stable surface complexes with two possible mutual arrangements of 25-OH and DPPC molecules. Interestingly, in the case of 25-OH mixtures with unsaturated PCs, the presence of a bulk hydrophobic moiety weakens interactions and enforces one specific orientation of oxysterol (with the C(3)-OH group) (Wnętrzak et al. 2021a). However, the strong attractive interactions are not characteristic for mixtures of sterols with all membrane lipids. For systems with phosphatidylethanolamines represented by DPPE, the interactions are weaker. In the case of DPPE mixtures with cholesterol or 25-OH, the thermodynamic analysis points to an almost ideal behavior (ΔG^{exc} values are close to zero). For DPPE mixed with 7-OH epimers, a slight negative (for 7α-OH) or positive (for 7β-OH) ΔG^{exc} values are observed, suggesting a weak attractive repulsive interactions, respectively. For negatively charged phosphatidylserines, exampled by DPPS, weak and mostly repulsive interactions with cholesterol and 7α-OH occur. In contrast, for 7β-OH and 25-OH, the magnitude of repulsive interactions is stronger (ΔG^{exc} values exceed 1300 J/mol and 800 J/mol for 7β-OH and 25-OH, respectively). This behavior can be described by the fact that the polar moieties of DPPE and DPPS can act as donors of intermolecular H-bonds per se (in the case of DPPS) and with water molecules (in the case of DPPS and DPPE). Incorporation of oxysterols into such densely packed film increases the distance between the phospholipid polar head-groups and disrupts the H-bonding network. Therefore, systems containing DPPE or DPPS exhibit lower stability attributed to slightly negative or positive ΔG^{exc} values. Differences in interactions between PCs and PEs and the studied oxysterols have important biological consequences. First, the observed differences in interactions may influence the distribution of cholesterol vs. oxysterols between inner (composed mainly of PEs and PSs) and outer (mainly PCs) membrane leaflets. Second, the translocation of some oxysterols between adjacent membrane leaflets may occur with different mechanism in comparison to cholesterol, e.g., the translocation of 25-OH occurs without changes in molecular orientation (Wnętrzak et al. 2021a). Such a facilitated transport was also described for other oxysterol substituted in isooctyl chain, i.e., 27-OH and will be discussed later in Sect. 1.4.6. Moreover, Olżyńska et al. have shown that chain-oxidized sterols promote the permeation of small charged particles, hydrophilic molecules, and water through the membrane (Olzynska et al. 2020).

Interesting results were obtained for mixtures with SM (see Fig. 1.6). Similarly to systems with PCs, the observed interactions are attractive for all studied sterols; however, their magnitude depends on molecular structure. Namely, for 25-OH/SM mixtures, the obtained ΔG^{exc} values are slightly more negative than in the case of the Chol/SM system. Although the exact values of ΔG^{exc} point to slightly stronger attraction in the 25-OH/SM system, it should be noted that interactions are generally comparable to those in the system with cholesterol (the same stoichiometry). This indicates a similar surface behavior of 25-OH and cholesterol in the SM environment. Indeed, in (Kobierski et al. 2021a), we have shown that SM enforces one specific orientation of 25-OH: being anchored with the C(3)–OH group to the water. A pronounced difference in the strength of interactions with SM is seen for two 7-OH epimers (Chachaj-Brekiesz et al. 2019). As mentioned in the previous section, both epimers differ in orientation at physiological

pressures. Namely, the hydroxyl group at C(7) in 7α-OH is detached from the water surface and therefore is accessible for hydrogen bonding with the hydroxyl group of the sphingosine backbone, contrary to 7β-OH, having both -OH groups immersed in water. Therefore, the interactions with SM are much stronger for 7-α-OH. This indicates that the structure of the interfacial region of membrane lipids plays a very important role in interactions. Interestingly, the attractive interactions were also observed between 7-K and SM isolated from the bovine brain (Wnętrzak et al. 2017), which indicates that the type of polar group is not a key factor in interactions with SM.

Another interesting issue, in particular in the context of the anticancer properties of oxysterols (discussed later in Sect. 1.4.5), is their strong affinity for ganglioside GM_1 (see Fig. 1.6). The comparable strength of interactions between GM_1 and Chol vs. some oxysterols (7-K and 25-OH) indicates that if both sterols are present, the oxysterols can compete with Chol for interaction with GM_1. Moreover, such a strong affinity may explain the facilitated selective incorporation of oxysterols into tumor cells, which are often characterized by gangliosides overexpression (Fish 1996).

As oxysterols are predominantly present in biosystems together with cholesterol, it also seems important to investigate interactions in cholesterol/oxysterol systems (see Fig. 1.7). As can be seen from ΔG^{exc} dependencies supported by the BAM images, 25-OH and Chol are mutually immiscible. For 7β-OH/Chol films, positive ΔG^{exc} values as well as inhomogeneous film textures also prove mutual immiscibility and repulsive interactions between lipids. In turn, 7-K attracts cholesterol molecules (ΔG^{exc} values are negative and film textures are homogeneous). These simple results allowed us to determine the influence of oxysterols on lipid rafts (cholesterol and sphingolipid-enriched domains). Namely, 25-OH does not have the ability to push cholesterol out of the lipid rafts (on the contrary, it has a stabilizing and raft-promoting effect). In turn, 7β-OH is capable of pushing cholesterol out of the model rafts by weakening of the strength of interactions. 7-K exerts a loosening effect; however, 7-K does not have the ability to push cholesterol out of the rafts (7-K probably competes with SM in the formation of cholesterol-containing surface complexes). The interactions in two-component systems described above show that even simplified systems, when studied in the right context, can provide a clear view of many physiological phenomena.

1.4 Investigation of Oxysterols Activity in Biomimetic Systems

Although analysis of interactions between biomolecule and chosen membrane constituent may provide some significant general information (as discussed in the previous section), the two-component systems are certainly oversimplification. Natural membrane is a complex system, therefore, to obtain more realistic picture the multicomponent artificial membrane models should be applied. In the following section, successive approaches for investigation of different aspects of the biological activity of oxysterols based on membrane models are presented.

1.4.1 Disturbing of Cholesterol Homeostasis

One of the most frequently studied aspects in the context of the membrane activity of oxysterols is the disturbance of cholesterol homeostasis. Cholesterol homeostasis is any mechanism that contributes to maintaining a balanced internal level of cholesterol in a living organism (Trapani et al. 2012). Cholesterol efflux can be inhibited by oxysterols in various ways, including nonstereospecific or apolipoprotein A-1 (apoA-1)-dependent interactions. It has been shown, using Langmuir monolayers and liposomes, that sterols oxidized in isooctyl chain (but not ring-oxidized sterols) contribute to the regulation of cholesterol homeostatic responses through non-enantiospecific interactions (Gale et al. 2009). The mechanism postulated by the authors is based on the observation that the sterane ring

system of 25-OH does not penetrate the membrane composed of DOPC as deeply as cholesterol. Instead, 25-OH can be incorporated into the membrane in an inclined orientation, so that both hydroxyl groups (C(3)-OH and C(25)-OH) interact with the polar heads of the phospholipids. Additionally, in the endoplasmic reticulum (ER), oxysterols regulate the level of cholesterol by competing with it in binding to membrane phospholipids. Studies on monolayer and bilayer models supported by cell line experiments have shown that treating the abovementioned systems with saturated palmitic acid leads to a reorganization of ER membrane phospholipids, thus reducing oxysterol activity in inhibiting cholesterol efflux (Gale et al. 2009). Cholesterol efflux may also be regulated by sterols oxidized in the sterane system, however, mainly in an apoA-1-dependent way (Gaus et al. 2001). ApoA-1 is the major protein component of high-density lipoproteins (HDL) that are considered as effective antiatherosclerotic agents. One hypothesis holds that HDLs antiatherosclerotic activity is due to their ability to remove excess cholesterol from peripheral cells in a transport system known as reverse cholesterol transport (RCT) (Ohashi et al. 2005; Griffiths and Wang 2021). According to the authors, 7-K by changing the composition and structure of the membrane lipid fraction, regardless of the presence of membrane proteins, reduces concentration-dependent cholesterol efflux and may contribute to foam cell formation and induction of atherosclerotic lesions (Gaus et al. 2001). Inhibition of apoA-1-dependent cholesterol efflux has also been also confirmed for 27-OH (Weingärtner et al. 2010). This does not seem surprising considering that elevated concentrations of these two oxysterols (7-K and 27-OH) have been found in atherosclerotic lesions (Poli et al. 2013). Phospholipid efflux may also be influenced by oxysterols (Gelissen et al. 1996, 1999). Other studies involving membrane models show that 7-K forms crystals in lipid vesicles and cell lines. Furthermore, cells exposed to 7-K develop extracellular crystals in the presence of an extracellular source of cholesterol, which also contributes to atherosclerotic lesions (Phillips et al. 2001). Interestingly, cells exposed to 25-OH do not produce crystals. According to the authors, 25-OH may not be able to form crystals in cells because it is not capable of self-association in membrane bilayers; however, the issue requires further research (Phillips et al. 2001). One of the key steps in HDL formation is lipidation of apoA-1 by the lipid transporter ABCA1. In (Massey and Pownall 2005a), it was shown that 7-K reduces the rate of apoA-1 insertion into phospholipid membrane interfaces. On the other hand, in (Massey and Pownall 2005b), the authors postulate that the oxysterol-regulated mechanism of inhibition of cholesterol efflux may be related to the disruption of the binding of apoA-1 to the lipid rafts of the cell membrane. The microsolubilization rate of multilayer lipid vesicles (MLV) composed of dimyristoylphosphatidylcholine (DMPC) to form discoidal high-density lipoproteins (rHDL) induced by apoA-1 strongly depends on both the concentration and the oxysterol structure. These studies partially reveal the complexity of sterol–lipid interactions, even in the presence of seemingly small differences in the structure of oxysterols, and point to 27-OH as the most potent oxysterol. In summary, the proatherosclerotic activity mechanism of oxysterols is complex and can be associated with modifying the interaction of apoA-1 with the plasma membrane, direct inhibition of ABCA1 lipid transporter activity, crystal formation, or control of the cholesterol substrate pool.

The next important aspect is the regulation of the efflux of oxysterols from cells. Increased efflux of oxysterols may be a consequence of: (1) their less efficient packing with membrane phospholipids compared to cholesterol (Fidalgo Rodríguez et al. 2019a; Szomek et al. 2020), (2) the activity of the above-mentioned ABCA1 (Tam et al. 2006), or (3) the action of unsaturated fatty acids, which—as shown in the 7-K example—can strongly bind to oxysterols and thus reduce their amount in the bloodstream (Fidalgo Rodríguez et al. 2019a, b, 2020). Interestingly, human cells are able to distinguish between oxysterols (for example, based on a single hydroxyl group in the side chain), which

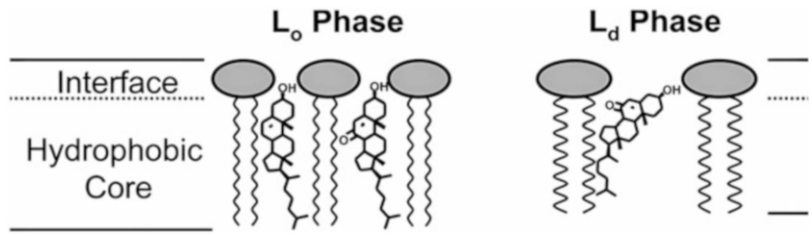

Fig. 1.8 Proposed model for the cholesterol and 7-ketocholesterol location within membrane domains. Reprinted with permission from (Massey and Pownall 2005a)

results in different transport as well as efflux dynamics and kinetics (Szomek et al. 2020).

1.4.2 Influence on Lipid Rafts

Taking into account the tendency of cholesterol to form so-called lipid rafts in biological membranes (Sezgin et al. 2017) raises the question of whether oxysterols are also capable of forming them? Due to the dynamics of the raft and the relatively short life time, it was convenient to conduct research on this issue in model systems (Wnętrzak et al. 2018). The most general conclusions drawn from these studies indicate that the type and localization of additional polar groups and, interestingly, their stereochemistry determine both raft formation and their arrangement. For example, analysis of the liposomes formed by DOPC/DPPC and DOPC/SM mixtures with the addition of cholesterol or 7-K, respectively, has shown that cholesterol increased lipid order and decreased bilayer polarity more effectively than 7-K (Massey and Pownall 2005a). 7-K is thus less effective in the formation of phospholipid-sterol complexes (and possibly also lipid-protein complexes) that determine the organization and dynamics of the lipid raft. Importantly, 7-K also showed a significant effect on the number and properties of phase boundaries, which—in turn—determine some interactions of the lipid phase with membrane proteins. Both cholesterol and 7-K can form tightly packed liquid ordered, L_o phases with phospholipids, such as sphingomyelin and saturated phosphatidylcholines. In consequence, in loosely packed liquid disordered, L_d bilayers formed by unsaturated phospholipids (above their melting temperature), the 7-K carbonyl group can be hydrated and form a hydrogen bond with the polar groups of the interface to move the sterol backbone further from the center of the bilayer (with respect to the cholesterol molecule). According to the authors, in mixtures of phospholipids where the L_o and L_d phases coexist, 7-K would have a stronger tendency than cholesterol to be incorporated into the L_d phase and thus destabilize the membrane rafts (see Fig. 1.8).

In another work (Wang et al. 2004), it was shown that 7-K induces the formation of DPPC enriched domains as effectively as cholesterol, although the DPPC/DOPC mixtures with 7-K showed greater solubility compared to those with cholesterol. Additionally, the packing of the domains was not as tight as those containing cholesterol (based on anisotropy measurements). On the other hand, Minzer and co-workers have shown that the ability of oxysterols to create detergent-resistant membrane (DRM) domains depends not only on the position of an additional polar group in the sterol backbone but also on the structure of phospholipids in model systems and the ratio of oxysterol to phospholipid (Mintzer et al. 2010). Furthermore, in these studies using Langmuir monolayers and liposomes formed by POPC and SM isolated from the bovine brain, it was shown that 25-OH exerts a stronger effect than 7-K and cholesterol on domain formation (Mintzer et al. 2010). Surprisingly, the condensation effect on the bovine brain SM monolayers was stronger for 7-K than for 25-OH. Furthermore, for a more complex system, supported lipid bilayer composed of DOPC/SM/Chol, it was shown that 25-OH makes bilayers less stiff compared to cholesterol (Domingues et al. 2021). In the same study, it has been postulated that the incorporation of 25-OH to model plasma

membranes or the replacement of some cholesterol with oxysterol leads to morphological changes and promotes heterogeneity of the lipid packing, which, according to the authors, confirms the raft-promoting effect of 25-OH. For comparison, the results obtained by Kobierski et al. have shown a different effect of 25-OH on lipid monolayers formed by egg SM, namely, increased condensation compared to analogous mixtures with cholesterol (Kobierski et al. 2021a). The strong condensation effect of 25-OH has also been described for films made by DMPC (Stottrup et al. 2014). Interestingly, oxysterols oxidized in the ring system (7-K and 5β,6β-epoxy) have the opposite effect, namely, they cause fluidization of films formed by saturated phospholipids (DPPC) (Telesford et al. 2015). Such differentiated results confirm that the formation of ordered domains is strongly influenced not only by the position and type of the polar group but also by the lipid environment of oxysterols. Interesting results can be found in (Massey and Pownall 2006). Model studies using MLV with different compositions (namely: DPPC, DOPC/SM, and DOPC/DPPC) have shown that 7β-OH does not induce L_o phase formation. Compared to cholesterol, 7β-OH also induces less changes in mechanical properties of the model membranes formed by the DOPC/SM and DOPC/DPPC mixtures. Furthermore, in none of the analyzed systems, 7β-OH did not form DRM. The authors postulate that 7β-OH is not able to take part in the attracting van der Waals interactions needed to tightly packed L_o phase formation and preferentially builds into the L_d phase. The weak DRM-promoting properties were also observed for 22(R)-OH. On the other hand, the best raft-promoting properties have been attributed to 25-OH and 27-OH. In both the DOPC/SM and DOPC/DPPC systems, they were more effective in forming DRM than cholesterol (Massey and Pownall 2006). The influence of oxysterols on the lipid raft stability appears to be as important as their formation. The results of many studies indicate that the presence of a polar group (i.e., hydroxyl or carbonyl) in the fused ring system and/or at C(20) in the isooctyl chain is a common structural feature of oxysterols disrupting the lipid domains (Wenz and Barrantes 2003). Also in (Li et al. 2003), the authors postulate that androsterol and 6-ketocholesterol are less effective than cholesterol in stiffening sphingolipid films; therefore, they may alter the stability of packed domains. On the other hand, 25-OH is again mentioned as oxysterol with the strongest raft stabilizing effect (Wenz and Barrantes 2003; Wnętrzak et al. 2022b). In (Wnętrzak et al. 2022b), the thermodynamic analysis of the interactions between the components in mixed Langmuir films showed that replacing cholesterol with 25-OH increases both the strength of the interactions and the stability of the mixed films. This is probably due to the formation of hydrogen bonds between 25-OH and the interfacial region of the SM molecules. In the same article, the results for 25-OH were compared with the results for two C(7)-oxidized sterols that have a different group (carbonyl vs. hydroxyl). Generally, it was found that, unlike 25-OH, which enhances the interactions between SM and Chol and thus stabilizes the raft, 7β-OH and 7-K have a fluidizing effect and weaken the interactions in the model raft. In the case of 7β-OH, this phenomenon can be explained by (i) weaker affinity of 7β-OH for SM molecules compared to the corresponding Chol/SM films (Chachaj-Brekiesz et al. 2019) and (ii) the repulsive nature of the interaction between cholesterol and 7β-OH (ΔG^{exc} approx. +760 J/mol at 30 mN/m). Therefore, replacing cholesterol by 7β-OH in model rafts (under forced conditions) may destabilize them, although 7β-OH itself has no affinity for incorporation in these domains. On the other hand, for 7-K, a strong interaction with cholesterol leads to the formation of 7-K/Chol complexes, which appears to be the key to disrupting lipid rafts. Interesting results were also obtained for 7α-OH (Wnętrzak et al. 2019b). This steroid, although it is a ring-oxidized cholesterol derivative, behaves similarly to chain-oxidized oxysterols (which may be due to a similar orientation at the water/air interface in a higher surface pressure range). Thermodynamic analysis combined with the analysis of rheological properties showed that the increased level of

7α-OH in the lipid raft causes a significant increase in molecular packing (over 100% compared to a normal raft with a composition of Chol/SM 1:2). Thus, taking into account the described results it can be concluded that the high concentration of oxysterols observed during pathological processes may lead to changes in the structure of microdomains and consequently destabilize membrane proper functioning.

1.4.3 Oxysterols in Neurodegeneration

Disruption of protein sorting and association may be an example of negative activity of oxysterols. Their effect on the interactions of amyloid β (Aβ) with biological membranes has been investigated in (Kim and Frangos 2008; Phan et al. 2013). In general, Aβ applied directly to the model lipid membranes in the presence of oxysterols causes dramatic changes in their physicochemical properties. First, it has been shown that 7-K and 25-OH, unlike cholesterol, do not inhibit Aβ binding to biological membranes. Experiments on cell-size liposomes containing 7-K showed a high tendency to associate with Aβ, while analog systems with 25-OH were more capable of morphological changes in response to the peptide (Phan et al. 2013). Differences in activity were related to the position of the additional polar group in the oxysterol molecule (in the ring system vs. chain). In (Kim and Frangos 2008), two oxysterols oxidized at C(7): 7-K and 7β-OH were compared. Both oxysterols reduced the thickness of the membrane, which facilitated the incorporation of amyloid. The presence of 7-K allowed the incorporation of the Aβ (1–42) peptide, while the 7β-OH allowed the insertion of both Aβ (1–42) and Aβ (1–40). The results obtained suggest an enhanced effect of oxysterols on the interaction of Aβ with membranes and represent an important step in elucidating the harmful effect of cholesterol oxidation on A-β-induced neurotoxicity. The role of oxysterols in the neurodegeneration process was also investigated in (Chachaj-Brekiesz et al. 2020). More specifically, the authors examined the effect of oxysterols with different structure on the myelin sheath modeled as Langmuir monolayer composed of Chol/PE/SM/PS/PC. In the presence of oxysterols, the fluidity of the model myelin sheath was observed to increase and the organization of lipids changes, which is reflected in the reduction of electric surface potential change (ΔV). When the additional hydroxyl group is located in the sterane moiety, the observed effect is related to the type of functional group (7-K vs. 7-OH), while the configuration (7α-OH vs. 7β-OH) seems to play a minor role. On the other hand, when the hydroxyl group is attached to the side chain (the case of 25-OH), the observed effect on the selected model is most significant. In conclusion, the strongest myelin–oxysterol interactions were observed for 7-K and 25-OH, which are known to be the most cytotoxic oxysterols in neurodegeneration. In other model studies using Langmuir monolayers that mimic the neuronal membrane (POPC/SM), it was shown that the affinity of 7-K to the model membrane is much higher than that of cholesterol (Wnętrzak et al. 2017). The authors postulate that the stronger intermolecular interactions observed due to the incorporation of 7-K may affect the physical properties of the neuronal membrane, thus altering synaptic transmission, receptor binding, and other processes that contribute to the neuronal dysfunction observed in neurodegeneration.

1.4.4 Oxysterols Effect on Erythrocytes

The negative effect of oxysterols was also observed in the case of erythrocytes. Their incorporation into the erythrocyte membrane has been studied with regard to both the outer (Targosz-Korecka et al. 2020) and inner (Lechner et al. 2022) leaflet of the membrane. The research was motivated by the observed changes in the morphology of red blood cells under the conditions of increased concentrations of oxysterols. Under these conditions, cholesterol loss can enhance membrane–cytoskeleton interactions by reorganizing the submembrane structure of the cytoskeleton, thereby increasing

cell stiffness. As is known, red blood cells must constantly deform as a result of flowing through blood vessels (including narrow capillaries), and any changes in their morphology caused by cholesterol oxidation can have a detrimental effect on the ability to flow and tissue oxygenation. In (Targosz-Korecka et al. 2020), a simple but common model of the erythrocyte membrane outer leaflet was used, namely, the Langmuir monolayer consisting of cholesterol and POPC (Chol/POPC). Then oxysterols were added to this system in various proportions (mole fractions $X = 0.1$ and $X = 0.5$). The results obtained indicate the diversification of the activity of selected oxysterols. Hence, 7α-OH causes fluidization of mixed films (in both concentrations), while its epimer—7β-OH—at a low concentration ($X = 0.1$) did not affect film packing, while at a higher concentration ($X = 0.5$) the monolayer stiffened. Similarly, stiffening behavior, but much more pronounced, was observed for 7-K (see figure). Studies on artificial models have been correlated with the results on erythrocyte cells taken from healthy patients. Again, the most significant changes in the modulus of elasticity were observed for cells incubated with 7-K (see Fig. 1.9).

This may be due to the conjugation of the C=C double bond with the ketone group at C (7), which reduces the conformational lability of the sterane backbone in 7-K, and causes flattening of the molecule compared to 7β-OH. Therefore, the stiffer and flatter structure of the 7-K molecule may hinder the free movement of the phospholipid acyl chains, causing an increase in condensation (decrease in elasticity) of the mixed film. In the case of the inner leaflet of the erythrocyte membrane (mimicked as DPPC/DPPE/DPPS/SM/Chol), it was also proved that cholesterol oxidation to 7-ketocholesterol leads to stiffening of the monolayer (under static and dynamic conditions), significant changes in organization of microdomains within the model membrane, disturbances in van der Waals, electrostatic and hydrophobic interactions between the sterol and other lipids, and an increase in the hydration of the lipid membrane (Lechner et al. 2022). All of this contributes to the dysfunction of red blood cells.

1.4.5 Beneficiary Effects of Oxysterols

However, the activity of oxysterols is not always harmful to our health. In recent years, their antiviral activity confirmed on a wide group of viruses has been intensively studied (Lembo et al. 2016; Marcello et al. 2020; Wang et al. 2020). It has been shown in (Gomes et al. 2018) that the presence of 25-OH instead of cholesterol can block membrane fusion catalyzed by HIV-FP, and thus the entry of HIV into the host cell. The changes observed in the secondary structure of HIV-FP indicate that the presence of 25-OH may reduce the ability of the peptide to form fusion pores by losing some of its conformational plasticity. Moreover, due to the use of a simplified model (cell membrane modeled as POPC/sterol; HIV membrane: POPC/sterol/SM/DPPC/POPE/POPS), it was shown that 25-OH targets the membrane lipids themselves and not another component of the viral or cell membrane. 25-OH can also modulate the behavior of viral membranes, which can affect not only viral entry but also the integrity of the viral envelope, since the cholesterol-depleted virus shows reduced infectivity. The effectivity of therapy can be further increased by combining 25-OH with the antiviral protein C-34 (Gomes et al. 2019). In other studies using multicomponent Langmuir monolayers, oxysterols (25-OH and 7β-OH) show preventive (fusion blockade) and protective (changes in the infected host membrane) antiviral activity against the ZIKA virus by modifying the lipid fraction in model systems (Wnętrzak et al. 2022a). A simple thermodynamic analysis showed that: (i) oxysterols strongly repel the virus model membrane, while showing a weak affinity for the dendritic cell membrane (target), which may facilitate blocking fusion and phase separation occurring and (ii) oxysterols show affinity for the infected membrane of the neuron without repelling the membrane of the model virus (therefore they do not block fusion in this

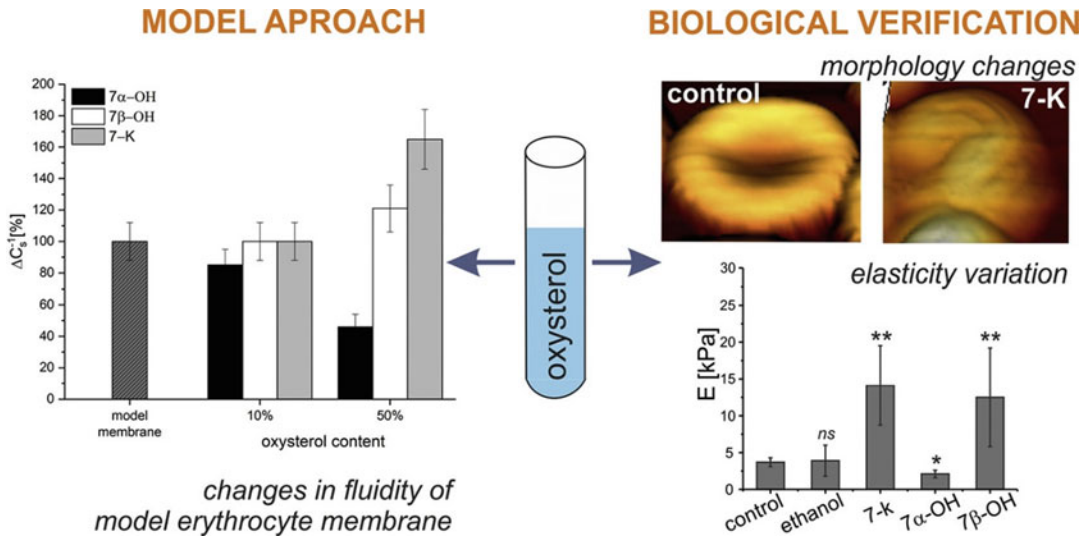

Fig. 1.9 Schematic representation of study considering the effect of selected B-ring-substituted oxysterols on artificial model erythrocyte membrane and isolated red blood cells. Reprinted with permission from (Targosz-Korecka et al. 2020)

system but act as therapeutics). Interestingly, the effects of both oxysterols (25-OH and 7β-OH) are comparable (so far, antiviral effects have been attributed mainly to oxysterols oxidized in the side chain). Moreover, since the lipid envelope of the ZIKA virus is morphologically similar to other enveloped viruses, the fusion mechanism may also be similar and therefore the results may be applied to other representatives of this group (such as SARS-CoV-2). Other beneficiary effects of oxysterols were described in (Cui et al. 2017). The authors explained the formation of stereosomes, nonphospholipid liposomes made of single-chain amphiphiles with a high content of sterols, with osteoinductive properties (inducing bone tissue formation from cartilage). It turns out that 20(S)-hydroxycholesterol in mixtures with stearylamine (SA) increases osteogenic differentiation of bone marrow stromal cells in the hydrogel environment. Such 20(S)-OH/SA systems also proved to be noncytotoxic over a wide concentration range. The study was confirmed in critical-sized mouse calvarial defects. Therefore, oxysterols may represent a promising nonphospholipid liposomal platform with osteoinductive properties.

One of the most promising mechanisms of action of oxysterols is their antitumor activity in therapy against glioblastoma multiforme. In the aspect of cancers, oxysterols are mainly associated as markers (their elevated level is observed in the broad aspect of neoplastic changes) and pathogenic factors (Vejux and Lizard 2009; Kloudova et al. 2017; de Freitas et al. 2021). However, recent studies show that these compounds can also be therapeutic. Their advantage in the treatment of glioblastoma is that they cross the blood-brain barrier. Among the selected oxysterols, which differed in the type of the polar group (carbonyl vs. hydroxyl) and its localization (C(7) vs. C(25)), 7-K turned out to be the most effective (Wnętrzak et al. 2021b). Its incorporation into the model glioblastoma membrane modeled as Chol/POPC/SOPE/SM/GM_1 resulted in significant stiffening of the membrane. Studies on the U-251 cell line confirmed this trend. In addition, incubation with oxysterols has been observed to lead to phospholipidosis (see Fig. 1.10).

The lipid disorder, in turn, inhibits cell proliferation and may play a key role in the induction of apoptosis by oxysterols. The antitumor activity of these compounds may furthermore be related to the immobilization of tumor cells due to the oxysterol-induced stiffening effect observed in both the model membrane and the cell line.

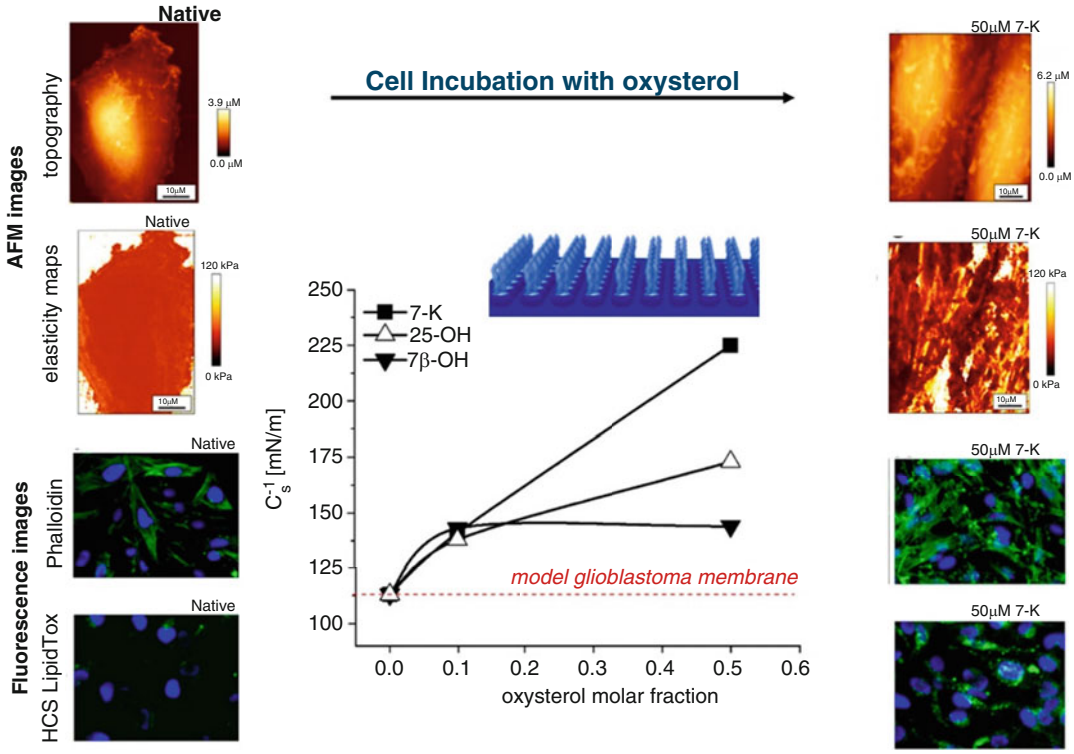

Fig. 1.10 Schematic representation of the study concerning oxysterols antiglioblastoma activity—Langmuir studies complemented with biological experiments. Reprinted with permission from (Wnętrzak et al. 2021b)

1.4.6 Investigation of Oxysterols Transport Between Membrane Leaflets

The cholesterol content in the cell membrane strongly depends on the type of cell. In the cell membrane of mammals, it makes about 20–30% of lipids (Casares et al. 2019). Since cholesterol interacts strongly with saturated lipids, its content is higher in membranes with a high content of these lipids (Róg et al. 2009; Mannock et al. 2010). This, in turn, explains the asymmetric distribution of cholesterol in cell membrane leaflets. In the outer leaflet, the cholesterol concentration can be up to 12 times higher than in the inner leaflet (Liu et al. 2017). However, thermodynamic fluctuations cause the movement of lipids, including cholesterol, from one leaflet to another. Such transverse movement of amphiphilic lipids between the layers of a biological membrane, first described in (McConnell and Kornberg 1971), is called a flip-flop and is understood as a migration of a molecule between the leaflets of a biological membrane (flop) with its reorientation by 180° (flip). Molecular dynamics simulations provide information on the speed of these processes. Computational studies report relatively fast interlayer transport of cholesterol compared to phospholipids, with a half-time of nanoseconds to minutes, depending on the membrane composition. In polyunsaturated bilayers, cholesterol flip-flops in the submicrosecond time scale (Gu et al. 2019). The process is slower, in the range of seconds, in bilayers with a high content of saturated lipids (Bennett et al. 2009). For the lipid raft model, a half-time of 30 min was obtained (Bennett and Tieleman 2012). Thus, it is clear that intermolecular interactions modify this parameter by orders of magnitude. Therefore, the question arises whether the oxidation of cholesterol or the delivery of its oxidized forms to the organism can cause a change in the flip-flop

frequency. Such a change could disturb the asymmetry of the distribution of sterols in the cell membrane, which, in turn, would lead to an alternation of its physical properties and would have serious biological consequences.

Parisio et al. (2012) found on the basis of umbrella sampling calculations that while the flip-flop time for cholesterol in the model consisting of DPPC and low concentration of sterol is about milliseconds and minor chemical modifications do not significantly change this parameter, the removal or addition of hydroxyl group shortens this time by orders of magnitude. Namely, they predicted that for 5-cholestene and 25-OH, the flip-flop time is below microseconds. Therefore, it seems that, in the case of oxysterols, the additional polar center, distant from the original one, significantly affects the time of the transverse movement.

As the next investigation showed, the free energy values for sterol translocation between leaflets for the Chol/POPC and 7β-OH/POPC models do not differ much and are 14 kJ/mol and 18 kJ/mol, respectively (Kulig et al. 2018). A higher free energy value for 7β-OH than for cholesterol suggests that the transverse movement between leaflets for this oxysterol is slower, probably due to the additional hydrogen bonds formed. The case of the POPC/27-OH system was different. The free energy value was approximately twice lower and equal to 8 kJ/mol.

In the same study, for a molecular dynamics simulation with an evolution time of 1 μs, interlayer translocation was not observed for either Chol/POPC or 7β-OH/POPC. For the simulation of 27-OH/POPC, in turn, a translocation with a time of 560 ns was identified. This displacement occurred without reorienting the sterol, which was described as a bobbing motion. However, such displacement causes that instead of the C(3)-OH, the C(27)-OH is exposed to the water phase. Such a process is possible only for oxysterols that have a polar group at the isooctyl chain. Because of the additional polar center, transverse motion, as opposed to flip-flop, without changing the orientation of the molecule, significantly reduces the potential barrier for such motion.

The molecular dynamics of cholesterol oxidized at the isooctyl chain has also been reported in the next two studies in which 25-OH (Nåbo et al. 2018) and 27-OH (Kulig et al. 2015) were investigated. In the POPC biomembrane model, it was found that these oxysterols most often orient in the so-called normal position, i.e., perpendicular to the interface with the C(3)-OH facing the water phase. This orientation is typical for cholesterol and sterols oxidized in the sterane ring system. However, oxysterol molecules parallel to the water surface, hydrogen bonded to the atoms of the polar phospholipid heads, were also observed. Most interestingly, the oxysterols were also located in a position between the layers of the biological membrane model, which was not observed in the case of cholesterol and oxysterols with hydroxyl groups at the ring. Taking such a position suggests that in the case of these oxysterols, the classic flip-flop process, i.e., transport between layers with a change in the orientation of the molecule, may occur more easily. These oxysterols were also in the flipped position, confirming the possibility of a bobbing motion. Furthermore, Szomek et al. (2020) observed a reorientation of 27-OH molecules from upright to horizontal in a membrane model consisting of POPC. Similar conclusions from molecular dynamics simulations were obtained in (Galiano and Villalaín 2020). For the membrane model consisting of POPC, POPE, POPC, Chol, and 25-OH, a flip of the 25-OH molecule initially oriented to the water with the C(3)-OH group was thoroughly observed. The reorientation lasted about 140 ns, with the molecule remaining parallel to the membrane surface in between the leaflets for 20 ns. The particle after rotation remained in the same leaflet.

In the study by Wnętrzak et al. (2021a), based on the simulation of the molecular dynamics of the membrane model from 25-OH and DPPC, it was shown that the systems in which the oxysterol molecules were oriented by either the C(3)-OH or C(25)-OH to water are energetically equivalent. This confirms again that the lipid migration can occur between leaflets without reorientation. Moreover, in the study by Kobierski et al. (2021a), where layers composed

of 25-OH and SM or GM_1 were simulated, a flip-flop of oxysterols was observed. Especially for 25-OH/SM the effect was significant, where 30% of the oxysterols with C(25)-OH facing the water surface changed orientation after 80 ns. For initial arrangement with C(3)-OH facing water surface groups 8% flipped. This means that also in the case of lipid rafts, the transverse movement, both flip-flop and bobbing, has a low potential barrier, which may cause these phenomena to occur more often as a result of cholesterol oxidation or oxysterol incorporation, which will affect the biological functions of the membrane.

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Association of ABCG5 and ABCG8 Transporters with Sitosterolemia

2

Sergio Paulo Bydlowski and Debora Levy

Abstract

Sitosterolemia is a rare genetic lipid disorder, mainly characterized by the accumulation of dietary xenosterols in plasma and tissues. It is caused by inactivating mutations in either ABCG5 or ABCG8 subunits, a subfamily-G ATP-binding cassette (ABCG) transporters. ABCG5/G8 encodes a pair of ABC half transporters that form a heterodimer (G5G8). This heterodimeric ATP-binding cassette (ABC) sterol transporter, ABCG5/G8, is responsible for the hepatobiliary and transintestinal secretion of cholesterol and dietary plant sterols to the surface of hepatocytes and enterocytes, promoting the secretion of cholesterol and xenosterols into the bile and the intestinal lumen. In this way, ABCG5/G8 function in the reverse cholesterol transport pathway and mediate the efflux of cholesterol and xenosterols to high-density lipoprotein and bile salt micelles, respectively. Here, we review the biological characteristics and function of ABCG5/G8, and how the mutations of ABCG5/G8 can cause sitosterolemia, a loss-of-function disorder characterized by plant sterol accumulation and premature atherosclerosis, among other features.

Keywords

Sitosterolemia · ABC transporter · Sterol · ABCG5 · ABCG8

Abbreviations

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette subfamily B1
ABCG5	ATP-binding cassette subfamily G5
ABCG8	ATP-binding cassette subfamily G8
ATP	Adenosine triphosphate
BSEP	Bile salt exporter protein
KO mice	Knock out mice
MDR	Multidrug resistance
NBD	Nucleotide-binding domain
P-gp	P-glycoprotein
TMD	Transmembrane domain

S. P. Bydlowski (✉)
Lipids, Oxidation and Cell Biology Team, Laboratory of Immunology (LIM19), Heart Institute (InCor), Faculdade de Medicina, Universidade de São Paulo, Sao Paulo, Brazil

National Institute of Science and Technology in Regenerative Medicine (INCT-Regenera) CNPq, Rio de Janeiro, Brazil
e-mail: sergio.b@fm.usp.br

D. Levy
Lipids, Oxidation and Cell Biology Team, Laboratory of Immunology (LIM19), Heart Institute (InCor), Faculdade de Medicina, Universidade de São Paulo, Sao Paulo, Brazil

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2.1 Introduction

Sitosterolemia is a rare genetic recessive disease in which an individual is unable to excrete xenosterols.

In 1974, Bhattacharyya and Conner first described a new syndrome, a lipid storage disorder, in two sisters who presented with tendon and tuberous xanthomas with normal plasma cholesterol levels and elevated plasma levels of plant sterols (phytosterols), such as sitosterol, campesterol, and stigmasterol. The disease was named—sitosterolemia—after the most abundant dietary xenosterol, sitosterol. In fact, some think that perhaps a more appropriate name for the disease would be xenosterolemia (Maguire et al. 2001). Anyway, this discovery initiated fundamental studies on how dietary sterols traffic and are eliminated by the body (Williams et al. 2021).

It took another 26 years before the sitosterolemia locus was mapped to chromosome 2p21 and the discovery that the genetic locus whose dysfunction leads to sitosterolemia encodes two genes, abcg5 and abcg8, whose proteins (ABCG5 and ABCG8) function as obligate heterodimers (Berge et al. 2000; Lu et al. 2001).

ABCG5 and ABCG8 are expressed only in hepatocytes, gallbladder epithelium, and enterocytes and are responsible for excretion of sterols, with xenosterols preferred over cholesterol (Patel et al. 2018). In fact, the naming of the disease has led to a bias: a vast range of other sterols, not just sitosterol, are accumulating in the body (Gregg et al. 1986). According to some researchers, a better name should be xenosterolemia.

Several missense mutations on either genes are the causative gene defect that lead to loss of function of the ABCG5/G8 transporter, which is associated with lipid phenotypes (Miettinen 1980; Salen et al. 1985; Berge et al. 2000; Lee et al. 2001; Lu et al. 2001; Brown and Yu 2010; Williams et al. 2021). Subsequent case reports established the recessive genetics of the disease and greatly expanded its potential clinical presentation.

2.2 Sterol Transport

All living cells depend on their ability to transfer molecules such as nutrients, hormones, metabolites, and across their membranes. Cell membrane is the natural barrier for intracellular constituents and the checkpoint of molecules and signals from the extracellular milieu. Lipids are the primary component of mammalian cell membranes, with cholesterol being a key component. Cholesterol accounts for ~40–50% of the total lipid content in the plasma membrane (Steck and Lange 2018). Cholesterol serves as the precursor molecule for steroid hormones that modulate gene regulation, for bile acids that enable for nutrient absorption, and for vitamin D which are vital for body health (Rezaei et al. 2023).

Translocation of cholesterol molecules on biological membranes plays an essential role in maintaining cholesterol homeostasis. However, relatively little is known about the mechanisms that control the sterol shuttling across lipid-bilayer membranes. Anyway, lipid-transport membrane proteins have been shown to be essential for the translocation of sterols and phospholipids to maintain lipid homeostasis, cellular functions, and the structural integrity of mosaic lipid bilayers (Abumrad et al. 2000; Sharom 2011; López-Marqués et al. 2015).

Cholesterol can be obtained by de novo cell biosynthesis or via dietary uptake in the intestine. In normal diets, the levels of cholesterol and non-cholesterol sterols from plants (xenosterols) or other dietary sources are usually equal. However, 50–60% of dietary cholesterol is absorbed, while xenosterols exhibit poor bioavailability, with <5% absorption (Salen et al. 1970). When more plant sterols are ingested, they compete with the bulk cholesterol for solubilization, thereby reducing dietary absorption of cholesterol and lowering plasma cholesterol.

Elimination of excess cholesterol is vital for life. Abnormal elevations in plasma cholesterol contribute to hyperlipidemia, a critical factor leading to cardiovascular diseases and other metabolic disorders (Salen et al. 1970). However, not all imported cholesterol is metabolized in the

cells. In fact, few cells have this capacity. Therefore, elimination of the excess amount of cholesterol has to be cleared from cells and tissues via two metabolic pathways that are essential to maintain homeostasis: reverse cholesterol transport through sterol acceptors in the circulation or direct cholesterol excretion through biliary and intestinal secretion (Vrins et al. 2012; Ouimet et al. 2019). Xenosterols have efficient biliary elimination (Salen et al. 1970).

In more advanced life forms, functions such as nutrient intake and the exchange of compounds between cellular organelles or tissues often take place against concentration gradients across cellular membranes (Schumacher and Benndorf 2017). It is therefore not surprising that in simple life forms like bacteria, almost 10% of the entire genome is dedicated to proteins that are involved in transport processes in the form of membrane-bound or soluble proteins (Blattner et al. 1997). Transport processes against chemical gradients always require free energy which is derived from either by simultaneous use of an opposing electrochemical potential difference (secondary active transport) or a coupled enzymatic reaction exploiting the chemical energy of adenosine triphosphate (ATP) hydrolysis (primary active transport) (Blattner et al. 1997; Schumacher and Benndorf 2017). This ATP-driven transport, which act through the activity of energy-dependent unidirectional, membrane-bound, compound-efflux transporter proteins, comprise a large superfamily, the ABC (ATP-binding cassette) transporters.

2.3 ABC Transporters

The ABC transporter superfamily comprises one of the largest families of evolutionarily conserved membrane proteins and is ubiquitously expressed in all domains of life, from nearly all prokaryotes to virtually all types of eukaryotic cells (Higgins 1992; Dean et al. 2001a, b; Dean and Annilo 2005; Locher 2016; Bilsing et al. 2023). ABC transporters are most abundantly expressed in organs with high metabolic rates and in endothelial cells that isolate organs like the blood–brain barrier and blood–testis barrier (Schumacher and Benndorf 2017).

These transporters are involved in a broad range of cellular processes, therein actively transporting a wide range of different substrates across the plasma membrane (Schumacher and Benndorf 2017). ABC transporters use the energy from ATP hydrolysis to drive the passage or flipping of various moieties across the bilayer membrane, from small inorganic and organic molecules to larger organic compounds (Schumacher and Benndorf 2017), including both hydrophilic and hydrophobic molecules such as sugars, peptides, drugs, phospholipids, and sterols (Dean and Allikmets 1995; Linton and Higgins 1998; Dean et al. 2001a, b; Hwang et al. 2016; Plummer et al. 2021).

ABC transporters are divided into three subclasses, two groups of importers and one group of exporters, according to their functional and architectural characteristics (Schumacher and Benndorf 2017). ABC importers are predominantly found in prokaryotes where they manage the nutrient and ion intake (Ferreira and de S-á-Nogueira 2010; Gisin et al. 2010); they only sparsely occur in eukaryotes. The vast majority of ABC transporters expressed in eukaryotes are ABC exporters, promoting functions such as secreting dietary substances and metabolites or even transport signaling molecules (Schumacher and Benndorf 2017). Also, ABC transporters are major sterol exporters responsible for both cholesterol efflux from peripheral cells and the elimination of excess cholesterol and dietary sterols such as sitosterols (Borst and Elferink 2002; Xavier et al. 2019).

There are currently 49 different genes known to encode ABC transporters in humans, which categorize subfamilies of ABC transporter proteins based on sequence similarity, sequence divergence and structural arrangement (Plummer et al. 2021; Huang and Ecker 2023). Since several of those genes are alternatively spliced during transcription, each of those 49 genes not only encode one single protein, but instead often a multiple of different ABC protein variants (Dean et al. 2001a, b). The subfamilies are named ABCA–ABCG. Five distinct families (A, B,

C, D, and G) display a wide array of substrate specificities and functionalities (Alam and Locher 2023). Of all ABC transporters that have been described so far, the three members ABCB1, ABCC1, and ABCG2 are less organ specific (Zhang et al. 2015).

The first human ABC transporter described, P-glycoprotein (ABCB1) (170 kDa) was discovered in 1976 by Juliano and Ling in Chinese hamster ovary (CHO) cells, selected for resistance to colchicine. These cells displayed pleiotropic cross-resistance to a wide range of amphiphilic drugs. Because the glycoprotein altered the membrane permeability (P), it was called P-glycoprotein (P-gp, encoded by the MDR-1/abcb1 gene) (Huang and Ecker 2023). ABCB1 is the first mammalian member of the large family of ABC transporters present in prokaryote (Davidson et al. 2008) and eukaryotes, from plants (Theodoulou 2000) to humans (Gottesman and Ambudkar 2001).

The division of ABC transportes in subfamilies is based mainly on similarity in gene structure, e.g., half vs. full transporters, and on sequence homology in the nucleotide-binding domains (NBDs) and transmembrane domains (TMD) (Schumacher and Benndorf 2017).

ABC transporters are organized as two symmetric halves that are expressed either (*a*) as separate subunits (half-transporters) that assemble as homodimers or heterodimers or (*b*) as monomers containing two nonidentical halves within a single polypeptide (full transporter) (Alam and Locher 2023). Each half comprises, at minimum, a NBD that is responsible for ATP binding and hydrolysis and a TMD that facilitates substrate export (away from the NBDs) or import (toward the NBDs) (Alam and Locher 2023). The human ABC transporters can be either full or half-transporters. In principle, full ABC transporter transcripts, such as members in the A- and C-subfamilies, comprise four domains within one polypeptide chain, namely 2 TMDs embedded in the lipid bilayer, and 2 NBDs facing the cytoplasmatic space (Plummer et al. 2021; Huang and Ecker 2023). The motifs are arranged as N-TMD-NBD-TMD-NBD-C, whereas the half ones have only one TMD and one NBD (Plummer et al. 2021). Hence, the half-transporters should form homodimers or heterodimers to perform their function (Huang and Ecker 2023). Among ABC transporter families, NBDs exhibit high sequence homology, with several canonical motifs. The transport function is generally believed to be driven by the NBD dimerization, in which ATP is bound and hydrolyzed. TMDs are structurally highly diverse, suggesting distinctive transport mechanisms for individual transporters (Ford and Beis 2019).

2.4 Main Diseases Related to ABC Transporters

Active in nearly all cells and tissues, ABC transporters play vital physiological roles ranging from lipid homeostasis to transport of diverse endogenous and exogenous compounds (Alam and Locher 2023). Several diseases result directly from dysfunction of these transporters, making them important targets for therapeutic intervention. Therefore, human ABC transporters hold tremendous biomedical and pharmacological relevance (Dean et al. 2001a, b; Borst and Elferink 2002; Leonard et al. 2003).

A major obstacle in cancer treatment is the development of cancer resistance to several structurally dissimilar cytotoxic substances (Huang and Ecker 2023). This phenomenon is termed as multidrug resistance (MDR), which renders the cancer cells ineffective in accumulating drugs, preventing their death. ABCB1, ABCC1, and ABCG2 were frequently observed with enhanced overexpression in multiple cancer types (Zhang et al. 2015). In fact, ABCB1 transporter is the most studied ABC regarding chemotherapy against cancer (Hwang et al. 2016; Seelig and Li-Blatter 2023). Subsequent to the discovery of P-gp, studies of cancer cells revealed other phenotypes, which showed multidrug resistance related characteristics. These multidrug resistance related proteins (MRPs) were later classified as the ABCC subfamily (Cole et al. 1992). Simultaneously, a novel half transporter member of the ABC superfamily was identified from a resistant

breast cancer cell line (Doyle et al. 1998), hence named as breast cancer resistance protein (BCRP), encoded by the ABCG2 gene. Additionally, other members of ABC transporter were reported to export at least one anticancer agent. For instance, ABCA2, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, and ABCC11 (Hwang et al. 2016). Others, such as ABCB11, also known as Spgp (sister of P-glycoprotein) or BSEP (bile salt exporter protein), which is predominantly expressed in liver, has the capacity to confer resistance to cytotoxic substrates like taxol and vinblastine (Childs et al. 1995, 1998).

With at least 20 human ABC transporters being related to the transport of lipids or lipid-like compounds, it is not surprising that some of these transporters have been linked to the pathogenesis of atherosclerotic vascular diseases (Schumacher and Benndorf 2017). Moreover, ABC transporters have also been associated with vascular endothelial homeostasis and blood pressure regulation, as well as platelet production and aggregation (Schumacher and Benndorf 2017).

Dysfunction of ABCA1 can lead to Tangier's disease (Alam and Locher 2023). ABCA2 dysfunction has been associated with intellectual and developmental deficiency, and also to amyloid homeostasis, thereby pointing to a potential role in Alzheimer's disease (Alam and Locher 2023). Genetic variations in the ABCA3 gene, involved in phospholipid transport from the cytoplasm to the lumen of lamellar bodies, can cause pulmonary surfactant metabolism dysfunction 3, a severe respiratory disorder (Alam and Locher 2023).

2.5 ABCG5/G8 Transporter

ATP-binding cassette subfamily G (ABCG) sterol transporters maintain the homeostasis of endogenous and exogenous sterol. A substantial part of exogenous sterols are undigestible phytosterols, which can lead to complications when accumulated. ABCG5/G8 is the main functioning protein to remove ingested plant sterols providing protection from their toxic effects, although the structural features behind substrate binding in ABCG5/G8 remain relatively poorly resolved.

2.5.1 ABCG5/G8 Transporter Genes

The ABCG subfamily comprises five genes encoding half-transporters. Both of the two genes *abcg5 and abcg8* are located on chromosome 2p21, adjacent to each other in a head-to-head fashion, on opposite strands, and are separated by only 374 base pairs (Berge et al. 2000).

Due to their proximity and opposite orientation, these two genes are regulated by a bidirectional intergenic promotor, which contains binding sites for hepatocyte nuclear factor 4α, GATA 4/6 (Sumi et al. 2007), and a liver receptor homolog 1 (Freeman et al. 2004). In addition, agonists for either liver X receptor or farnesoid X receptor regulate ABCG5/G8 mRNA levels (Repa et al. 2002).

Each ABC transporter (ABCG5/G8) comprises two nucleotide-binding composite sites, where the Walker A motif of one NBD is paired with the ABC signature motif of the other NBD. Therefore, one of the ATP-binding sites presents a degenerate motif, while the other presents a conserved motif, which is the only one able to support ATP hydrolysis.

2.5.2 Characteristics of the ABCG5/G8 Transporter Structure

The members of the G-subfamily are half transporters with only one NBD and one TMD. The ABCG transporters are also characteristic. They are the only human ABC transporters with their inverted domain topology that contain an N-terminal NBD followed by a C-terminal TMD (Alam and Locher 2023).

G subfamily members must form homo- or heterodimers for functionality (Dean et al.

2001a, b). Although two transportes, ABCG1 and ABCG4, have been proposed to also function as heterodimers, most studies support the notion that both are homodimers (Cserepes et al. 2004; Hegyi and Homolya 2016). In contrast, ABCG5 and ABCG8 form obligate heterodimers (Lee et al. 2001; Wang et al. 2006; Brown and Yu 2010).

Each gene ABCG5 and ABCG8 encodes a "half-transporter" protein that is nonfunctional in the monomeric state (Brown and Yu 2010). However, assembly of an ABCG5/G8 heterodimer, driven by the adipocyte-derived hormone leptin, leads to the formation of the fully functional transporter ABCG5/G8 (Brown and Yu 2010).

Structural information of the ABCG5/G8 heterodimer was revealed by X-ray crystallography, establishing a new molecular framework toward a mechanistic understanding of ABC sterol transporters (Graf et al. 2002, 2003). This structure contained an asymmetric unit with two heterodimers that interact through their TMDs with NBDs at opposite sides of the membrane. Without bound nucleotide or lipid substrate inside the transporter, the ABCG5/G8 structure exhibits an inward-facing conformation (Plummer et al. 2021). Three helices from both ABCG5 and ABCG8, form a three-helix bundle, which bridges the TMD and NBD.

In addition, a sterol-binding site was postulated at the membrane-transporter interface based on the crystal structure of ABCG5/G8 (Farhat et al. 2022), solving the crystal structure of ABCG5/G8 in complex with cholesterol. The structure shows that an orthogonal cholesterol molecule fitting horizontally in front of A540, a conserved ABCG5 residue at this orthogonal sterol-binding site.

2.5.3 Observations on ABCG5/G8 Mutations

Mutations present in some sitosterolemia patients impair heterodimer trafficking (Graf et al. 2004), suggesting that these mutations disrupt ABCG5/G8 cellular localization rather than reducing ABCG5/G8 transport activity (Plummer et al. 2021). However, this seems not to be a rule. The Ala540Phe mutant in ABCG5, a residue that putatively binds cholesterol, resulted in reduced biliary cholesterol transport (Lee et al. 2016).

On the triple-helical bundle or the transmembrane polar relay, several residues have been shown to bear disease-causing missense mutations from patients with sitosterolemia or other lipid metabolic disorders. Notably, several disease-causing mutations are clustered in the membrane-spanning region or at the NBD–TMD interface. This suggests the roles of these structural motifs in regulating the ABCG5/G8 function.

ABCG5/G8 has also been studied through overexpression of either wild-type or mutant ABCG5/G8 in *abcg5*/*abcg8* KO mice (Plummer et al. 2021). Mice lacking *abcg5*, *abcg8*, or both show increased plasma levels of sterols and reduction of sterol secretion into the bile. In contrast, overexpression of ABCG5/G8 showed the opposite effect, with reduced sterol absorption and increased biliary sterol levels (Yu et al. 2002a, b). These effects are dependent on ABCG5/G8 expression levels. Moreover, using *mdr2* KO mice, a lack of secretion was observed, suggesting that the function of ABCG5/G8 is dependent on a functional ABCB4 transporter (Plummer et al. 2021).

Homodimers of either ABCG5 or ABCG8 are likely nonfunctional, evident from low ATPase activity relative to the native heterodimer (Wang et al. 2006), impaired trafficking (Graf et al. 2003), and low biliary cholesterol transfer in KO mice (Graf et al. 2003; Zhang et al. 2006). Surprisingly, *abcg8* KO in mice results in continued expression of ABCG5 at the apical membrane and secretion of sitosterol into the bile, suggesting that the ABCG5 homodimer may be functional or that an alternative pathway of sterol secretion into the bile exists (Klett et al. 2004).

2.5.4 Function and Regulation of ABCG5/G8

Physiologically, ABCG5/G8 plays an essential role in controlling sterol homeostasis in our bodies.

The ABCG5/G8 transporter is predominantly expressed on the apical surface of hepatocytes along the canalicular membranes of the bile ducts in the liver and on the brush-border membranes of enterocytes in the small intestines (Patel et al. 1998; Berge et al. 2000; Graf et al. 2002, 2003; Yu et al. 2002a, b; Hirata et al. 2009; Brown and Yu 2010; Zein et al. 2019).

ABCG5/G8 is the primary transporter and sterol-efflux pump that selectively exports excess cholesterol, noncholesterol sterols, and dietary plant sterols from hepatocytes into bile canaliculi and in the intestine back to the intestinal lumen (Lee et al. 2001, Wang et al. 2006; Rezaei et al. 2023), by translocating sterols within the plasma membrane and in endosomes (Sano et al. 2014; Pandzic et al. 2017; Xavier et al. 2020). Expression level of ABCG5 and ABCG8 is further modulated by bile acid levels in both the liver (Dean et al. 2001a, b) and intestine (Kamisako et al. 2007).

One proposed mechanistic mode for the sterol transfer to bile acid suggests that ABCG5/G8 translocates sterol across the bilayer membrane, functioning as a liftase (Small 2003; Lee et al. 2016). However, there is little experimental evidence to support this hypothesis.

Another model suggests that ABCG5/G8 functions only as a sterol floppase, increasing the sterol concentration in the outer leaflet and allowing for extraction of sterol by the bile salt micelle (Kosters et al. 2006). This hypothesis is supported by the observation that the function of ABCG5/G8 depends on ABCB4, a phosphatidylcholine transporter, and sterol is potentially extracted from the outer leaflet via sterol-phospholipid vesicles (Crawford et al. 1997). Multidrug-resistant protein 3 (MDR3), also known as ABCB4, is a phospholipid translocase embedded in the canalicular membrane. Although it actively flips inner leaflet phospholipids and sterols to the outer leaflet, there is evidence supporting its subsequent role in substrate secretion toward the bile. Furthermore, MDR3 is found to be essential for the proper function of ABCG5/G8, which believed to be caused by its involvement in the formation of mixed micelles.

Finally, ABCG5/G8 may directly transfer sterols to bile salt micelles following sterol flipping across the plasma membrane (Plummer et al. 2021). Spontaneous cholesterol flipping between leaflets is a common event in the plasma membrane, capable of undertaking intermediate horizontal orientation within the membrane core. It is thus possible that ABCG5/G8 catalyzes cholesterol flipping from inner to outer leaflets, peripherally through its exterior surface.

In addition, ABCG5/G8 exhibits cholesterol efflux activity in the presence of bile acid micelles (Vrins et al. 2007). Mixed micelles are very charged, small aggregates of phospholipids, cholesterol and bile salts, and these micelles form the basis of currently known ABCG5/G8 acceptor particles. Accordingly, it has been suggested that mixed micelles of bile acids dock onto ABCG5/G8 to induce conformational changes and stimulate ATPase for the transport of cholesterol (Johnson et al. 2010). Acceptor particles will then intake the exported lipids from ABCG5/G8.

Differently from homodimeric ABCG1, the heterodimeric ABCG5/G8 carries out selective sterol excretion. ABCG5/G8 is unique in its capability of preferential efflux for dietary plant sterols over cholesterol, preventing the abnormal accumulation of plant sterols in human body (Berge et al. 2000; Lee et al. 2001; Graf et al. 2003; Yang et al. 2004; Schumacher and Benndorf 2017). Intestinal ABCG5/G8 acts as a first-pass gate, pumping xenosterols back into the intestinal lumen, whereas liver ABCG5/G8 pumps xenosterols into the bile (Patel et al. 2018). There are numerous reports that ABCG5/G8 exhibits a preference for the transport of noncholesterol sterols over cholesterol (Yu et al. 2004; Plummer et al. 2021). The preference for noncholesterol sterols in either intestine- or liver-localized *abcg5/abcg8* KO mice was slightly reduced compared to whole-animal *abcg5/abcg8* KO models, suggesting that expression of ABCG5/G8 in both liver and intestine is required for full selectivity (Wang et al. 2015). The mechanisms that govern such substrate selectivity within this protein subfamily, however, remain elusive.

2.6 Sitosterolemia

This very rare autosomal recessive disorder is characterized by drastically elevated plasma and tissue levels of plant sterols (Schumacher and Benndorf 2017). As stated before, missense mutations in both genes, ABCG5 and ABCG8, are related to sitosterolemia (Berge et al. 2000; Hubacek et al. 2001; Lu et al. 2001). ABCG5/G8 exhibits substrate specificity, particularly for plant sterols (e.g., sitosterol and campesterol) over cholesterol (Matsumura et al. 2007; Michaki et al. 2012). In fact, sitosterolemia patients exhibit increased absorption of numerous plant xenosterols, not just sitosterol (Morita and Imanaka 2012).

The clinical presentation may include elevated low-density lipoprotein (LDL) cholesterol and tuberous tendon xanthomas. Hypercholesterolemia-induced premature atherosclerosis was particularly observed to affect male patients at a young age, leading to CVD-like angina pectoris, myocardial infarctions, and sudden cardiac death (Kwiterovich et al. 1981; Brown and Yu 2010). Hematologic manifestations (hemolytic anemia, macrothrombocytopenia, splenomegaly, and bleeding disorders) can result from the accumulation of plant sterols in platelet membranes, producing hypertrophic and hyperplasic dysfunctional platelets. Adrenal dysfunction, arthritis, elevated liver function tests, and cirrhosis (in rare cases hepatic failure) are other features (Shulman et al. 1976; Miettinen 1980; Kwiterovich et al. 1981; Lin et al. 1983; Salen et al. 1985; Beaty et al. 1986; Nguyen et al. 1990; Bhattacharyya et al. 1991; Rees et al. 2005; Mushtaq et al. 2007; Wang et al. 2014; Bazerbachi et al. 2016). Clinical studies in individuals with sitosterolemia revealed reductions in cholesterol synthesis, biliary cholesterol secretion, plasma clearance, and fecal elimination of neutral sterols (Salen et al. 1989; Nguyen et al. 1990; Bhattacharyya et al. 1991; Cobb et al. 1997). Due to similar clinical presentations, sitosterolemia has been, in many cases, inaccurately diagnosed as familial hypercholesterolemia or idiopathic liver cirrhosis.

The ABCG5/G8 loss of function in animal models, as well as in humans, shows that accumulation of xenosterols leads to dramatic phenotypes, such as macrothrombocytopenia and platelet dysfunction, liver disease, appearance of gallstones, elevation of low-density lipoprotein cholesterol levels and cholesterol accumulation with xanthoma formation and atherosclerosis (Grass et al. 1995; Yu et al. 2002a, b, 2005; Kajinami et al. 2004; Acalovschi et al. 2006; Buch et al. 2007; Wang et al. 2007; Chen et al. 2008; Kuo et al. 2008; Katsika et al. 2010; Patel and Salen 2010; Srivastava et al. 2010; Renner et al. 2013; Von Kampen et al. 2013; Jiang et al. 2014). In mouse models (but not humans), infertility, immune dysfunction, and cardiomyopathy have been reported (McDaniel et al. 2013; Solca et al. 2013; Wilson et al. 2013). This begs the question whether a lifetime of low-level exposure to dietary bioactive xenosterols, whose levels of entry and retention may be altered by polymorphisms in ABCG5 and ABCG8, may have biological consequences.

Despite the absorptive phenotype and metabolism of phytosterols to bile acids, the clinical management of these patients with low sterol diets and bile acid-binding resins resulted in modest and inconsistent reductions in plasma phytosterols (Lin et al. 1983; Nguyen et al. 1991). Treatment of sitosterolemia involves a diet that is low in plant lipids and the administration of the drug ezetimibe, which acts as an inhibitor of Niemann-Pick C1-like protein 1 (NPC1L1), that functions in intestinal sterol absorption Morita et al. 2007).

In conclusion, although sitosterolemia is a rare genetic disease, this disorder should be considered in the differential diagnosis, due to its early clinical manifestations and relatively difficult treatment.

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Part II

Bio- and Chemical- Synthesis, and Analytical Methods

Chemical and Biochemical Features of Spinasterol and Schottenol

3

Farid Khallouki, Mohamed Ksila, Imen Ghzaiel, Soukaina Essadek, Mounia Tahri Joutey, Samah Maaloul, Wafa Zennouhi, Laila Benbacer, Mohamed Bourhia, Lhoussain Hajji, Amira Zarrouk, Leila Rezig, Sandrine Rup-Jacques, Raoudha Abdellaoui, Taoufik Ghrairi, Olfa Masmoudi-Kouki, Boubker Nasser, Pierre Andreoletti, Mustapha-Cherkaoui-Malki, Mohammad Samadi, Anne Vejux, and Gérard Lizard

F. Khallouki
Biology Department, Faculté des Sciences et Techniques, Moulay Ismail University of Meknes, Errachidia, Morocco

Biology Department, Faculty of Sciences, Moulay Ismail University of Meknes, Meknes, Morocco

M. Ksila
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Neurophysiology, Cellular Physiopathology and Valorisation of Biomolecules, (LR18ES03), Department of Biology, Faculty of Sciences, University Tunis El Manar, Tunis, Tunisia

I. Ghzaiel
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Faculty of Medicine, Laboratory 'Nutrition, Functional Food and Vascular Health' (LR12ES05), University of Monastir, Monastir, Tunisia

S. Essadek · M. T. Joutey
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Biochimistry, Neuroscience, Natural Resources and Environment, Faculty of Science and Technology, University Hassan I, Settat, Morocco

S. Maaloul
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

W. Zennouhi
Biology Department, Faculté des Sciences et Techniques, Moulay Ismail University of Meknes, Errachidia, Morocco

L. Benbacer
Unité de Biologie et Recherches Moléculaires, Département Sciences du Vivant, Centre National de l'Energie, des Sciences et Techniques Nucléaires (CNESTEN), Rabat, Morocco

M. Bourhia
Faculty of Medicine and Pharmacy, Ibn Zohr University, Laayoune, Morocco

L. Hajji
Biology Department, Faculty of Sciences, Moulay Ismail University of Meknes, Meknes, Morocco

A. Zarrouk
Faculty of Medicine, Laboratory 'Nutrition, Functional Food and Vascular Health' (LR12ES05), University of Monastir, Monastir, Tunisia

L. Rezig
LIP-MB 'Laboratory of Protein Engineering and Bioactive Molecules', National Institute of Applied Sciences and Technology, LR11ES26, University of Carthage, Tunis, Tunisia

High Institute of Food Industries, University of Carthage, Tunis, Tunisia

S. Rup-Jacques · M. Samadi
Laboratory of Chemistry and Physics Multi-Scale Approach to Complex Environments, Department of Chemistry, University Lorraine, Metz, France

R. Abdellaoui
Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

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Abstract

Phytosterols, which are produced in plants, are structurally similar to cholesterol. Their basic structures consist of a cyclo pentano-perhydrophenanthrene nucleus composed of 3 hexane rings and of a pentane ring with an alkyl side chain. There are around more than 250 phytosterols and related compounds that have been identified in natural resources. Among them, spinasterol and schottenol, its dihydro analog, are often found in seeds, and consequently in seed oils, and in other botanical parts of some plant families such as Sapotaceae, Cactaceae, and Cucurbitaceae. Spinasterol and/or schottenol has been identified in dietary and cosmetic argan oil, milk thistle seed oil, nigella seed oil, and pumkin seed oil. These phytosterols that have several bioactive properties make them potentially attractive molecules in pharmacology. Their chemical and biochemical features are summarized and the analytical methods used to characterize and analyze these compounds are presented.

Keywords

Phytosterols · Δ7-sterols · Spinasterol · Schottenol · Seed oils

T. Ghrairi · O. Masmoudi-Kouki
Laboratory of Neurophysiology, Cellular Physiopathology and Valorisation of Biomolecules, (LR18ES03), Department of Biology, Faculty of Sciences, University Tunis El Manar, Tunis, Tunisia

B. Nasser
Laboratory of Biochimistry, Neuroscience, Natural Resources and Environment, Faculty of Science and Technology, University Hassan I, Settat, Morocco

P. Andreoletti · Mustapha-Cherkaoui-Malki · A. Vejux · G. Lizard (✉)
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France
e-mail: gerard.lizard@u-bourgogne.fr

Abbreviations

GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
HPLC-UV-DAD	HPLC-ultra violet-diode array detector.
HPLC-UV-VWD	HPLC-ultra violet-variable wavelength detector
NMR	Nuclear magnetic resonance
RP-HPLC	Reverse-phase HPLC
TMS	Trimethylsilyl

3.1 Introduction

Phytosterols are a class of lipids structurally similar to cholesterol, from which they differ in the substituents on the side chain. Their trivial names are derived from their plant origins. They are formed from a basic perhydro-cyclopenta[a] phenanthrene backbone composed of three hexagonal rings in the phenanthrenic position (A, B, C) and a pentagonal ring (D). They also bear one or two double bonds on the B ring. Two angular methyl group located on carbon 10 and 13, respectively, as well as a branched side chain grafted on the ring D at C-17 and a hydroxyl group on C-3 of ring A and a methyl or ethyl group at the position C-24, the basic numbering of the sterol backbone (**I**) is depicted in Fig. 3.1. In the fully saturated perhydro-cyclopenta[a]phenanthrene (known as Gonane skeleton), rings B/C are trans-linked, as are rings C/D. Substituents above the plane of the ring are designated as β, and below as α. The β positions of the H atoms at C-8, C-9, C-10, C-13, and C-14 are already included in the trivial name Gonane. The methyl groups, the group hydroxyl, and the side chain are above the plane of the sterane backbone, in the cis position, and are qualified as in beta configuration. Such configurations have nothing to do with the plane subspaces above and below the ring plane of the sterol backbone, rather, this notation is based on the Fischer projection of the side chain. Such

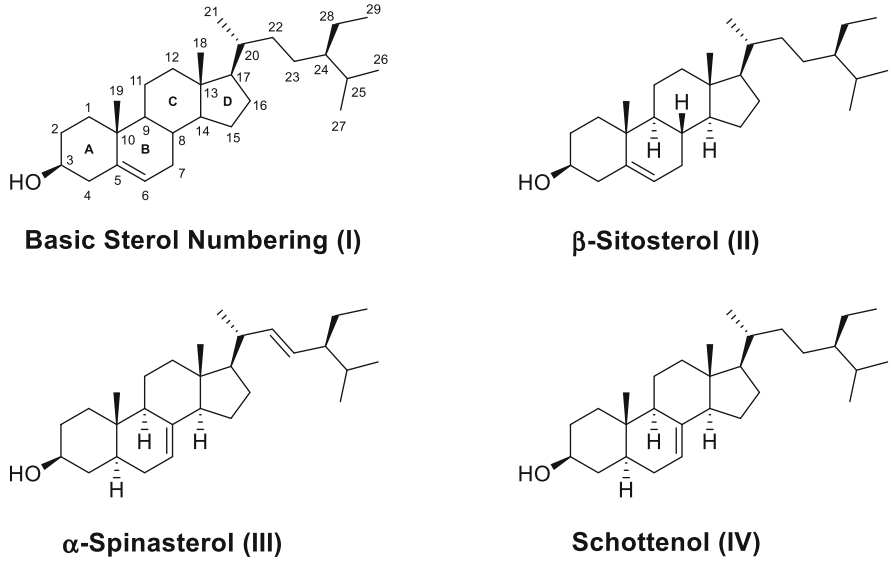

Fig. 3.1 Basic sterol numbering and structures of β-sitosterol, α-spinasterol, and schottenol. PubChem-related information; β-sitosterol: compound CID 222284; α-spinasterol: compound CID 91751675; schottenol: compound CID 441837

systems bear some structural variations of position and number of double bonds, C-24 substitution ethylation broadly occurs as 24α-epimer and is more common than the methyl substitution, which more frequently arise as both 24α- and 24β-epimers (Garg and Nes 1984). Phytosterols are highly liposolubles and are amphiphilics due to the presence of hydroxy group. Free phytosterols and phytosteryl esters are soluble in non-polar solvents such as hexane, while polar modifiers are needed to solubilize their steryl glycoside forms (Hartmann and Benveniste 1987). To date, about 250 different phytosterols were described (Nes 2011), which in the plant kingdom are mainly found in the form of conjugates, within the 3-OH group is either covalently linked with fatty acids or cinnamoyl moiety or glycosylated with hexoses. Very common, esterification occurs also at the C6 hydroxyl group of the carbohydrate moiety with a fatty acid of the steryl glycosides which are termed acyl sterol glucoside (Moreau et al. 2002, 2018). Glycosylated sterols and acylated steryl glycosides are ubiquitous in plants and occur in some microorganisms, fungi, algae, and certain animal tissues (Grille et al. 2010).

The most predominant form of phytosterols corresponds to β-sitosterol (Stigmasta-5-en-3-β-ol or 24β-ethylcholest-5-en-3β-ol, **II**, Fig. 3.1), it differs only from cholesterol by the presence of an ethyl group at the level of carbon 24 of the side chain (Moreau et al. 2002; Nes 2011). Depending on the double-bond position in the B ring, phytosterols are divided into three main groups: a large number of sterols are Δ5 sterols as exemplified by β-sitosterol; there are also Δ7-sterols with a double bond between C7 and C8 which are less commonly present in nature compared to Δ5-sterols, other types of steroids could be rearranged and get two double bonds in the B-ring as well as stanols with fully saturated rings of the sterane moiety. Other structures include monomethyl and dimethyl sterols, those are metabolic intermediates of 4-desmethylsterols and are generally minor components in most plant sources.

Phytosterols are present in very low concentrations in the unsaponifiable fraction of vegetable oils, fats, cereals, vegetables, fruits, and berries. In addition to herbaceous plants, microalgae (Francavilla et al. 2012) and seed oils (argan oil, milk thistle seed oil, nigella seed oil, olive oil, *Pistacia lentiscus* seed oil) (Zarrouk

et al. 2019; Meddeb et al. 2018; Ghzaiel et al. 2021; Rezig et al. 2022), the waste of industrial processing of wood are also important sources of phytosterols (Ceylan et al. 2022). In food sector, phytosterols can be added in margarine, mayonnaise, milk, meat, cheeses, and juices (Choudhary and Tran 2011) and could contribute to reduce the cholesterol level (Chen et al. 2019). There are lot of positive arguments in favor of the health benefits of phytosterols in human health especially for the prevention of cardiovascular diseases since they can act on the intestinal absorption of cholesterol and on the immune system (Plat et al. 2015; Makhmudova et al. 2021). However, the risk of phytosterolemia/sitosterolemia, which can be associated with an elevated consumption of phytosterols, must not be ignored especially mainly with sitosterol (Lizard 2008; Ajagbe et al. 2015). As phytosterols are more and more present in human diet, their incidence on human health and diseases needs to be better understand (Plat et al. 2019). Phytosterols are also useful as raw materials for the synthesis of hormones and related pharmaceuticals and cosmetics and as additives to thermoplastic resins used in the manufacture of rubber materials (Levasseur et al. 2020; Farag et al. 2022).

3.2 Δ7 Sterols Occurrence

This chapter will focus on the Δ7-sterols, especially spinasterol and schottenol, Δ7-sterols are sterol lipids with a structure based on the stigmastane skeleton, which consists of a cholestane moiety bearing an ethyl or methyl group at the carbon atom C24. An interesting fact is while most plants are rich in Δ5-sterols, Δ7-sterols appear to be restricted only in a some specific plants families including Cucurbitaceae and Amaranthaceae (Breinhölder et al. 2002; Moreau et al. 2002, 2018; Schlag et al. 2022), Sapotaceae (Khallouki et al. 2003), and Cactaceae (de Araújo et al. 2021). Δ7-sterols occur in traces in other families such as Asteraceae, Plumbaginaceae, Tamaricaceae, and Theaceae (Fernández-Cuesta et al. 2014; Lam et al. 2021; Rozentsvet et al. 2022), as well as in Saururaceae (Yang et al. 2022). Some other species such as Chenopodiaceae were also able to accumulate Δ^7-Sterols (Rozentsvet et al. 2022), other examples list them in rice bran (Poaceae) (Määttä et al. 1999), in ginseng (*Panax quinquefolium* L., Araliaceae) (Beveridge et al. 2002), pine (Pinaceae) (Kovacheva et al. 1990), and in *Sesamum indicum* (Pedaliaceae) (Mohamed and Awatif 1998).

α-Spinasterol or (24E)-5α-Stigmasta-7,22-dien-3β-ol (**III**, Fig. 3.1) is found in various plant sources such as spinach, from which it takes its name. It is specifically found, as the major sterol of only a few plant families such as Caryophylaceae, Sapotaceae, Cucurbitaceae, Cactaceae, Fabaceae, and Amaranthaceae (Khallouki et al. 2003; Badreddine et al. 2015; Montesano et al. 2018; Aljohani 2022).

α-Spinasterol was also detected at low concentration in some fruits and higher plants including berry seeds in *Baccharis illinita* (Asteraceae) (Freitas et al. 2009), in *Kalimeris integrifolia* (Asteraceae) (Wang et al. 2018), in *Jatropha mollissima* (Pohl) Baill (Euphorbiaceae) (da Silva Araújo et al. 2021), in *Callicarpa macrophylla* Vahl (Verbenaceae) (Lam et al. 2021), as well as in *Herba Houttuyniae* (Saururaceae) (Yang et al. 2022).

Both α-spinasterol and its dihydro analog schottenol (5α-Stigmast-7-en-3β-ol, 22-dihydrospinasterol, **IV**, Fig. 3.1) were the main sterols identified in the wood and bark extracts of Acacia species (Fabaceae) (Thotathil et al. 2022), *Baccharis coridifolia DC.* (Asteraceae) (Arisawa et al. 1985), in A*rgania spinosa* (*Sapotaceae*) (Khallouki et al. 2003), and in Cactus pear seed oils (El Kharrassi et al. 2014).

3.3 Δ7 Sterols Chemistry

Efficient extraction of phytosterols from the lipid fraction of the plant matrix is the most important step in the sample preparation procedure. When comparing extraction methods, attention should be paid to the various conjugates in which phytosterols may exist, either in free forms or as

sterides, glycosides or acylated glycosides (Schlag et al. 2022). Nonpolar lipid solvents, medium polar and polar solvents, and their mixtures are used to release steroid derivatives from materials. Because most phytosterols in oil seeds exist as free alcohols or fatty acid esters, they are readily isolated in non-polar solvents, while more polar solvents are used for other plant materials containing glycosylated phytosterols. Subsequent separations of the substance mixture were made by liquid-liquid extraction, thin-layer chromatography, as well as by column chromatography.

Soxhlet extraction is also among the conventional extraction methods. However, the demand for new green techniques is increasing and the technics are extensively reviewed with the emphasis on the extraction of phytosterols from plant origins (Jun 2013) (Uddin et al. 2015). One approach to improve the extractability of phytosterols from complex plant matrices is to hydrolyze the matrix with alkali or acids. Hydrolytic procedures can, however, have a negative effect on Δ7-conjugated phytosterols. Exposure to heat, light, and air have to be avoided. Δ7-sterols with also an ethylidene side chain are particularly prone to acid-catalyzed isomerization (Münger and Nyström 2014) and produce unknown artifactual compounds, hence, acid hydrolysis should be skipped in the analysis of these materials and replaced by enzymatic hydrolysis which is more convenient to help examine Δ7-sterols in their free forms (Breinhölder et al. 2002; Nyström et al. 2012).

In the presence of heat, light, metal contaminants, and oxygen, preformed radicals easily attack the double bond in Δ7-sterols, beginning an autocatalytic free radical chain reaction following the same chemistry as the oxidation of unsaturated lipids both in ring backbone or at the side chain level (Dragoun et al. 2022). Noteworthy, whereas cholesterol oxide derivatives have cytotoxic effects on human monocytic cells, no cytotoxicity was observed with oxyphytosterols (Vejux et al. 2012). Phytosterols extraction can be improved with an ultrasonic bath (de Figueiredo et al. 2018), pulsed electric field-assisted extraction, microwave-assisted extraction (Xiao et al. 2013), supercritical fluid extraction (Roiaini et al. 2016), or pressurized liquid extraction (Moreau et al. 2003). Other alternatives to reduce the use of organic solvents in sample preparation is solid-phase micro-extraction (Balme and Gülaçar 2012) as well as to use supercritical CO_2 procedure (Winkler et al. 2007). Such techniques remarkably increase the efficiency of the extraction process (Azmir et al. 2013).

3.4 Δ7-Sterols Characterization

Identified Δ7-sterols have the basic structure of stigmastane (24α-ethyl) or poriferastan (24β-ethyl). Stigmastane derivatives are the most detected; although according to Breinhölder et al. (2002) and Rodriguez et al. (1996) investigations, the 7,25- and 7,22,25-unsaturated compounds are in the poriferastan configuration. Rodriguez et al. (1996) also mentions 24-methylcholest-7-en-3β-ol, with no statement being made about the stereochemistry at C-24.

Analytics of determination and especially identification of Δ7-sterols is very complex precisely because there are few commercial standards of this group of sterols compared to Δ5-sterols. Moreover, they are easily oxidable with usually artifacts formation hence preventing the determination of correct phytosterols profiles (Kamal-Eldin et al. 1998; Phillips et al. 2005). The use of preparative chromatography methods as well as cleanup is recommended prior to the analytical methods especially for mass spectrometry detections both in high and low resolution for determination of phytosterols in samples. Nonderivatized phytosterols evaporate poorly and are relatively unstable. Therefore, prior to gas chromatography coupled with mass spectrometry (GC-MS), Phytosterols were analyzed as their silyl derivatives with or without further sample cleanup, respectively, in refined and crude oils. To improve evaporation, stability, and GC resolution, phytosterols are usually converted to their trimethylsilyl (TMS) ethers. TMS derivatives are more suitable for quantitative analysis, and they are easier and faster to obtain

than other esterifications. N,O-Bis (trimethylsilyl)trifluoroacedamide with trimethylchlorosilane or tert-butyl methyl ether is the most used derivatization techniques (Khallouki et al. 2003; Junker et al. 2019). Δ7-sterols tend also to have longer retention times than Δ5-sterols (Oliveira et al. 2005; Phillips et al. 2005). The mass spectrometric characteristics of Δ7-sterols are well documented. In GC–MS, the electronic impact mass spectra of phytosterol derivatives often exhibit a significant $[M-15]^+$ ion, which is very useful for structural elucidations (Harvey and Vouros 2020). Moreover, in mass detection, the Δ5 and Δ7 sterol analogs were easily differentiated, indeed, the ion $[M-129]^+$ is the characteristic base peak of the TMS derivatives of Δ5-sterols, whereas it is absent from the mass spectra of the Δ7 sterols; Δ7 sterols present also a strong molecular ion and lost their side chain much more easily, inducing an intense peak at m/z 255 (Freire et al. 2005), which is a fragment of a four-ring structure; stanols on the other hand showed a base peak at m/z 215, formed after the removal of the side chain and part of the sterane ring.

Furthermore, although high-performance liquid chromatography (HPLC) technologies are less commonly used for the separation of free phytosterols than GC with specific separation and detection capabilities, attempts have been made to apply such technique for the analysis of free phytosterols (Abidi 2001). In analytical HPLC, reverse phase (RP)-HPLC is more selective for separating homologues of free sterols and unsaturated analogs than normal (N)-HPLC. Nevertheless, the analysis with HPLC–MS as well as HPLC–MS/MS enable much more detailed statements about the analytes than common methods such as HPLC-ultra violet (UV)-variable wavelength detector (VWD) or HPLC-UV-diode array detector (DAD). Both hyphenated techniques represent an extremely sensitive process with highest selectivity and are therefore ideally suited for use in quality control (Esche et al. 2013; de Figueiredo et al. 2018). Other reports on the separation of Δ7-phytosterols were described (Zhang et al. 2006; Münger et al. 2018). In HPLC, Δ7-sterols elute earlier than their Δ5 analogs in Polaris C8-A column (250 mm × 10 mm internal diameter stainless steel) (Zhang et al. 2006).

3.5 Spinasterol and Schottenol (Also Named Dihydrospinasterol) Characterization

α-Spinasterol ($C_{29}H_{48}O$) is a doubly unsaturated sterol with other names such as (3β,5α,22E)-Stigmasta-7,22-dien-3-ol, (E)-5α-Stigmasta-7,22-dien-3β-ol, 5α-Stigmasta-7,22-dien-3β-ol, Bessisterol or as hitodesterol. α-Spinasterol was identified mainly by MS detection by having recourse to either its literature mass data comparison or with special software databases; its structural information may also be ascertained using manual characteristic fragment ions. In GC–MS, the TMS ether derivatives of spinasterol among other Δ7-phytosterols were characterized by flamme ionization and mass detectors (Garg and Nes 1984; Kamal-Eldin et al. 1998).

α-Spinasterol has been earlier synthesized by hydrogenation of 7-dehydrostigmasteryl benzoate over platine (Pt) in ethyl acetate or by hydrogenation of 7-dehydrostigmasteryl acetate with tris(tripheny1phosphine) chlororhodium in benzene-ethanol over Raney/Ni in dioxane. Schottenol synthesis was obtained via α-spinasterol hydrogenation over Pt in ether first by Barton and Cox (1948). More recent study described its synthesis by hydrogenation over Pt in ether or Raney/Nikel (Badreddine et al. 2015).

In GC–MS and due to their rigid structures, both phytosterols schottenol and α-spinasterol generally showed a distinct molecular peak $[M]^{+\cdot}$. For both, loss of the 3-hydroxy groups as water produce the common fragment at $[M-18]^{+\cdot}$. The ion with the mass $[M-15]^+$ is also a good signal due to splitting off a methyl group (usually the C-19). If the side chain of each phytosterol is dissociated, the fragment $[M-SC]^+$ with m/z = 273 is obtained. Other signal include $[M-SC-2H]^+$ (m/z = 271), $[M-SC-H_2O]^+$ at m/z = 255, $[M-SC-42]^+$ (m/z 231) as well as $[M-SC-60]^+$ (m/z 213). Since the study of Brooks

et al. (1980), m/z = 213 and 255 are considered as characteristic fragments of Δ7-sterols.

As regard of schottenol ($C_{29}H_{50}O$, IV, Fig. 3.1), named also (24R)-ethyl-5α- cholesta-7-en-3β-ol or 5α-Stigmast-7-en-3β-ol 7-Stigmastenol with Monoisotopic Molecular Weight 414.386. In its mass spectrum, the molecular ion at m/z = 414 is the base pic (100%). Its fully saturated side chain is more stable than its unsaturated analogue α-spinasterol; therefore in GC–MS, the molecule remains intact during electron impact ionization more often than α-spinasterol. All the fragments with their respective intensities of both phytosterols are summarized in the Table 3.1 which also includes some other physico-chemical properties such as melting points, infrared wavenumbers, and Kovats index in capillary GC columns. α-Spinasterol and schottenol have been identified on the basis of nuclear magnetic resonance (NMR) (Kojima et al. 1990; Badreddine et al. 2015; Strobl 2004) and the whole related spins are summarized in the respective ^{1}H NMR (Table 3.2) and ^{13}C NMR (Table 3.3). Assignments were made on the basis of mono- and bi-dimentional NMR including COSY, HMQC, and HMBC correlations. The signals for C-11 and C-21 were confirmed by HMQC, HMBC, and by other 1D pulse sequence such as DEPT135 experiment.

3.6 Conclusion

Spinasterol and schottenol can be considered as rare phytosterols since they are present in few plants. Important progresses have been made in their chromatographic separations and detection analysis resulting in complete assignments and clearly resolutions. New procedures for their encapsulation could improve their bioavailability, their chemical stability, and their oral administration. At the moment, it is considered that spinasterol and schottenol may exhibit valuable pharmacological capacities in human health.

Table 3.1 Spectroscopic data and physical properties of spinasterol and schottenol

	Alpha-spinasterol (PubChem 91751675)	Schottenol (PubChem 441837)	References
Molecular formula	$C_{29}H_{48}O$	$C_{29}H_{50}O$	
Melting points (°C)	171–173	150–152	Badreddine et al. (2015)
HRMS (found)	412.3716	414.386166214	Badreddine et al. (2015)
IR (neat) (in cm^{-1})	3424, 3314, 2960, 2937, 2868, 1447, 1039, 969	3538, 2930, 2910, 2863, 2850, 1446, 1038	Badreddine et al. (2015)
EI-MS: m/z fragments (relative intensities)	**412** (18; $M^{+\cdot}$); **397** (8; M^{+}-CH_3); **394** (1; $M^{+\cdot}$-H_2O); **379** (2; M^{+}-CH_3-H_2O);**369** (8; M^{+}-C_3H_7); **351** (4); **300** (13); **273** (23; M^{+}-SC); **271** (100; M^{+}-SC-2H); **255** (32; M^{+}-SC-H2O); **253** (6; M^{+}-SC-H_2O-2H); **246** (15); **231** (10; M^{+}-SC-C_3H_6); **229** (14; M^{+}-SC-C_3H_8); **213** (13; M^{+}-SC-C_3H_6-H_2O); **211** (2; M^{+}-SC-C_3H_8-H_2O)	**414** (100; M^{+}); **399** (34; M^{+}-CH_3); **396** (2; M^{+} -H_2O); **381** (4; M^{+}-CH_3-H_2O); **273** (15; M^{+}-SC); **255** (37; M^{+}-SC-H_2O); **231** (14; M^{+}-SC-C_3H_6); **229** (9; M^{+}-SC-C_3H_8); **213** (13; M^{+}-SC-C_3H_6 -H_2O)	Strobl (2004)
Kovats index (GC columns)	**3245** (OV-1) **3325** (standard nonpolar)	**3344** (in SE-30) **3500** (in OV-17)	Brooks (1980)

HRMS: high-resolution mass spectrometry; IR: infrared; EI-MS: electron ionization mass spectrometry

Table 3.2 ^{1}H NMR (400 MHz) spectra of most characteristic spins of spinasterol and schottenol

	Spinasterol (Strobl 2004)				Schottenol (Badreddine et al. 2015)			
H-Atom	δ [ppm]	Multiplicity	J [Hz]	Integration	δ [ppm]	Multiplicity	J [Hz]	Integration
18	0.54	s		3	0.55	s		3
19	0.79	s		3	0.81	s		3
27	0.79	d	6.4	3	0.83	d	6.8	3
29	0.80	t	7.3	3	0.86	t	7.6	3
26	0.84	d	6.5	3	0.85	d	7.2	3
21	1.02	d	6.6	3	0.94	d	6.4	3
3	3.58	m		1	3.61	m		1
23	5.02	dd	15.2; 8.7	1				
7	5.15	m		1	5.18	dd	4.36; 2.33	1
22	5.15	dd	15.0; 8.7	1				

s: singlet; d: doublet; t: triplet; m: multiplet; dd: doublet-doublet

Table 3.3 ^{13}C NMR (in $CDCl_3$ at 125 MHz) of total characteristic spins of schottenol and spinasterol

	Schottenol		Spinasterol		
C-atom	^{13}C NMR δ (ppm)		^{13}C NMR δ (ppm)		
	Badreddine et al. (2015)	Kojima et al. (1990)	Kojima et al. (1990)	Badreddine et al. (2015)	Strobl (2004)
18	11.85	11.8	12.0	12.04	12.07
29	11.98	11.9	12.3	12.24	12.25
19	13.04	13.0	13.0	13.04	13.05
27	19.05	19.0	19.0	19.00	18.95
26	19.83	19.8	21.1	21.09	21.10
11	21.56	21.5	21.5	21.38	21.57
21	18.91	18.9	21.4	21.55	21.57
15	23.08	23.0	23.0	23.02	23.03
28	22.98	22.9	25.4	25.40	25.41
16	27.97	27.9	28.5	28.51	28.40
6	29.66	29.6	29.6	29.65	29.66
2	31.49	31.4	31.4	31.47	31.51
25	29.17	29.1	31.9	31.88	31.84
10	34.21	34.2	34.2	34.22	34.25
1	37.15	37.1	37.1	37.15	37.17
4	37.99	37.9	38.0	37.99	38.03
12	39.57	39.5	39.4	39.47	39.49
5	40.27	40.2	40.2	40.27	40.30
20	36.59	36.6	40.8	40.83	40.76
13	43.39	43.4	43.3	43.29	43.31
9	49.46	49.4	49.4	49.46	49.48
24	45.85	45.8	51.2	51.25	51.27
14	55.05	55.0	55.1	55.13	55.15
17	56.10	56.0	55.8	55.91	55.94
3	71.06	71.0	71.0	71.06	71.07
7	117.43	117.4	117.4	117.46	117.47
23	26.21	26.1	129.4	129.45	129.47
22	33.91	33.8	138.7	138.17	138.12
8	139.62	139.6	139.5	139.56	139.57

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LC–MS Approaches for Oxysterols in Various Biosamples

4

Kristina S. Kømurcu, Steven R. Wilson, and Hanne Røberg-Larsen

Abstract

Oxysterols are involved in a plethora of biological processes, including a wide variety of diseases. Therefore, monitoring oxysterols is important for obtaining a deeper understanding of their biological roles and utilizing them as, for example, biomarkers. However, oxysterols can be challenging compounds to study, as they can be very similar in chemical structure but still have distinct biological roles. In addition, oxysterols may be difficult to detect, even with advanced analytical instrumentation. We here focus on the use of liquid chromatography–mass spectrometry (LC–MS) for the analysis of oxysterols, with an additional focus on the steps needed to prepare oxysterols for LC–MS. Steps can include chemical modification of the oxysterols for improving LC–MS sensitivity and adding chemicals that can reveal if the oxysterol levels have been perturbed during preparation. We then round off with descriptions and applications of various sample preparations for different biological matrices, from blood to cells, and biosamples with emerging attention, for example, exosomes and organoids. Taken together, oxysterol analysis is highly compatible with a wide variety of biosamples, allowing for a deeper understanding of these challenging analytes.

Keywords

Oxysterols · Biosamples · Liquid chromatography–mass spectrometry · Derivatization · Sample preparation · Exosome · Organoids

Abbreviations

APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BHT	Butylated hydroxytoluene
CZE	Capillary zone electrophoresis
DESI	Desorption electrospray ionization
EADSA	Enzyme-assisted derivatization for sterol analysis
ER−	Estrogen receptor negative
μLESA	Microscale liquid extraction for surface analysis
ER+	Estrogen receptor positive
ESI	Electrospray ionization
GC	Gas chromatography
GPCR	G protein-coupled receptors
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
LXR	Liver X receptor

K. S. Kømurcu · S. R. Wilson · H. Røberg-Larsen (✉)
Department of Chemistry, University of Oslo, Oslo, Norway
e-mail: hannero@kjemi.uio.no

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m/z	mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSI	Mass spectrometry imaging
NAFLD	Nonalcoholic fatty liver disease
RPLC	Reversed phase liquid chromatography
SFC	Supercritical fluid chromatography
SMO	Smoothened
TMS	Trimethylsilyl
UHPLC	Ultra-high pressure liquid chromatography

4.1 Introduction

Oxysterols are metabolites of cholesterol that are formed enzymatically (e.g., by CYP450 enzymes) or by autoxidation. A variety of oxysterols exist (for some examples of oxysterols and their pathway, see Fig. 4.1) with various biological activities, for example, as ligands of nuclear receptors such as the liver X receptor (LXR) and estrogen receptor (ER), G protein-coupled receptors, and smoothened (Griffiths and Wang 2019). Oxysterols are involved in a plethora of diseases, such as cancer, Epstein–Barr, nonalcoholic fatty liver disease (NAFLD), Alzheimer's disease, and others. Therefore, the identification and quantification of the different oxysterol isomers are important for understanding the biological mechanisms behind the diseases and for biomarkers/diagnostics. The oxysterols can be present in biological samples as free sterols (nonesterified) or conjugated to, for example, a fatty acid (esterified).

The analysis of oxysterols can be challenging. For example, many oxysterols with distinct biological roles are structural isomers (e.g., different hydroxycholesterols or dihydroxycholesterols). Mass spectrometry (MS) is often the method of choice for analyzing biosamples, but for oxysterols, this is far from straightforward. For example, the aforementioned isomers (see also Fig. 4.1) share the same mass-to-charge ratio (m/z) and will then result in identical signals in the mass spectrometer; this can even be the case when performing tandem MS (MS/MS), which essentially consists of fragmenting the parent mass and applying MS to the resulting fragments. This lack of distinct analytical features becomes a bottleneck for distinguishing compounds with different biological and diagnostic roles. To secure the identification of the different isomers, a separation step upstream of the MS analyzer is often needed. This separation step is very often based on chromatography, for example, liquid chromatography (LC), gas chromatography (GC), or supercritical fluid chromatography (SFC, also known as convergence chromatography, UPCC). In addition, other separation approaches can be utilized, such as capillary zone electrophoresis (CZE). In chromatography separation, a sample is driven by a mobile phase through a column that contains an immobilized stationary phase. The mobile phase can be a liquid (LC), gas (GC), or supercritical fluid (SFC). For CZE, the solvent is an electrolyte but does not have a stationary phase (and is hence not defined as a type of chromatography but an electrophoretic approach). The different components in a sample are separated based on their different degrees of affinity for a stationary phase, based on, for example, polarity, charge, or size, as they are driven through the column with the mobile phase. For example, one compound in a sample that has little affinity for a stationary phase is poorly retained by the column and will hence exit the column before a compound that has a stronger affinity for the stationary phase and is more retained. Compounds are then separated and enter the mass spectrometer at different time points, called retention times.

Traditionally, oxysterol separations have been performed by GC after a derivatization step to make the oxysterol volatile enough for GC separation (a major prerequisite but also a limitation of GC analysis). Typically, oxysterols are derivatized with trimethylsilyl. The derivatization steps most often include an alkaline hydrolysis step, allowing the determination of total oxysterol content (nonesterified and esterified). The

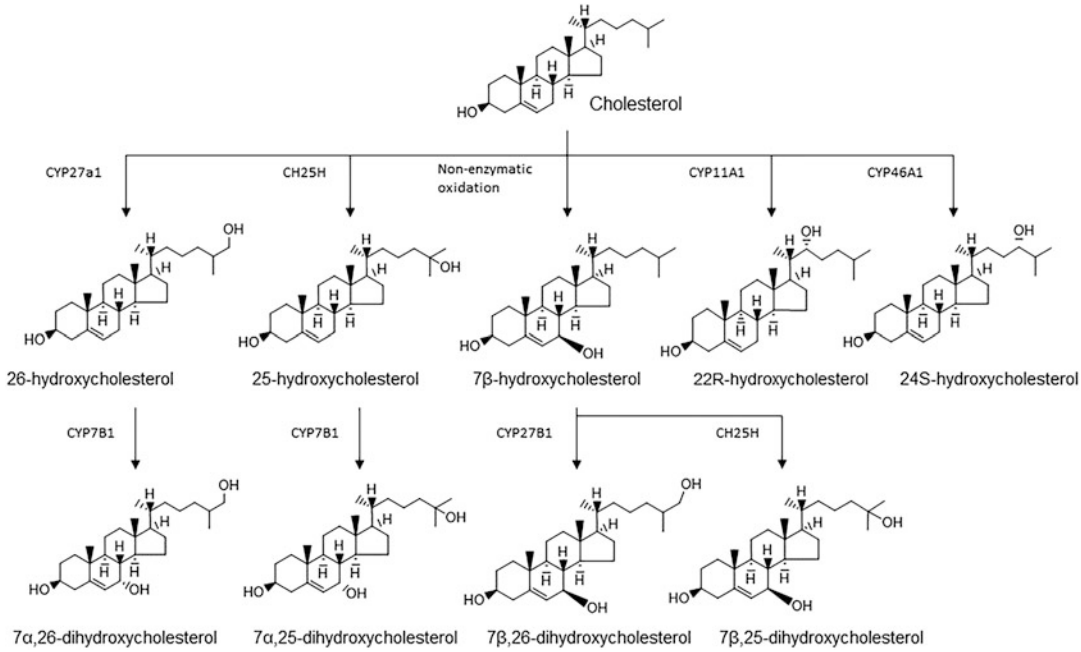

Fig. 4.1 Structure of some oxysterol isomers and the precursor cholesterol. All the hydroxycholesterols (and also the dihydroxycholesterols) have the same molecular mass, resulting in the same signal in the MS. A separation, for example, by chromatography, is important for correct isomer identification

hydrolysis and derivatization steps are often performed at high temperatures, which might cause the autoxidation of cholesterol (the precursor) into oxysterols during the sample preparation (see textbox 1), creating false positive results. In addition, GC can have limitations regarding limited samples, and derivatization steps may not always be applicable to all oxysterol types. Other separation approaches may have limitations. For example, a CZE separation is largely dependent on the charge and hydrodynamic size of the compounds that are to be separated, which may also be very similar for many oxysterols. SFC is limited to compounds that have solubility in supercritical fluids, which can also be a limiting factor for several oxysterol variants, both known and unknown. LC is, however, arguably a more versatile approach, as it is not dependent on elevated temperature or analyte volatility and enjoys a host of separation principles suited for the wide range of oxysterol variants. In addition, LC is a current go-to approach for the separation of very limited samples, for example, metabolites and proteins, even down to single cells and beyond (Røberg-Larsen et al., 2021). Due to these reasons, we here focus on LC–MS approaches for analyzing oxysterols in various biosamples. First, we describe some general aspects of the separation and detection of oxysterols with LC–MS, followed by considerations of whether one wants to analyze specific target oxysterol analytes or undertake a more global approach. We then continue with a focus on how the sample cleanup is done for securing sensitive analysis of oxysterols in biological samples, and finally, we discuss how these parts are applied for analysis of different types of samples, such as cells, exosomes, tissues, and blood.

Autoxidation

Stemming from their precursor cholesterol, oxysterols are formed by enzymatic oxidation or nonenzymatic oxidation

(continued)

(autoxidation). Although autoxidation is controlled in a biological system, it may be less straightforward to control during the time from sample preparation to analysis as the oxysterols are present in low concentrations in our body against a high background of the precursor cholesterol. For example, cholesterol in the samples can be oxidized into oxysterols at elevated temperatures. Autoxidation artifacts cannot be separated from oxysterols; hence, they are quantified as oxysterols. This might be one of the reasons for the large variation in oxysterol concentration reported in biosamples between studies (the mass spectrometer cannot distinguish between an oxysterol produced in the body and one unwantingly created during sample handling). Cholesterol can be removed from the sample early in the sample handling process (e.g., by solid-phase extraction). However, such steps may come with the potential of losing oxysterols as well, compromising the sensitivity of the method (not unlike proteins that may be lost when performing a depletion of the most abundant proteins in a blood proteomics sample). Other approaches, such as adding heavy cholesterol to the sample for autoxidation monitoring, are also possible. If cholesterol is oxidized during sample preparation, the heavy cholesterol is oxidized to heavy oxysterols, which can be monitored in the MS. This will not remove the autoxidation problem, but samples that have proven autoxidation through the monitoring of heavy oxysterols can be discarded. Even with approaches as described here, autoxidation is a key concern one must have during the monitoring of oxysterols and the development of novel methodologies.

4.2 LC–MS of Oxysterols

4.2.1 How Are Oxysterols Separated?

In liquid-based separations, the most common separation principle is reversed-phase LC (RPLC). In RPLC, the stationary phase is hydrophobic (e.g., alkyl chains covalently bound to porous silica particles) and the mobile phase is polar, consisting of water and a water-mixable organic solvent, for example, methanol or acetonitrile. A buffer or an acid is added to the mobile phase for pH control. Separation is based on the different degrees of hydrophobic interactions between the analytes and the stationary phase. RPLC is often the preferred separation technique when handling biosamples due to its robustness and high compatibility with aqueous biosamples and MS.

The separation step is, as mentioned, of high importance when isomers are to be detected and measured. Most separations of oxysterols are performed using octadecyl alkyl chain (C18)-bonded silica stationary phases. With RPLC, more polar oxysterols elute first, for example, dihydroxy cholesterols, followed by more hydrophobic side-chain hydroxylated and ring-hydroxylated sterols. Different C18 columns might provide somewhat different retention and selectivity of the isomers depending on, for example, the carbon load of the column and modifications to the stationary phase chemistry. The choice of organic modifier in the mobile phase (methanol, acetonitrile, or combinations of several organic solvents) also affects the selectivity of the separation. For a comprehensive comparison of columns for derivatized and nonderivatized oxysterol determination, see Dias et al. (2018b).

4.2.2 How Are Oxysterols Transferred to the Mass Spectrometer?

To transfer the analytes (often thermolabile) from the liquid phase into charged gas-phase ions (the form in which compounds are analyzed by MS), a

transitional step is needed between the LC and MS. The most common approach is applying electrospray ionization (ESI). Other ionization sources for LC–MS, for example, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), have also been successfully applied for oxysterol analysis, but ESI is by far the most widely used. ESI is essentially placing an electric potential between the LC outlet and the MS inlet. Instead of solvent dripping out of the LC columns, the applied voltage generates charged droplets that fly toward the MS inlet. Due to the surplus charge of the droplets, the repulsion results in the droplets exploding into smaller droplets, reaching the final stage where analytes dissociate from the droplets into gas phase ions without the aid of temperature. This process requires that the analytes be charged/chargeable. Oxysterols are, due to their neutral nature, not ESI-friendly. Therefore, various approaches such as derivatization to enhance detection sensitivity have been applied, for example, enzyme-assisted derivatization for sterol analysis (EADSA) with Girard P or T, picolinyl ester, or N,N-dimethylglycine esters. The common goal for all these approaches is to incorporate a charge or chargeable group into the oxysterol to enhance sensitivity when applying ESI. In addition, most of these derivatization approaches result in MS/MS fragmentations that are highly specific, further enhancing sensitivity and identifying (new) oxysterols. For a more comprehensive review of derivatization strategies for LC–MS determination of oxysterols, see, for example, Griffiths et al. (2016). Most of these derivatization reactions are performed at ambient temperature, decreasing the risk of autoxidation of cholesterol into oxysterols during sample preparation compared to GC derivatization reactions.

Together with the development of higher-sensitivity MS instruments, LC–MS determination of oxysterols without derivatization is possible. McDonald et al. (2012) created adducts with ammonium, charging the oxysterols (and other sterols), resulting in detection levels of 1 ng/mL from 200 μL of plasma. This approach is highly instrument-dependent (e.g., dependent on ionization source temperature; Mendiara et al. 2018). In contrast to many GC-based approaches, most derivatization strategies for LC–MS allow for either free (nonesterified) or total (esterified and nonesterified) quantification of oxysterols.

Most MS instruments are solely based on measuring the mass-to-charge (*m/z*) ratio of compounds and their fragments, including both high-resolution units such as time-of-flight mass spectrometers and triple quadrupole mass spectrometers, which have a lower resolution but traditionally superior quantitive traits. We have here set a premise that MS is dependent on a chromatography-related step. However, there are variants of MS that can assist with the separation of compounds, for example, ion mobility MS. Ion mobility MS is increasingly used in bioanalysis, both in proteomics and metabolomics. Here, analytes are separated as a function of molecular shape in addition to the mass-to-charge separation in the MS. The drift time is dependent on charge, size, shape, polarity, and collision cross-section and can provide isomer separation. Kylli et al. (2017) showed that the native oxysterol isomers did not separate well in ion mobility, but after derivatization with p-toluenesulfonyl isocyanate, separation was achieved for some of the oxysterol isomers (direct infusion to the MS, no chromatography in front). The derivatization also greatly enhanced the oxysterol signal in ESI, allowing for better detection limits. The combination of ultra-high pressure LC (UHPLC) and ion mobility MS allowed quantification down to 1 ng/mL from fibroblast cells. Overall, the results from Qiu et al. (2021) and Kylli et al. (2017) show the possibility of performing untargeted analysis of oxysterols using derivatization, LC, ion mobility, and MS for secure identification of new and known oxysterols.

MS can be operated on with several degrees of specificity. An untargeted analysis is to record compounds without preset filters. Targeted analysis implies a pre-set filtration, that is, a finite number of masses that are to be measured. The targeted approach can often lead to increased sensitivity and specificity, while untargeted "sterolomics" allows for a more versatile

approach and can be used to scout for novel oxysterols.

4.3 Sample Cleanup Before LC–MS Analysis

Different biosamples require various sample preparation techniques to extract the oxysterols and prepare the analytes for LC–MS analysis. After extraction (and possible derivatization), a sample cleanup is needed before chromatography, either to remove components from the sample that can interfere with the chromatographic separation and/or detection (matrix effects that can lead to ion enhancement or ion suppression, which can affect sensitivity and/or quantification) or to avoid undissolved material (e.g., cell debris or protein precipitate) that can clog the LC column, ESI unit, or even the mass spectrometer. Salts that have no retention when using RPLC will not interfere with the hydrophobic oxysterols but should not be introduced into the MS as salt contamination will increase the need for maintenance (e.g., cleaning of the MS inlet and lenses) and should therefore also be removed.

Solid-phase extraction is a selective sample preparation method that is used before LC. The sample is applied to a small cartridge, containing a stationary phase of choice, for example, a reversed-phase material. Non-retained compounds (e.g., salts, derivatization reagents) can be washed out using a non-eluting solvent. The oxysterols can then be eluted from the column using a stronger mobile phase, while more retained components (e.g., cholesterol or more hydrophobic materials) are retained in the column. Such one-time-use solid-phase extraction columns are widely used in bioanalysis and are available as single cartridges or in 96-well format.

The removal of cholesterol using these types of columns is effective, but to avoid autoxidation, cholesterol should be removed at an early stage (e.g., before derivatization steps), often resulting in the need to perform two different solid-phase extraction steps: one before and one after derivatization. Solid-phase extraction for the removal of excess derivatization reagent can also be performed online by the LC instrument by using multitimes-use small LC columns.

4.4 Sample Preparation of Various Biosamples for LC–MS Analysis

4.4.1 Plasma and Serum

There are numerous examples of the determination of oxysterols in plasma and serum samples. To avoid the oxidation of cholesterol into oxysterols (autoxidation), which creates sample preparation artifacts and compromises the quantification, several careful steps should be included. The general procedure (see Fig. 4.2). seems to be to collect the blood sample on ethylenediaminetetraacetic acid (EDTA)-containing vacutainers, and plasma or serum is prepared by centrifugation. EDTA chelates Fe^{2+} and prevents the formation of free radicals and possible oxidation. The next step usually includes the addition of an antioxidant, such as butylated hydroxytoluene (BHT). The sterols are then extracted from the serum or plasma using either an alcohol such as ethanol or 2-propanol or versions of traditional lipid extraction methods such as the Folch method (2:1 v/v chloroform/methanol) or the Bligh and Dyer method (1:2 v/v chloroform/methanol). An additional effect of extraction with organic solvents is the precipitation of proteins. After collecting the resulting supernatant, cholesterol can be removed from the oxysterols, or autoxidation can be monitored by adding heavy isotope-labeled cholesterol to the sample: If autoxidation occurs, heavy isotope-labeled cholesterol will create heavy isotope-labeled oxysterols that can be monitored in the MS, and the sample can be discarded. Internal standards for quantification should also be added to the sample at an early stage, preferably before the removal of cholesterol.

Depending on the choice of analyzing total (nonesterified and esterified) or free (nonesterified) oxysterols, the sample preparation can include an alkaline hydrolysis step. Oxysterols are then (usually) derivatized into more

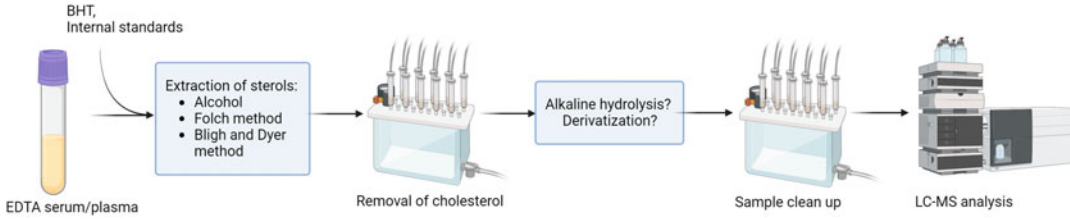

Fig. 4.2 Workflow for preparation of serum or plasma samples for oxysterol analysis. The sample is collected on ethylenediaminetetraacetic acid-containing vacutainers, and serum or plasma is created by centrifugation. Butylated hydroxytoluene is added to avoid oxidation, together with isotope-labeled internal standards. Oxysterols are extracted using a solvent (e.g., alcohol), and cholesterol can be removed. If total oxysterols are to be determined, alkaline hydrolysis is included, often followed by derivatization to enhance MS sensitivity. Excess reagents and components that can compromise chromatography are removed during sample cleanup before LC–MS analysis for detection and quantification. Created with BioRender

MS-friendly derivates, followed by a sample cleanup and LC–MS analysis.

4.4.1.1 Analysis Without Derivatization

The comprehensive method developed by McDonald et al., with sterol determination without derivatization, has been attempted to be adopted by others (Roberg-Larsen et al. 2015; Mendiara et al. 2018), but as the author states, the formation of ammonium adducts for enhanced detection is highly instrument (or, more precisely, ionization source) dependent. Nevertheless, the direct analysis of oxysterols using LC–ESI–MS after protein precipitation and sample cleanup is possible. The MS signal is usually detected with the loss of one or two water molecules ($[M + H-H_2O]^+$) (Roberg-Larsen et al. 2015; Mendiara et al. 2018; Dias et al. 2018a).

The approach can be used to determine both free (nonesterified) and total (nonesterified and free) oxysterols. Mendiara et al. (2018) used the enzyme *cholesterol esterase* to avoid alkaline hydrolysis, resulting in improved recovery and less background noise as the enzyme is more specific and avoids the release of triglycerides and fatty acids that can interfere with the chromatography. Overall, the utilization of enzymes for hydrolysis reduces the need for sample cleanup and elevated temperatures during sample preparation and is an approach that should be further investigated and adapted for total (esterified and nonesterified) oxysterol determination.

Helmschrodt et al. (2013) use an alternative ionization source to ESI, APCI, to determine oxysterols in plasma after fast and simple sample preparation. APCI is less dependent on the analytes´ charge-readability and is arguably a suitable component in the analysis of underivatized oxysterols. Proteins were precipitated and sterols were extracted using methanol and 2-propanol, followed by evaporation of the solvent, re-desolvation, and centrifugation before the analysis. The method was applied for the determination of free 7-keto, 7α/β-hydroxy, 5,6α-epoxy, 5,6-β-epoxycholesterol, cholestane-3β-,5α,6β-triol and cholesterol in both human plasma and atherosclerotic plaques, which are all linked to the initiation and progression of atherosclerosis. The authors used isotope-labeled cholesterol for autoxidation monitoring, showing no autoxidation. The isomers were separated on a monolithic column (Chromolith SpeedROD RO-18e), which is a one-piece polymer synthesized within the column, typically associated with a lower back pressure compared to conventional LC and UHPLC, an LC variant with smaller particles (high back pressure but can allow for more rapid separations). The method's robustness with this simple sample preparation was not discussed. The method has been further developed to determine nonesterified cholesterol, 17 nonesterified oxysterols, and 17 free and conjugated bile acids in plasma and cerebrospinal fluid (Reinicke et al.

2018). The extended method also contains an additional sample cleanup step using an automated online solid-phase extraction column switching system, which allows large injection volumes (200 μL) to enhance detection limits. The method was applied to study patients with blood–brain barrier disturbances, but no significant differences were detected compared to controls. The method has also been adopted by Kloudova-Spaenkova et al. to study the circulating levels of seven free oxysterols during the progression of luminal subtype breast cancer. They found that the circulating levels of 7-α-hydroxycholesterol, 26-hydroxycholesterol (also referred to as 27-hydroxycholesterol), cholesterol-5β,6β-epoxide, and cholestan-3β,5α,6-β-triol were lower in patients with small tumors compared to large and later-stage cancers.

4.4.1.2 Analysis with EADSA Derivatization

Griffiths established that EADSA is a popular choice for analyzing oxysterols and is applied in plasma and serum samples as well (Griffiths et al. 2013). The approach enjoys excellent sensitivity and characteristic MS/MS spectra, aiding the detection, identification, and elucidation of sterols. This method has been adapted by others and can be used with both Girard P and T reagents. A drawback with this method is the creation of syn and anti forms during the derivatization, resulting in double peaks on traditional C18 stationary phases. This seems not to be an issue for hydroxycholesterols when a phenyl-based reversed-phase stationary phase is applied. In addition, the method does not separate oxysterols with naturally occurring ketone groups at position 3 from those with a hydroxyl group. Hence, samples should be divided into two, prepared with and without the enzyme cholesterol oxidase, and analyzed separately. The development of different derivatization tags for EADSA allows multiplexing, reducing the analysis time on the LC–MS. Nevertheless, the need for two sample preparations on the same sample complicates the analysis. The method has been used extensively in the group of Griffiths and Wang but has also been adapted by others (DeBarber et al. 2010, 2011; Roberg-Larsen et al. 2014, 2017; Soroosh et al. 2014; Solheim et al. 2019).

4.4.1.3 Other Derivatization Approaches

The derivatization of oxysterols into picolinyl esters (a method established by Honda et al. 2008) is suitable for the determination of oxysterols in small-volume plasma samples. Xu et al. (2013) modified the method to determine the total (esterified and nonesterified) concentration of 4β-hydroxycholesterol and cholesterol in 5 μL of human and mouse plasma. The ratio between 4β-hydroxycholesterol and cholesterol was suggested as an endogenous biomarker for CYP3A4/5 activity. After alkaline hydrolysis, the oxysterols were derivatized into picolinyl esters, followed by a liquid–liquid extraction step using hexane for sample cleanup. In contrast to Honda et al., Xu et al. created sodium adducts in their MS, emphasizing the importance of MS method optimization. The 4β-hydroxycholesterol was also derivatized in two positions (3 and 4), while the Honda approach resulted in the addition of one group. The addition of one or two groups should be optimized, as these derivates will give different retention times in reversed-phase chromatography and can make the chromatographic separation challenging (Nadarajah et al. 2017) or even lead to false quantifications.

Marelli et al. have also adopted the Honda method for the determination of oxysterol related to spastic paraplegia type 5. Aliquots of 20 μL of EDTA plasma were added to internal standards and BHT in methanol, followed by alkaline hydrolysis and derivatization into picolinyl esters. Interestingly, the ESI was set to negative mode, usually most suited for anions and not cations, which the derivatized oxysterols were in this approach. The authors, however, do not state if they monitored sodium adducts or the precursor ions or fragments monitored in the MS/MS analysis. Nevertheless, Marelli et al. could conclude that 25-hydroxycholesterol and 26-hydroxycholesterol and their ratio to total cholesterol could discriminate between spastic

paraplegia type 5 patients and healthy controls with 100% sensitivity and specificity.

N,*N*-dimethylglycine derivatives of oxysterols have been shown to have good chromatographic traits. Pataj et al. (2016) separated the isomers on a biphenyl column using a fast gradient, resulting in baseline separation of the isomers in 7.8 min. The method was applied to human plasma and red blood cells with the aim of doing an extensive investigation into rare diseases such as Niemann-Pick type C, cerebrotendinous xanthomatosis, and neurodegenerative or cardiovascular diseases.

4.4.2 Cultured Cells

For measuring cell content, LC–MS of oxysterols is fully applicable in several formats. For example, conventional columns (>1 mm ID) can be used for ample samples, or more down-scaled variants can be employed for limited samples (Roberg-Larsen et al. 2012). For example, Lundanes and co-workers applied nano-LC columns for the analysis of oxysterols in cancer cell samples. The nano-LC system allowed for measurements at the thousand-cell scale (Roberg-Larsen et al. 2014) (Fig. 4.3). However, there is currently a major push toward single-cell analysis. Although fairly conventional preparation methods were applied in Roberg-Larsen et al. (2014), that is, ethanol-based extraction from cells, in-vial derivatization, etc., a single-cell analysis will undoubtedly require dramatic miniaturization of the sample preparation with precise robotic systems for ultra-small samples, including lysis, derivatization, and sample cleanup, as seen in, for example, single-cell proteomics (Røberg-Larsen et al. 2021).

4.4.3 Tissue

4.4.3.1 Brain Tissue

Oxysterols in both human and mouse brains have been studied with different approaches in relation to both Alzheimer's disease and development. Dias et al. (2022) studied oxysterols and oxysterol sulfates in Alzheimer's disease brains after homogenization and extraction with methanol and BHT. Sample cleanup and enrichment of oxysterols were performed on an offline polymeric SPE column, followed by LC-MS analysis without derivatization. With the developed method, they observed that enzymatically generated 26-hydroxycholesterol and nonenzymatically generated 7-oxycholesterol were significantly elevated in Alzheimer's disease brain tissue. Ahonen et al. (2014) studied oxysterols and vitamin D metabolites in mouse brains using APPI for enhanced detection. Meljon et al. (2012) use EADSA to analyze oxysterols in newborn mouse brains. Oxysterols were extracted from the brain using methanol:chloroform (1 + 1), followed by removal of cholesterol and derivatization with Girard P reagent. The method was used to map bioactive oxysterols in a newborn mouse brain, identifying several oxysterols derived from demisterol and high levels of 24S,25-epoxycholesterol, a potent LXR and Insig ligand.

MS imaging (MSI) has also been used to map cholesterol in the brain after on-tissue derivatization following the EADSA method and matrix-assisted laser desorption ionization or desorption electrospray ionization (Angelini et al. 2021). The method showed how the cholesterol distribution changes during development in mouse brains using isotope-labeled standards for quantification. This approach has also been applied to oxysterols and sterols (Yutuc et al. 2020); however, the oxysterol isomers require separation before MS detection, and microscale liquid extraction for surface analysis (μLESA) was applied to extract spots with 400 μm spatial resolution and transfer them to the LC–MS for separation, identification, and detection (Fig. 4.4).

4.4.3.2 Breast Cancer Tissue

26-Hydroxycholesterol (also referred to as 27-hydroxycholesterol) has been linked to breast cancer (Wu et al. 2013; Nelson et al. 2013), and this has been studied in tumor tissue, both ER-positive (ER+) and negative (ER−). Wu et al. (2013), using the McDonald method, showed that 26-hydroxycholesterol increased in

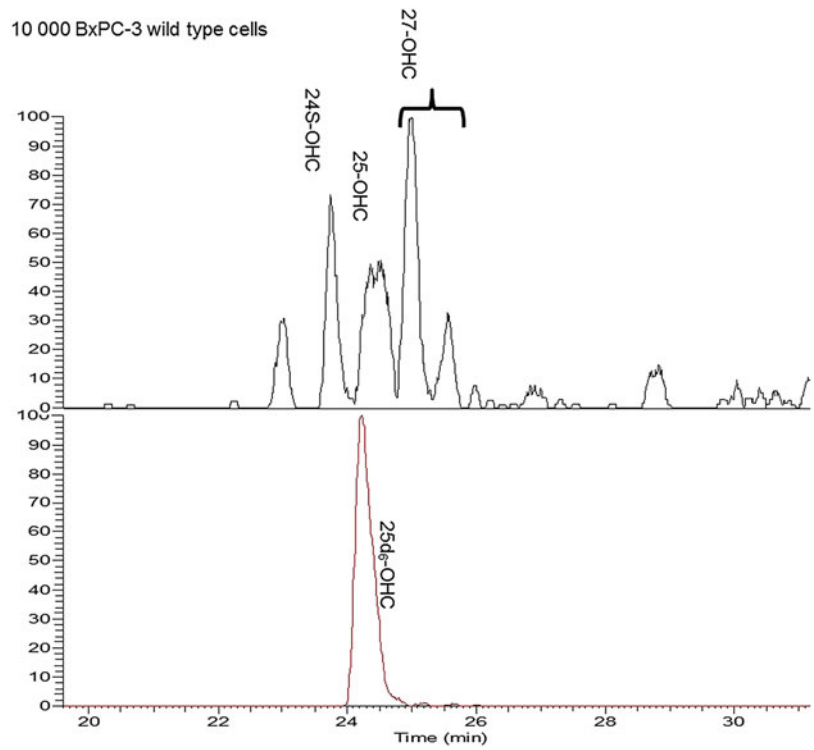

Fig. 4.3 Chromatogram of side-chained hydroxycholesterols extracted from cultured pancreatic adenocarcinoma cells using nano-LC–MS. Analytes were extracted from the cells using ethanol and Girard T-derivatization. Reprinted with a CC-BY license from Roberg-Larsen et al. (2014)

human ER+ breast cancer tissue compared to normal breast tissue. Solheim et al. (2019) used the EADSA method with Girard T and online sample cleanup in combination with fast LC–MS to examine both free and total oxysterol concentrations in breast cancer tissue from both ER+ and ER− tumors. The study revealed large intratumor variations in the concentration of different enzymatically formed oxysterols. The variation was observed both in absolute and relative concentration, implying that the extraction efficiency of sterols from the tissue was not the issue. The findings linking 26-hydroxycholesterol with breast cancer are also in contrast to the circulation of 26-hydroxycholesterol in the blood, where a large study (involving 530 individual patients) revealed that higher circulation of 26-hydroxycholesterol was associated with a lower risk of breast cancer in postmenopausal women (Lu et al. 2019). This finding highlights the importance of studying smaller sample selections, for example, tissue or exosomes (see below), to understand the role of oxysterols in, for example, breast cancer.

4.4.4 Exosomes

Exosomes are small vesicles that are produced by cells and released into the extracellular space. They are involved in intercellular communication and play a role in various physiological and pathological processes. Cancer cells may also release exosomes with cargo that can promote further disease development. Exosomes are typically 30–150 nm in size and contain a variety of biomolecules, including proteins, and nucleic acids. In addition, exosomes may feature small molecules such as lipids. Røberg-Larsen and co-workers applied LC–MS methodology with online solid-phase extraction for measuring oxysterols in exosomes from cell lines, including breast cancer cell lines MCF-7 (ER+) and MDA-MB-231 (ER−). While side-chain hydroxylated oxysterols did not have outstanding levels in the cells compared to healthy cells, the level of 26-hydroxycholesterol was significantly higher in MCF-7 exosomes (Fig. 4.5). This result could be interesting regarding disease

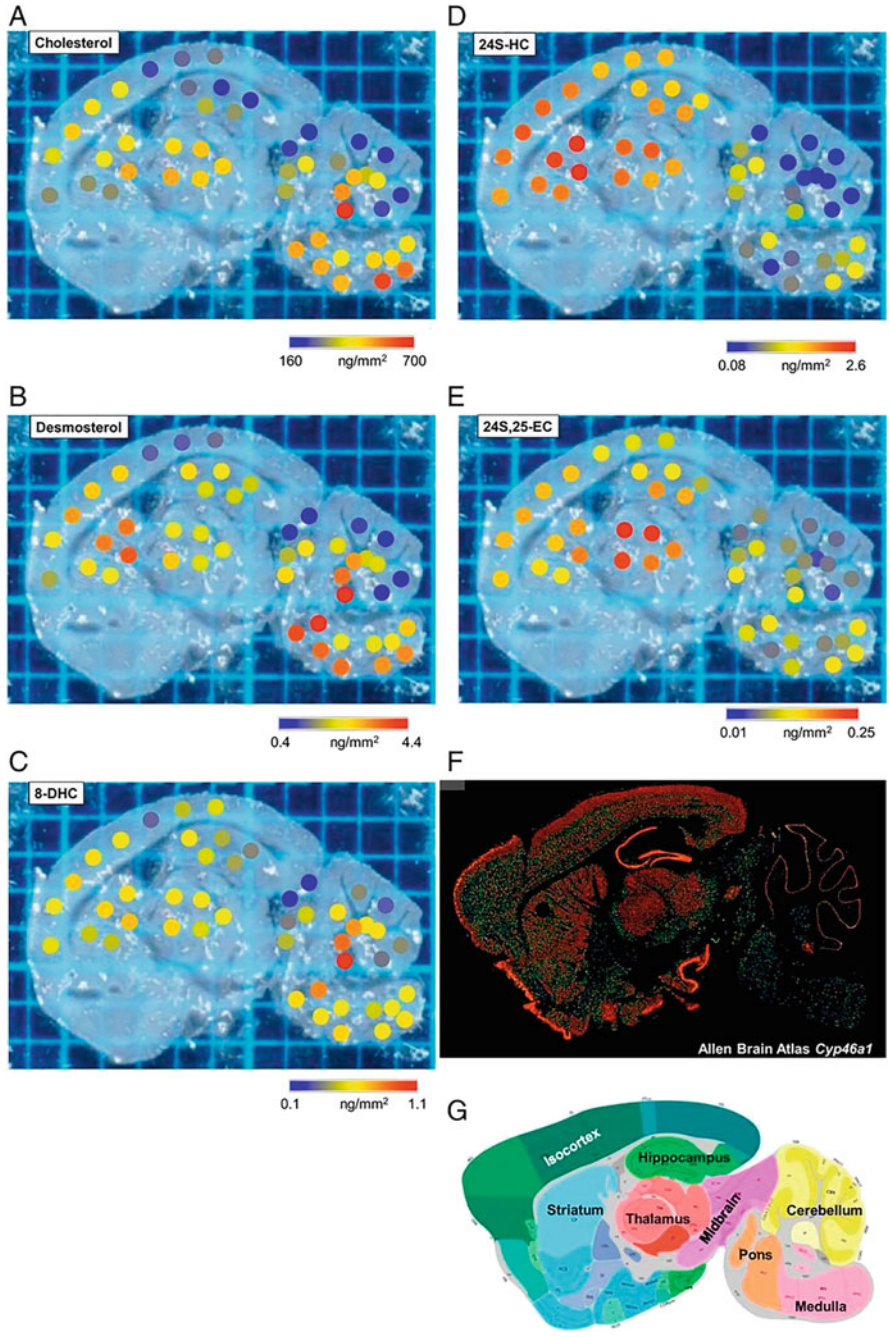

Fig. 4.4 Mouse brains were analyzed using μLESA-LC–MS after EADSA for sterol detection and isotope-labeled quantification. Reprinted with a CC-BY license from Yutuc et al. (2020)

development, as 26-hydroxycholesterol has been shown to be associated with cancer proliferation in ER+ cancers (Wu et al. 2013; Nelson et al. 2013). A key challenge in exosome analysis is their availability; extraction from biosamples will only result in limited amounts (e.g., <1 μg

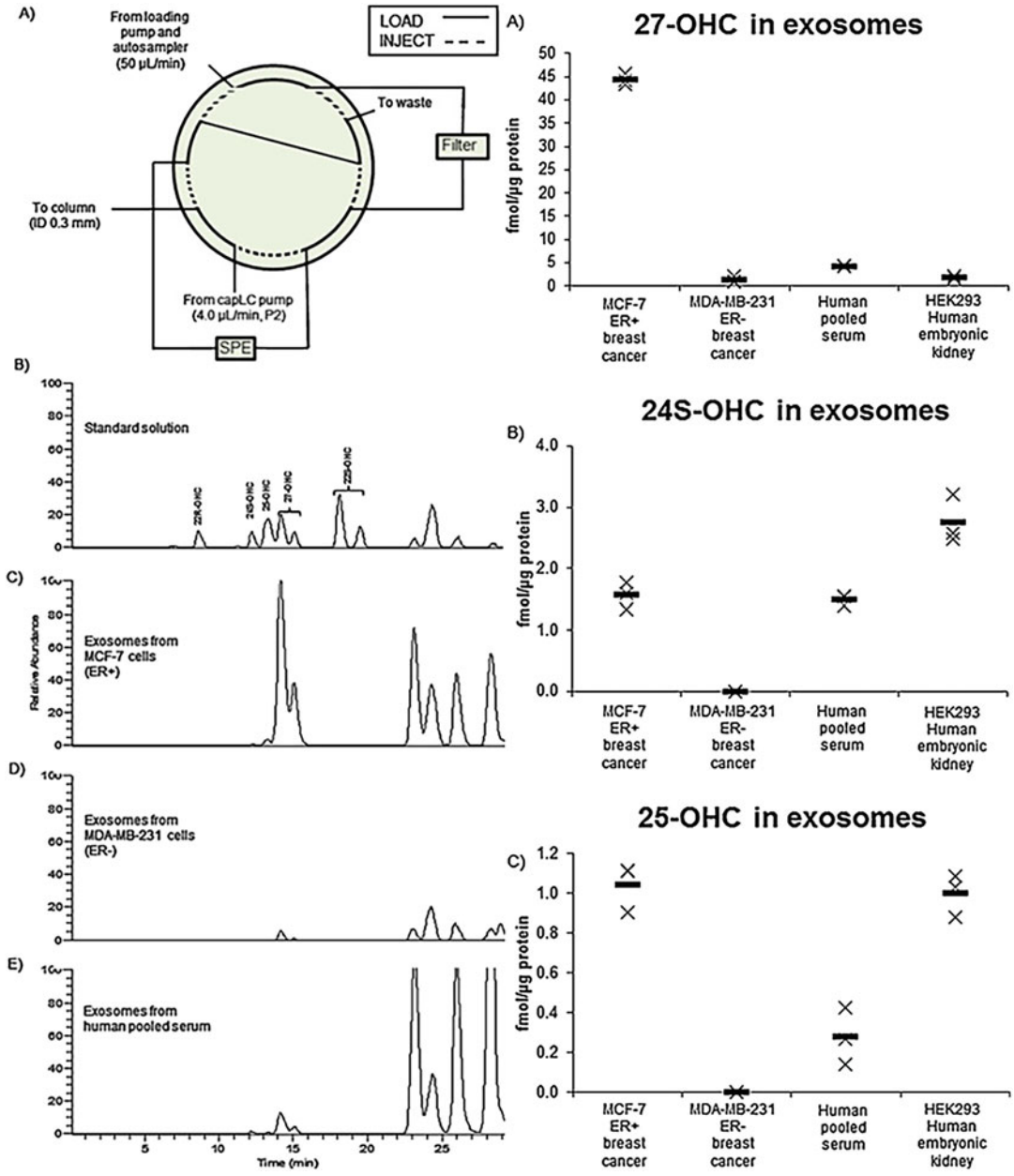

Fig. 4.5 Upper left: an overview of the automatic filtration and filter back flushing-solid-phase extraction-liquid chromatography system plumbing applied for the sample preparation and analysis of oxysterols in exosomes. Lower left: representative chromatograms of oxysterol analytes from exosomes of cancer cells and exosomes from human serum. Right: levels of oxysterols revealed a significant increase of 26-hydroxycholesterol (also referred to as 27-hydroxycholesterol) in exosomes from estrogen receptor-positive cells, compared to similar oxysterols. Reprinted with permission from Roberg-Larsen et al. (2017)

exosome protein/million cells). Therefore, it is imperative to have access to highly sensitive analytical platforms. For the study described here, capillary LC was employed (a downscaled

variant of conventional LC using sub 1 mm inner diameter columns). For lysing and extraction of oxysterols, the same sample preparation approach used for nano-LC–MS of cells was applied, showing that the simple ethanol extraction-based protocol can be used for several kinds of related samples. Perhaps, a greater challenge with exosomes is securing the contamination-free isolation of these vesicles; the exosomes analyzed in this study (obtained commercially) had been isolated using a polymer precipitation approach. Exosomes were characterized by Western blot and NanoSight for size and intactness. Size distribution analysis did not reveal a significant presence of lipoproteins.

4.4.5 Organoids

Organoids are laboratory-grown organ models and are emerging tools for developmental biology, drug discovery, and disease modeling, to mention a few of their many applications. Organoids can be grown from, for example, induced pluripotent stem cells into miniature models of, for example, the liver, kidney, and even brain. Liver organoids can, for example, be used to study nonalcoholic fatty liver disease by subjecting liver organoids to excessive amounts of lipids, resulting in the liver organoids developing various stages of the disease (see Fig. 4.6). A key challenge with organoids can be that a single organoid is <1 mm in size, which can present issues with sensitivity, as with other limited sample amounts and sizes. Organoids have not been widely explored regarding oxysterols. However, an early study by Kømurcu et al. showed that oxysterols, both dihydroxcholesterols and hydroxycholesterols, were found in elevated levels in the medium of treated organoids compared to controls, implying that the organoids secrete oxysterols as a response to infection (Kømurcu et al. 2023). Approximately 90 µL of the organoid medium was mixed with internal standard cholesterol-25,26,27-13C for autooxidation monitoring. Samples were evaporated to dryness and redissolved in 20 µL 2-propanol before Girard T-derivatization. These results were also in accordance with a few other studies of NAFLD using blood from patients, suggesting that oxysterols may have a potential for serving as biomarkers of the disease and calling for further studies. The sample preparation was similar to that of tissue samples (Solheim et al. 2019) and also featured automatic filtration and filter flush-solid-phase extraction-LC–MS for the sample cleanup, separation, and detection of the analytes. However, the system was not capable of quantifying the low levels in the control samples, and additional oxysterols may be present in the liver organoids with diagnostic value. Therefore, the sample preparation may need to be downscaled for further gains in sensitivity.

4.5 Conclusion

The preparation and analysis of oxysterols have been explored, focusing on approaches centered around LC and MS. Oxysterols required substantial attention in every step, including: addressing the possibility of autooxidation during sample preparation, which can perturb accurate quantification; considering a derivatization step, which may enhance sensitivity and mass spectra quality, but may require several additional steps in the procedure; how to remove potentially interfering compounds and materials, for example, through the use of solid-phase extraction, either in an online or offline manner; how to separate the oxysterols in a selective fashion, to not mix structurally similar but biologically distinct isomers; addressing potential adducts that may form due to the addition or absence of sample preparation steps. Finally, these steps must be applied and modified for a range of biomaterials, from ample amounts of blood samples to potentially limited samples such as exosomes.

However, oxysterol sample preparation has been vigorously explored and optimized in several directions. Today, we can measure oxysterols in a highly sensitive and selective manner, unraveling biological functions and biomarkers. In this regard, one can say that the field is a

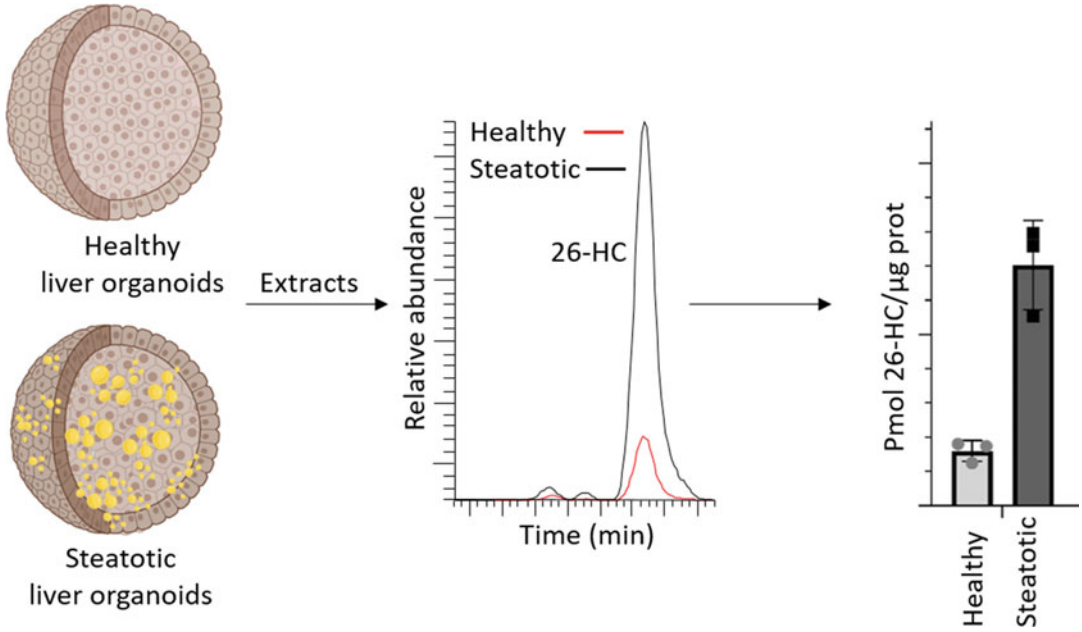

Fig. 4.6 Steaotis was induced in liver organoids, followed by measurements with LC–MS, which revealed that 26-hydroxycholesterol (also referred to as 27-hydroxycholesterol) was significantly upregulated in the secretion of treated organoids. As these samples are quite limited, efforts are being made to downscale the sample preparation. Reprinted from Kømurcu et al. (2023)

success. Still, improvements regarding ease of preparation and throughput would be very welcome as we continue to seek new oxysterol variants in complex and minute samples, potentially of clinical interest and for the health of all of us.

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Mass Spectrometry Imaging of Cholesterol and Oxysterols

5

William J. Griffiths, Eylan Yutuc, and Yuqin Wang

Abstract

Mass spectrometry imaging (MSI) is a new technique in the toolbox of the analytical biochemist. It allows the generation of a compound-specific image from a tissue slice where a measure of compound abundance is given pixel by pixel, usually displayed on a color scale. As mass spectra are recorded at each pixel, the data can be interrogated to generate images of multiple different compounds all in the same experiment. Mass spectrometry (MS) requires the ionization of analytes, but cholesterol and other neutral sterols tend to be poorly ionized by the techniques employed in most MSI experiments, so despite their high abundance in mammalian tissues, cholesterol is poorly represented in the MSI literature. In this chapter, we discuss some of the MSI studies where cholesterol has been imaged and introduce newer methods for its analysis by MSI. Disturbed cholesterol metabolism is linked to many disorders, and the potential of MSI to study cholesterol, its precursors, and its metabolites in animal models and from human biopsies will be discussed.

Keywords

Mass spectrometry · Mass spectrometry imaging · Cholesterol · Oxysterol · Neurodegeneration · 24S-hydroxycholesterol

5.1 Introduction

Pioneering work by the groups of Caprioli, Spengler, Clench, and Murphy has moved mass spectrometry imaging (MSI) from the preserve of the mass spectrometry (MS) specialist into the realm of the analytical biochemist (Stoeckli et al. 2001; Spengler 2015; Trim et al. 2008; Berry et al. 2011). At its simplest, a probe (e.g., a laser or ion beam) samples the surface of a tissue section pixel by pixel, with mass spectra being recorded of the sampled surface at each pixel. An image of any ion recorded by the MS can then be generated across the sampled surface, with ion abundance pixel by pixel illustrated on a color scale (Fig. 5.1) (Ellis and Soltwisch 2023; Angelini et al. 2021). As an ion's *mass/charge* (*m/z*), as recorded by MS, is related to its parent compound's molecular mass and hence to a particular compound, MSI, at least to a first approximation, gives a molecular image of a compound in a tissue. There are many differing sampling probes providing different degrees of spatial resolution, ranging from 100 to 400 nm or smaller for secondary ion MS (SIMS) at the high spatial resolution end of the spectrum (Brunet and Kraft

W. J. Griffiths (✉) · E. YutucY. Wang
Swansea University Medical School, Swansea, Wales, UK
e-mail: w.j.griffiths@swansea.ac.uk; eylan.yutuc@swansea.ac.uk; y.wang@swansea.ac.uk

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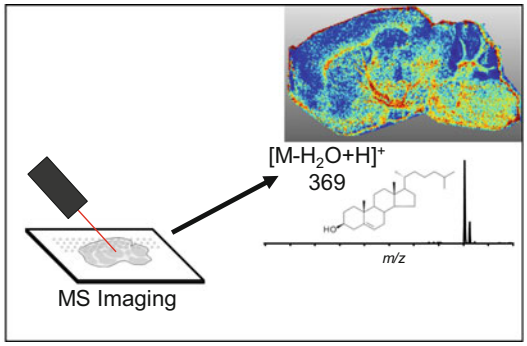

Fig. 5.1 Schematic of MSI. A probe, for example, a laser light or an ion beam, samples the surface of a tissue section pixel by pixel. Mass spectra are recorded at each pixel. An image of any recorded ion is generated from its MS-abundance data at each pixel. Illustration of MALDI-MSI of cholesterol in a sagittal section of the mouse brain. Modified from Angelini et al. Analytical Chemistry 2021 93 (11), 4932–4943, reference (Angelini et al. 2021), Copyright © 2021, The Authors. Published by the American Chemical Society under CC-BY license. *MALDI* matrix-assisted laser desorption ionization, *MSI* mass spectrometry imaging

2023; Lazar et al. 2013); 50 to 100 μm typical for matrix-assisted laser desorption ionization (MALDI) (Berry et al. 2011; Ellis and Soltwisch 2023; Angelini et al. 2021) and desorption electrospray ionization (DESI) (Wang et al. 2019) and 20 μm or less for the more sensitive MALDI2 (Soltwisch et al. 2015; Ellis et al. 2017); to 300–1000 μm for liquid extraction for surface analysis (LESA) at the low spatial resolution end of the spectrum (Almeida et al. 2015; Hall et al. 2017). However, as spatial resolution is increased, the pixel size gets smaller and the available tissue to be analyzed is reduced, meaning that low-abundance metabolites are discriminated against at high spatial resolution. This is less of a problem for highly abundant cholesterol than for its lower abundance precursors and metabolites.

A challenge for MSI of sterols is their comparatively poor ionization characteristics in MALDI-MSI and DESI-MSI, two of the dominant MSI technologies of today (Berry et al. 2011; Wang et al. 2019). Phospholipids are more readily ionized and tend to dominate the MSI spectra of animal tissues. Cholesterol is usually observed as the $[M + H\text{-}H_2O]^+$ ion at *m/z* 369.35 and cannot be differentiated from its isomers lathosterol or zymostenol (Fig. 5.2), although the latter two isomers are almost always of very minor abundance in comparison to cholesterol and unlikely to significantly contribute to the signal at *m/z* 369.35 (Lütjohann et al. 2004). Isomer differentiation is more of a problem for desmosterol, 7-dehydrocholesterol (7-DHC), and 8-dehydrocholesterol (8-DHC), precursors of cholesterol, which are often found in similar abundance in tissue and can lead to misidentifications in the literature. MSI of oxysterols (oxidized forms of cholesterol) is even more challenging on account of the fact that they can be formed in vivo from cholesterol enzymatically and nonenzymatically but also ex vivo in the air (Schroepfer 2000), and tissue sections for MSI are prepared in the air. The situation is further complicated by the fact that oxysterols formed ex vivo are isomeric to those formed in vivo, and most MSI technologies are unable to resolve isomers. Despite these challenges for the analysis of sterols and oxysterols, technologies are being developed to allow their imaging.

5.2 MALDI-MSI and DESI-MSI of Cholesterol and Its Precursors

Both MALDI and DESI are desorption ionization methods (Ellis and Soltwisch 2023). In MALDI-MSI, the tissue section to be analyzed is coated with a matrix, usually a UV-absorbing matrix, by spraying the matrix from a pneumatic sprayer or by sublimation of the matrix onto the tissue surface in a vacuum chamber. A matrix is not required for DESI. The tissue itself is usually 5–20 μm thick and cryosectioned using a microtome. The tissue is thaw-mounted onto a microscope slide, which often has a conductive surface of indium tin oxide for MALDI-MS (Ellis and Soltwisch 2023). In the MALDI process, the exact ionization mechanism is still a subject of debate but can be imagined in simple terms as the excitation of the matrix by the absorption of laser light (usually 337, 349, or 355 nm) leading to

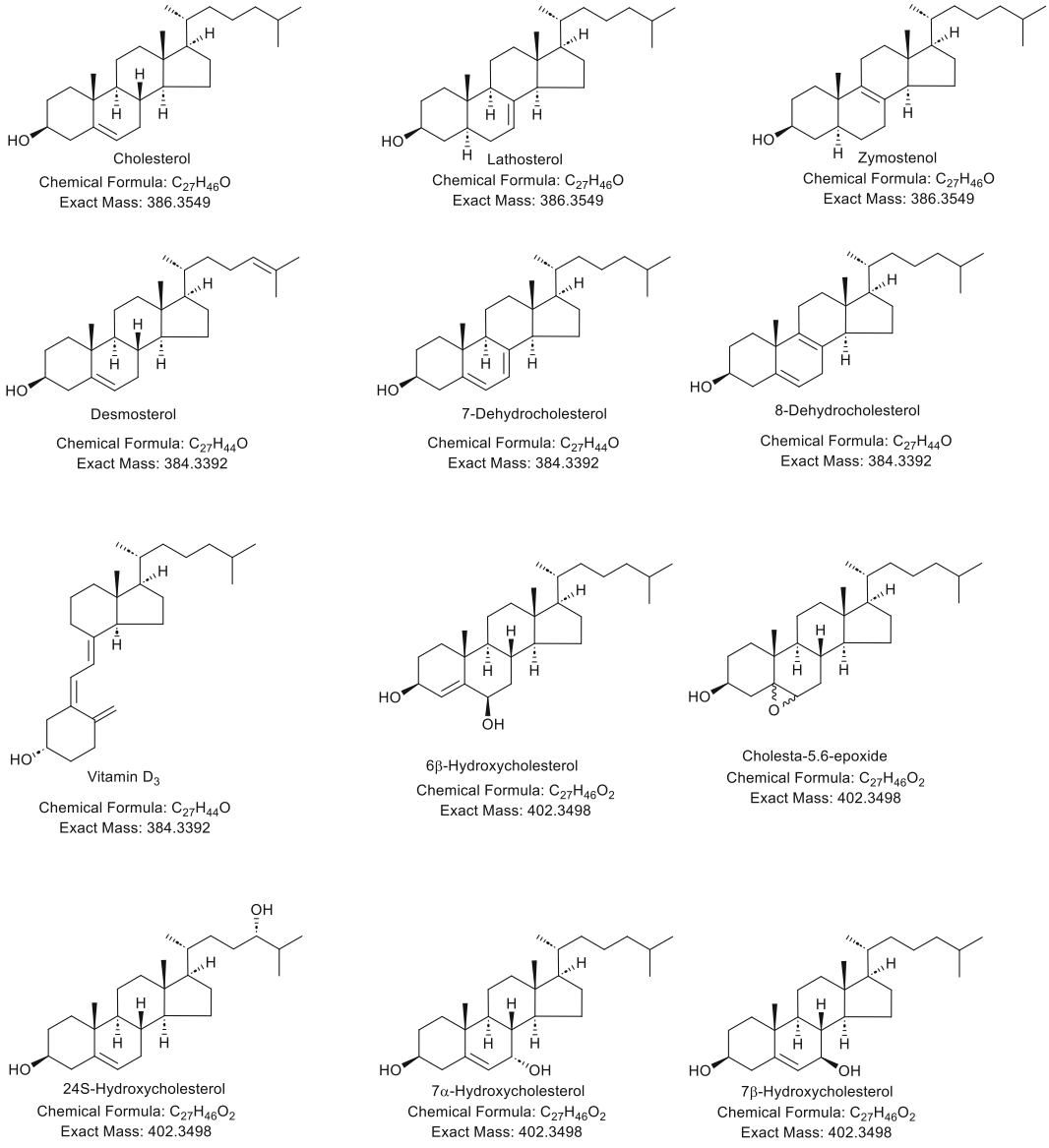

Fig. 5.2 Structures of cholesterol, desmosterol, and their isomers, and of the oxysterols 24S-hydroxycholesterol formed enzymatically and of 7α-hydroxycholesterol and 7β-hydroxycholesterol which can both be formed in vivo enzymatically or nonenzymatically and ex vivo by autoxidation. The structures of 6β-hydroxycholesterol and cholesta-5,6-epoxide are also shown

vaporization and ionization of matrix and surface analytes, and in the positive ion mode, the transfer of a cation, often a proton or alkali metal, from the matrix to the analyte. This mechanism favors the analysis of Brønsted and Lewis bases. In the negative-ion mode, the matrix acts as a Brønsted or Lewis base, and ionization favors proton donors. The ionization process can occur under vacuum (vacuum MALDI) or at atmospheric pressure (AP-MALDI), but in both cases, it does not favor the ionization of cholesterol in either the positive or negative-ion mode, as it is a weak

proton acceptor and weak proton donor. Nevertheless, in tissues where cholesterol is abundant, images can be generated. The brain is a good example of an organ rich in cholesterol, with average levels of about 20 μg/mg wet weight (Dietschy and Turley 2004). MALDI-MSI has been used to image cholesterol in rodent brains and highlight its differential abundance in distinct anatomical regions (Tobias et al. 2018; Cologna 2019). Although aromatic small molecules usually constitute the MALDI matrix, graphene oxide has also been used in the MSI of cholesterol as the $[M - H]^-$ ion at *m/z* 385.35 in breast cancer tissue (Fig. 5.3) (Zhou et al. 2017).

Silver ions (Ag^+) are known to complex to double bonds (Fig. 5.3) (Nikolova-Damyanova 2009). This chemistry has been exploited for the MSI of sterols. In an early study, $AgNO_3$ was sprayed as a "matrix" onto an etched silicon wafer and tissue thaw mounted ready for a "nano-structure initiator"—MSI (Patti et al. 2010). Images were generated for cholesterol as $[M + {}^{109}Ag]^+$ at *m/z* 495 and for dehydrocholesterol, a combination of 7-DHC (probably also 8-DHC) and desmosterol, at *m/z* 493 from the brains of 1-day-old mice. In brain tissue from a mouse model of Smith-Lemli-Opitz syndrome (SLOS), where *7-dehydrocholesterol reductase* (*Dhcr7*), the gene that codes the enzyme that converts 7-DHC to cholesterol, is deleted, a buildup of dehydrocholsterols was observed in the cerebellum and brain stem (Patti et al. 2010). The application of Ag^+ to enhance MSI can be achieved in different ways. $AgNO_3^+$ solution can be pneumatically sprayed onto tissue (Yang et al. 2020), or alternatively, silver can be sputtered onto tissue (Xu et al. 2015; Nezhad et al. 2022). The end result is the same, an improvement in the sensitivity for MSI of double bond-containing sterols.

A modification of MALDI called MALDI-2 has been developed that improves the ionization of certain lipid classes, including sterols. Here, a second laser pulse interacts with the plume of matrix and analyte generated by a primary laser, producing more matrix ions available to transfer charge to the analyte (Soltwisch et al. 2015). Using MALDI-2 cholesterol, monitored as the $[M + H - H_2O]^+$ at 369.35, has been imaged in a sagittal section of the rat brain and at high spatial resolution (15 μm) in the mouse cerebellum, as has somewhat strangely vitamin D_3 as the $[M + H]^+$ ion at *m/z* 385.35 (Soltwisch et al. 2015; Ellis et al. 2017). Vitamin D_3 is the light-induced ring-opened product of 7-DHC. Its presence in the circulation is extremely low, and it is unlikely to be present in the brain. It is more likely that the authors were detecting 7-DHC, 8-DHC, their isomer desmosterol, a dehydrated cholesterol autoxidation product, or perhaps vitamin D_3 artifactually generated by laser light (Fig. 5.2). Interestingly, MALDI-2 has been used to image cholesterol esters in multiple sclerosis brain tissue at a small pixel size of 6 μm (Ellis and Soltwisch 2023). Cholesterol esters, if present in mammalian brains, are present at very low levels (Dietschy and Turley 2004), but perhaps their presence in multiple sclerosis brains is a consequence of the disease itself.

Like MALDI, DESI is a desorption ionization method. In DESI, an electrospray of charged solvent droplets is directed at the target surface. The charged droplets sample the surface, extracting analytes, and scatter in the direction of the inlet capillary of the mass spectrometer. Ionization occurs in an ESI-like process, with analytes having a high gas phase basicity being most preferentially ionized in the positive ion mode and those with a high gas phase acidity being preferentially ionized in the negative ion mode (Cooks et al. 2006). DESI can be used for MSI, with the dimensions of the primary electrospray beam dictating the pixel size. Pixel sizes of 50–150 μm are most common. Due to its lack of strong basic or acidic groups, cholesterol tends to be poorly ionized by DESI; however, ionization can be improved by incorporating trifluoroacetic acid in the DESI spray solvent (Wang et al. 2019). DESI-MSI has been nicely exploited for the imaging of mouse brains and in a mouse model of Alzheimer's disease (AD) (Wang et al. 2019). Infrared (IR)–MALD-ESI is a hybrid of IR-MALD and ESI where analytes are desorbed by IR-MALD and ionization is achieved in a plume of charged droplets from an ESI emitter. Using IR-MALD-ESI-MSI cholesterol has been

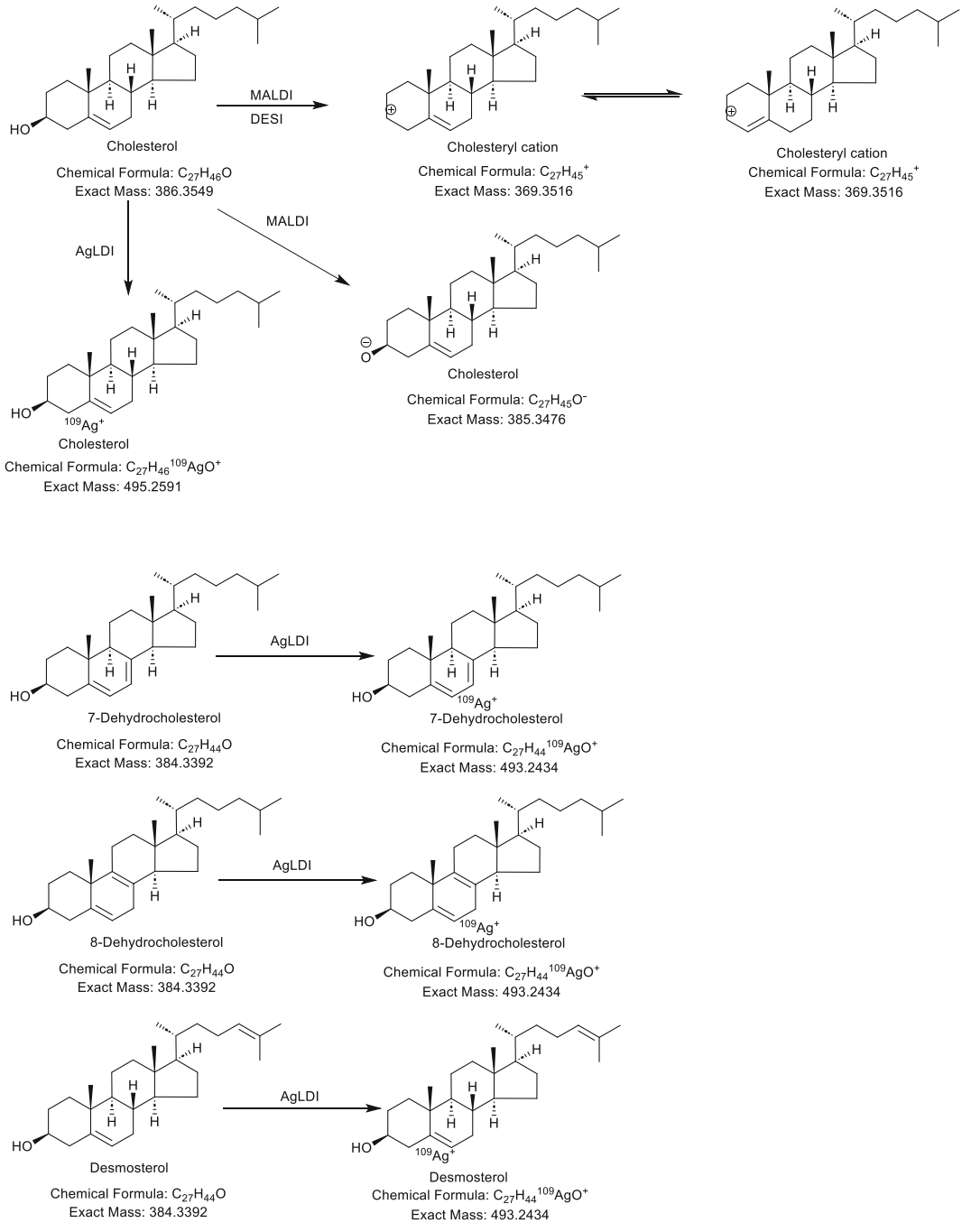

Fig. 5.3 Ionization of cholesterol by matrix-assisted laser desorption ionization and of other double bond-containing sterols by silver-laser desorption ionization (AgLDI)

imaged in the skin and found to be high in the epidermis (Bai et al. 2021).

5.3 MSI of Sterols Exploiting Chemical Derivatization

The concept of derivatization is embedded in the history of sterol and steroid analysis (Sjovall 2004). Although primarily used to enable gas chromatography—MS of sterols and steroids, derivatization has also been employed to improve the sensitivity of LC–MS and MALDI-MS analysis (Wang and Griffiths 2020). More recently, derivatization methods have been used to enhance the MSI of sterols and steroids (Huang et al. 2022; Griffiths et al. 2020). Many steroids possess a keto (oxo) group; this function is readily derivatized by hydrazine reagents to give a hydrazone, and this form of derivatization has been widely exploited in steroid biochemistry for decades to enhance substrate polarity (Fig. 5.4) (Girard and Sandulesco 1936; Wheeler 1968). Two popular derivatization reagents are the Girard T (GT) and Girard P (GP) reagents, which contain a positively charged nitrogen group. These have been used for on-tissue derivatization to enhance the signals for steroids in a number of different tissues. The derivatization reagent can be sprayed onto a tissue slice using a pneumatic sprayer, then the tissue slice is incubated in an acidic atmosphere to catalyze the reaction, and after drying in a desiccator and application of matrix, it is ready for MALDI-MSI or, in the absence of matrix, DESI-MSI. This form of technology has been used for MSI of steroids in rodent adrenal tissue (Takeo et al. 2019; Cobice et al. 2013), testis (Shimma et al. 2016; Cobice et al. 2016), lung (Zecchi et al. 2021), cartilage (Barre et al. 2016), tumor xenografts (Mackay et al. 2021), and brain (Cobice et al. 2018).

Cholesterol and most oxysterols, however, do not possess an oxo group and are not suitable for direct derivatization with hydrazine reagents. This obstacle is overcome for in-solution derivatization by incorporating an enzymatic oxidation step prior to treatment with Girard reagent (Griffiths et al. 2006; Roberg-Larsen et al. 2012) and can be similarly adopted as part of the on-tissue derivatization approach (Angelini et al. 2021; Yutuc et al. 2020). This on-tissue "enzyme-assisted derivatization for sterol analysis" (EADSA) approach has been successfully adopted for imaging cholesterol in rodent and human brains, liver tissue, and in developing zebrafish. In brief, fresh frozen tissue from, for example, a rodent brain is cryo-sectioned to 10–15 μm thickness and thaw-mounted onto glass slides (ITO coated for MALDI-MSI) and dried in a vacuum desiccator, then isotope-labeled standards in ethanol are pneumatically sprayed onto the tissue to allow for normalization of the signal. Bacterial cholesterol oxidase is next sprayed onto the tissue in phosphate buffer, and the tissue is then incubated for 1 h in a humid atmosphere. The cholesterol oxidase enzyme oxidizes 3β-hydroxy-5-ene sterols to their 3-oxo-4-ene analogs (Fig. 5.4) (MacLachlan et al. 2000), and after drying the tissue again in a desiccator, it is sprayed with GP reagent in aqueous acidic methanol, then incubated in an atmosphere of aqueous acidic methanol (Angelini et al. 2021; Yutuc et al. 2020). The tissue is dried once more. For MALDI-MSI, the matrix is sprayed onto tissue prior to analysis; this is not required for DESI-MSI or LESA-MSI (see below).

Shown in Fig. 5.5 (top panel) is an image of cholesterol in a sagittal section of the mouse brain, generated following on-tissue EADSA. This image can be matched to plate 17 of the Allen Mouse Brain Atlas: https://mouse.brain-map.org/static/atlas (Fig. 5.5, bottom panel) (Lein et al. 2007). The image was recorded at 50 μm resolution using AP-MALDI with *m/z* measurement to an accuracy of 10 ppm on an Orbitrap IDX mass spectrometer. Cholesterol is seen to be abundant in fiber tracts, particularly those of the corpus callosum, arbor vitae of the cerebellum, and pons and medulla (see https://mouse.brain-map.org/static/atlas). Images with higher spatial resolution can be recorded, but with the penalty of acquisition time. Here the assumption is made that the ion at 518.4105 ± 10 ppm (Fig. 5.5, top panel) corresponds to cholesterol; this is reasonable as its isomers, lathosterol and zymostenol,

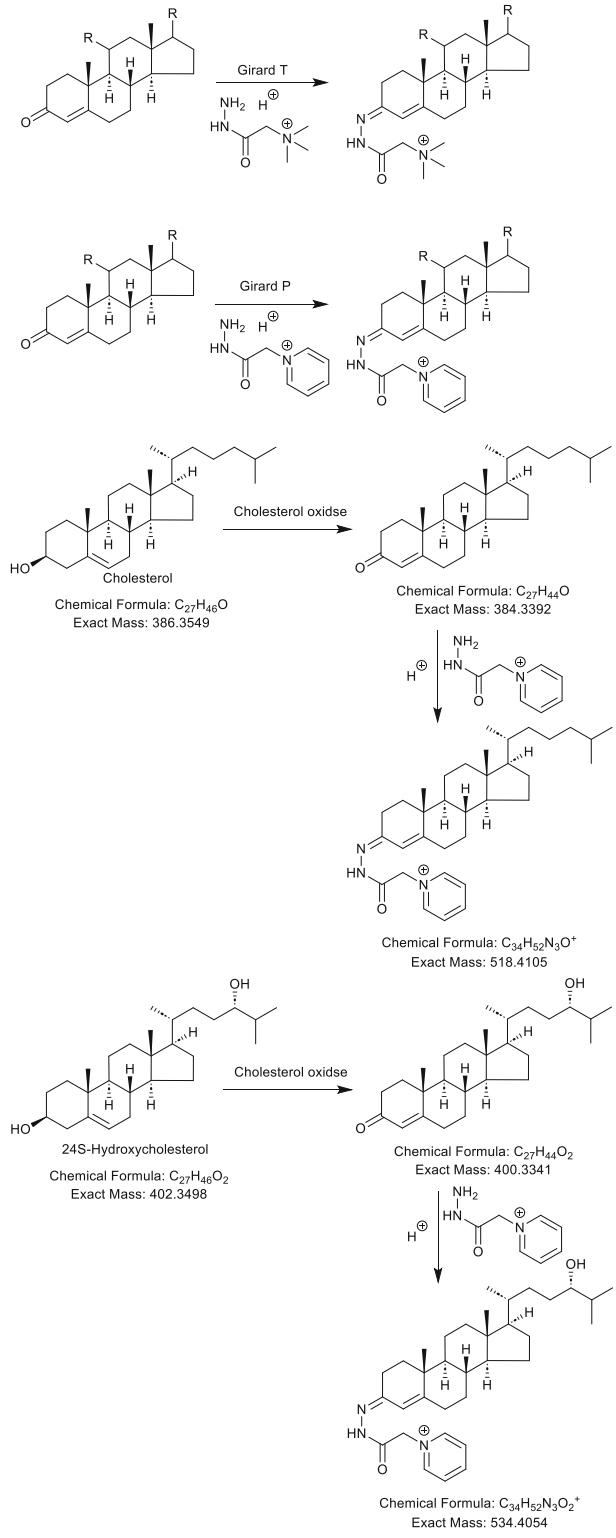

Fig. 5.4 Derivatization of steroids and sterols with the Girard T (GT) and Girard P (GP) reagents. To derivatize sterols without a keto (oxo) group but with a 3β-hydroxy group, cholesterol oxidase is used to oxidize the hydroxy group to a ketone ready for subsequent reaction with Girard reagent

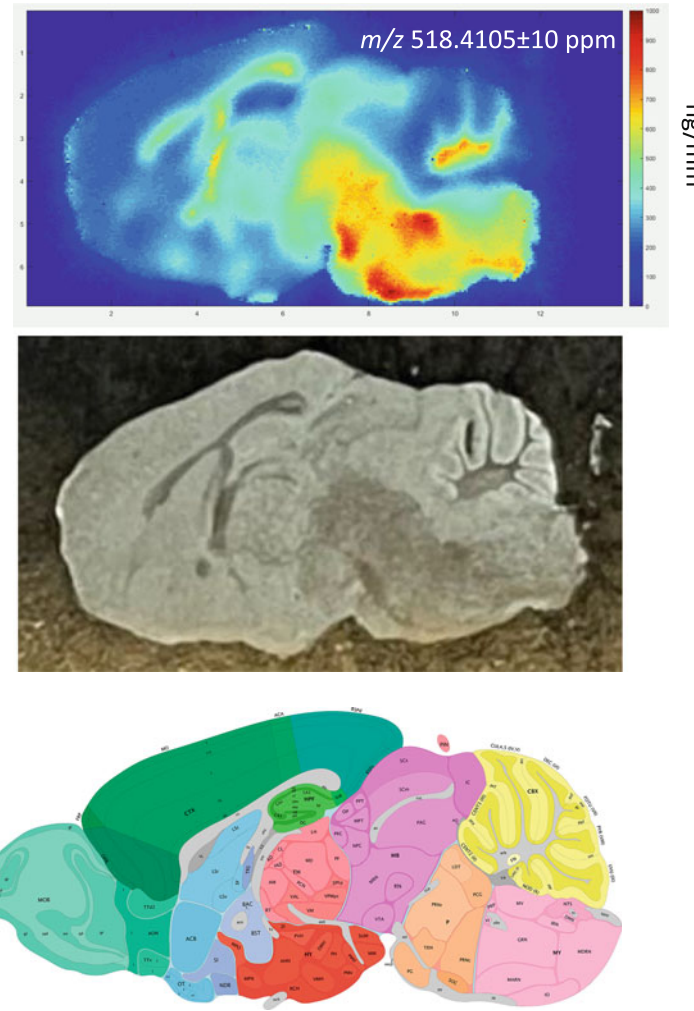

Fig. 5.5 Atmospheric pressure-matrix-assisted laser desorption ionization-mass spectrometry imaging of cholesterol in a sagittal section of the mouse brain following on-tissue EADSA with derivatization using GP reagent. The upper panel shows the image of GP-derivatized cholesterol at *m/z* 518.4105 ± 10 ppm. The central panel depicts an optical image of the same tissue section, and the bottom panel is plate 17 from the Allen Mouse Brain Atlas https://mouse.brain-map.org/static/atlas (Allen Institute for Brain Science) (Lein et al. 2007). *EADSA*, enzyme-assisted derivatization for sterol analysis, *GP* Girard P

are of much lower abundance in the brain (Lütjohann et al. 2004). An added advantage of Girard derivatization is that the derivatized sterols give informative fragmentation patterns in tandem MS (MS/MS or MS^2) and even more information when multistage fragmentation is applied (MS^n) (Yutuc et al. 2021). Where available, this allows the use of MS^3 to generate an image in MALDI-MSI and to confirm that the ion being monitored is in fact derived from cholesterol (Fig. 5.6) (Angelini et al. 2021; Griffiths et al. 2020). A problem encountered in MALDI-MSI is that the tissue surface is exposed to air during sample preparation; this is almost unavoidable as the tissue needs to be cryosectioned and manually mounted on a slide. Exposure to air can lead to the oxidation of cholesterol and the artifactual formation of oxysterol. This makes MALDI-MSI of oxysterols almost impossible unless a fragment ion can be found that is unique to an endogenous oxysterol and absent from MS/MS or MS^n spectra of autoxidation artifacts, in which case MALDI-MSI could be carried out using a multiple-reaction monitoring (MRM) like "scan."

A way to circumvent the problem of distinguishing endogenous compounds from

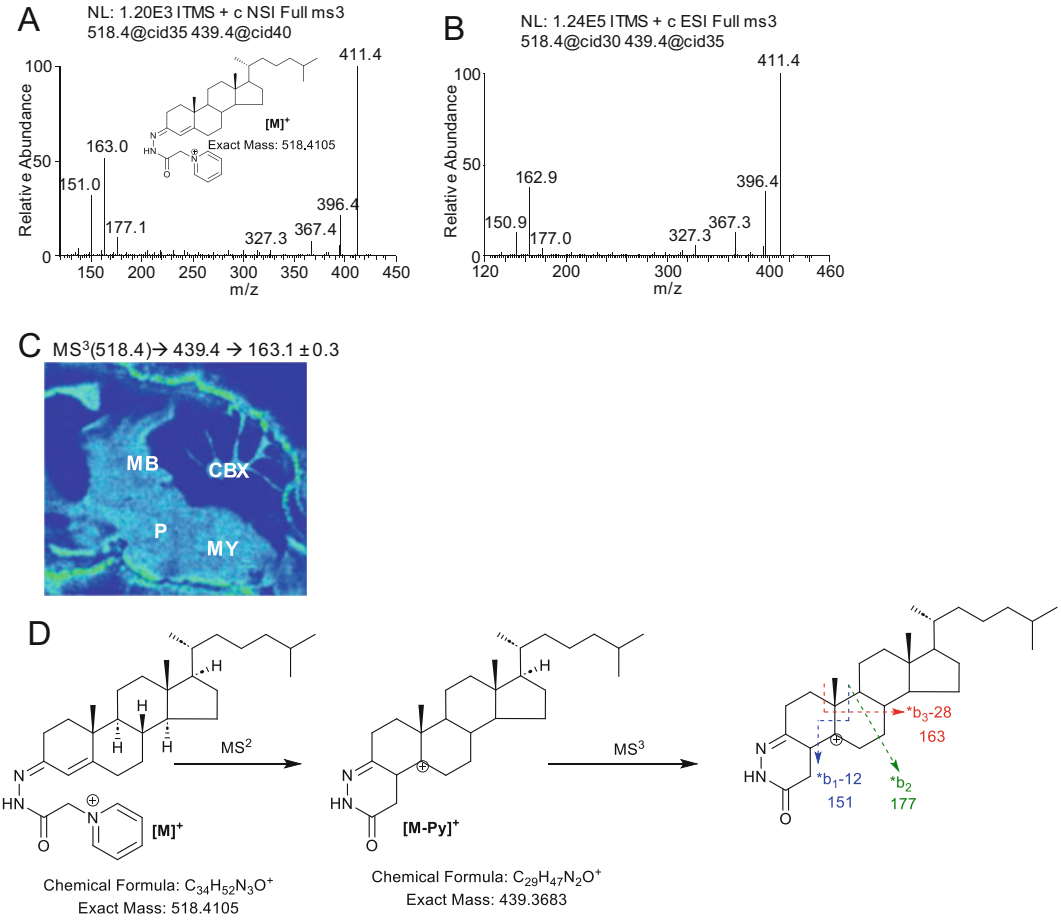

Fig. 5.6 AP-MALDI-MSI of cholesterol following GP derivatization exploiting MS^3 targeting the cerebellum in a sagittal section of the mouse brain. (**a**) MS^3 spectrum of endogenous cholesterol from a single pixel. (**b**) MS^3 spectrum of authentic cholesterol standards. (**c**) Image of cholesterol generated using the MS^3 transition 518 → 439 → 163. (**d**) Fragmentation pattern for GP-derivatized cholesterol. *MB* midbrain, *P* pons, *MY* medulla, *CBX* cerebellum. Modified from Angelini et al. Analytical Chemistry 2021 93 (11), 4932–4943, reference (Angelini et al. 2021), Copyright © 2021, The Authors. Published by the American Chemical Society under CC-BY license. *GP*, Girard P, *MS*, mass spectrometry

isomers potentially formed by autoxidation is to introduce a separation step between surface sampling and MS analysis. One possibility is ion mobility separation, where after ionization ions are separated based on their size, shape, and charge (Kirkwood et al. 2023); however, this has yet to show the separation of sterol isomers. A different strategy is to decouple surface sampling from MS analysis. We have recently taken this approach, where the surface is sampled by a robotic probe as in the LESA technique (Almeida et al. 2015; Hall et al. 2017), and the extracted analytes are separated by LC prior to ESI–MS analysis (Yutuc et al. 2020). We have adopted this strategy following on-tissue EADSA to image mouse brains using a grid format where each pixel corresponds to a LESA extraction followed by LC separation and $MS(MS^n)$ analysis (Yutuc et al. 2020). The pixel size can be 300 μm, but the penalty of chromatographic resolution is

in analysis time, where each LESA-LC-MS(MS^n) run may take 1 h; hence, spatial resolution is usually degraded to 800 μm requiring fewer pixels to cover a mouse brain section, which is the order of about 100 pixels at 800 μm resolution (Fig. 5.7). Despite the long analysis time and comparatively low resolution, a surface can be imaged for individual oxysterol isomers and for cholesterol and its precursors in a single analysis (Yutuc et al. 2020). Shown in Fig. 5.7 is the layout for pixel-by-pixel analysis of a sagittal section of the mouse brain and the resulting images for 24S-hydroxycholesterol (24S-HC), 24S,25-epoxycholesterol (24S,25-EC), cholesterol, and its precursors, desmosterol and 7-DHC/8-DHC. These images correspond to plate 17 in the Allen Mouse Brain Atlas (see https://mouse.brain-map.org/static/atlas). Note that the nose is absent from the tissue section analyzed, and the pixels in the far left column are likely to have been over-sampled due to tissue folding during the mounting process. Nevertheless, 24S-HC is found to be most abundant in the thalamus and stratum regions of the mouse brain, while cholesterol is most abundant in the pons and medulla (Fig. 5.7) (Yutuc et al. 2020). The LESA-LC-MSI method incorporating GP-derivatization has recently been used to support PET imaging studies of CYP46A1, the enzyme that converts cholesterol to 24S-HC in the brain (Haider et al. 2022).

An alternative route for on-tissue derivatization of sterols is to exploit chemical (rather than enzymatic) derivatization of the sterol hydroxy group to a ketone followed by derivatization with hydrazine reagent and MALDI-MSI (Wang et al. 2023) or to derivatize the hydroxy group directly with betaine aldehyde. The latter has been used for DESI-MSI, where betaine aldehyde is included in the ESI spray solvent (Wu et al. 2009).

5.4 Secondary Ion Mass Spectrometry (SIMS): MSI

SIMS provides the highest spatial resolution of MSI. Here a fast-moving ion beam (keV) of metal ions or clusters, for example, C_{60}^+, is focused to give a pixel size of less than 1 μm and results in surface ionization. Primary ion beams tend to lead to high fragmentation of lipids, which is a disadvantage for MSI (Ellis and Soltwisch 2023). NanoSIMS is a variant of SIMS that uses Cs^+ or O^- as the primary ion beam, leading to extensive fragmentation of lipids on the tissue surface but spatial resolutions of 50–100 nm (Brunet and Kraft 2023). A clever application of NanoSIMS is when cells are enriched with ^{18}O-cholesterol by culturing in a medium containing ^{18}O-cholesterol. The cells are then analyzed by NanoSIMS and the $^{18}O^-$ fragment ion monitored (Brunet and Kraft 2023). As plasma membranes are rich in cholesterol, it can be assumed that when imaging these membranes, the $^{18}O^-$ ions detected are derived from the labeled cholesterol. By incubating fibroblasts with ^{18}O-cholesterol, ^{15}N-sphingosine, and ^{15}N-sphinganine, Kraft and colleagues could show that plasma membranes contain domains enriched in ^{15}N-sphingolipids but that ^{18}O is more uniformly distributed, indicating that sphingolipids and cholesterol do not form domains rich in both cholesterol and sphingolipids (Frisz et al. 2013). This argues against the lipid raft hypothesis (Lingwood and Simons 2010).

When SIMS uses a liquid metal ion gun filled with bismuth, a primary beam of Bi_3^+ cluster ions is generated, which provides a softer form of ionization with $[M + H]^+$ and $[M + H - H_2O]^+$ ions being generated from a tissue surface. Lazar et al. (2013) used this form of SIMS-MSI to image cholesterol in brain tissue from human donors and found that cholesterol is elevated in cortical layers III and IV of the cerebral cortex of tissue from AD donors.

5.5 Potential Use of MSI to Study the Involvement of Sterols in Disease

For many years, disordered cholesterol metabolism has been linked with neurodegenerative diseases including AD, Huntington's disease, Parkinson's disease, motor neuron disease, and multiple sclerosis (Corder et al. 1993; Valenza

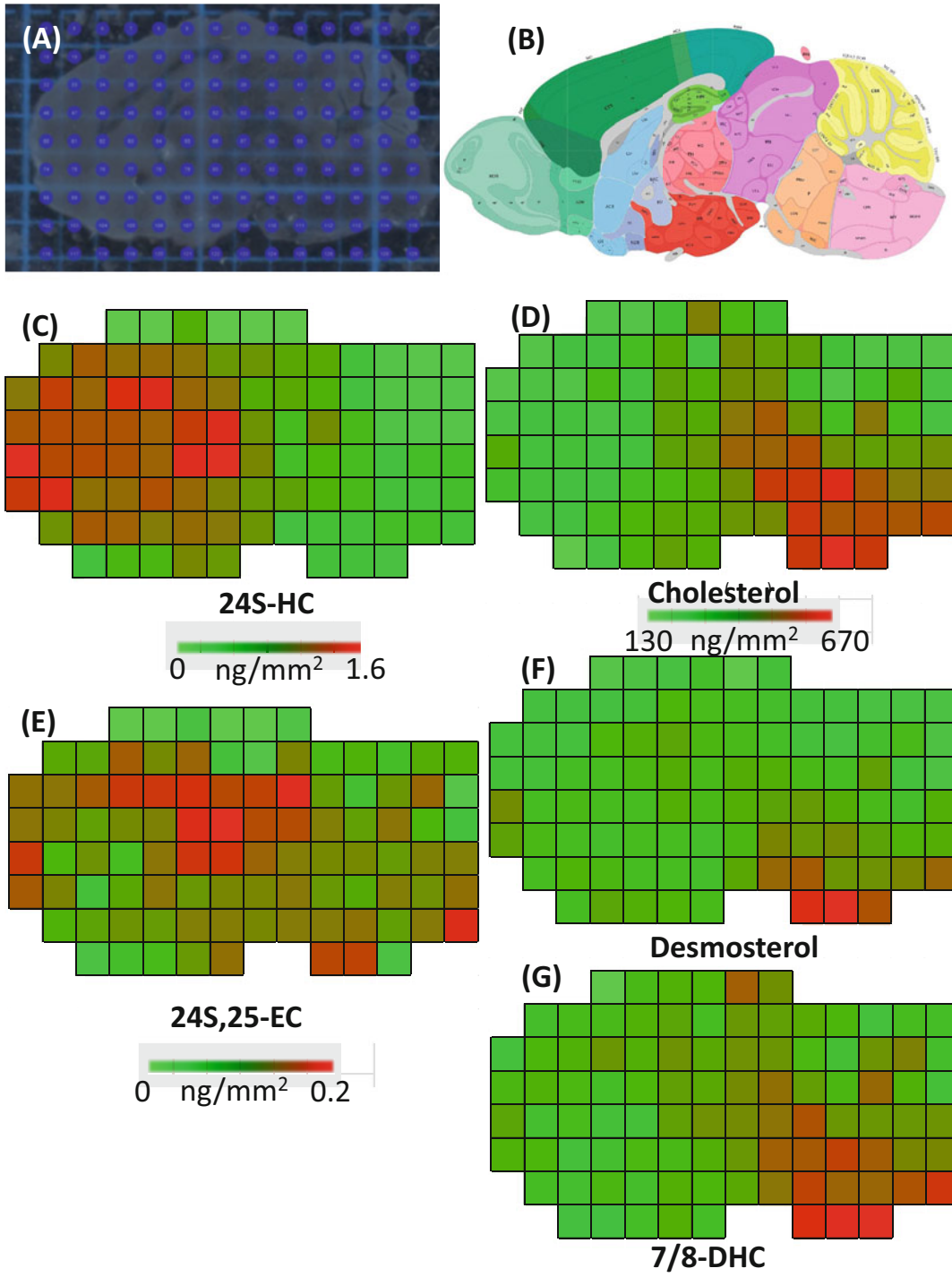

Fig. 5.7 LESA-LC-MSI of cholesterol, its precursors, and oxysterols after on-tissue EADSA with GP derivatization. Identification of analytes was confirmed by retention time, accurate mass, MS^3 spectra, and reference to authentic standards. Quantification was made by isotope dilution against isotope-labeled sprayed-on standards. (**a**) Grid showing each pixel to be analyzed in a sagittal section of the mouse brain. (**b**) Plate 17 in the Allen Mouse Brain Atlas (Allen Institute for Brain Science) (see https://mouse.brain-map.org/static/atlas) (Lein et al. 2007). (**c**) 24S-hydroxycholesterol, (**d**) cholesterol, (**e**) 24S,25-epoxycholesterol, (**f**) desmosterol, and (**g**) 7/8-dehydrocholesterol *EADSA*, enzyme-assisted derivatization for sterol analysis, *GP* Girard P, *LESA* liquid extraction for surface analysis, *LC* liquid chromatography, *MSI* mass spectrometry imaging

et al. 2005; Wang et al. 2021a; Crick et al. 2017; Abdel-Khalik et al. 2017). MSI provides an exciting tool to investigate these disorders in both human donors and mouse models. However, most of the cholesterol in the brain is located in the myelin sheaths (70–80% in rat and mouse brains) surrounding axons and is metabolically inactive (Dietschy and Turley 2004), so while gross changes in oligodendrocyte cholesterol are likely to be detected by MSI, for example, in multiple sclerosis, this is unlikely to be so for metabolically active cholesterol in neurons. MSI for cholesterol precursors and metabolites may prove a better approach to studying neurodegenerative disorders. However, cholesterol precursors and metabolites are about 100–1000 times less abundant than cholesterol in the brain, making their imaging a challenge (Wang et al. 2021b). Perhaps, the LESA-LC-MSI approach incorporating derivatization will prove most successful, as this allows cholesterol and its immediate precursors and metabolites to be analyzed in the brain in a single assay, although admittedly at low spatial resolution (Yutuc et al. 2020).

Sterols are not only implicated in neurodegeneration but also in fatty liver disease, diabetes, atherosclerosis, and inflammatory disorders. MSI will no doubt be used in the study of these diseases and provide a much-needed correlation between sterol concentrations and location.

5.6 Conclusion

MSI has great potential for the analysis of sterols and oxysterols and for investigations of their importance to human health. While most of the studies to date have been performed on rodent models, human tissue can also be analyzed. The major advantage of MSI is that when utilized with appropriate isotope-labeled standards, absolute concentrations of analytes can be determined at defined anatomical locations. While it is possible to generate images of cholesterol at sub-μm pixel sizes, MSI of less abundant sterols and oxysterols is more challenging. The challenge is accentuated by the existence of multiple oxysterol isomers and the prevalence of cholesterol becoming oxidized ex vivo in the air to give oxysterols of identical structure to those generated in vivo. Nevertheless, separation methods are being developed to link with MSI, which will allow isomer separation, although the problem of autoxidation may be more difficult to overcome. A major advantage of MSI is that, besides the analyte of interest, images can be generated for other molecules that ionize and are recorded in the MS scan. From a health perspective, we foresee MSI offering real advantages in the study of neurodegenerative disease, fatty liver, diabetes, and immunity.

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Declaration of Competing Interests

The authors declare the following financial interests, which may be considered potential competing interests: WJG and YW are listed as inventors on the patent "Kit and method for quantitative detection of steroids" US9851368B2. WJG, EY, and YW are shareholders in CholesteniX Ltd.

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Part III

Implication in Aging and Human Diseases

Oxysterols in Central and Peripheral Synaptic Communication

6

Alexey M. Petrov

Abstract

Cholesterol is a key molecule for synaptic transmission, and both central and peripheral synapses are cholesterol rich. During intense neuronal activity, a substantial portion of synaptic cholesterol can be oxidized by either enzymatic or non-enzymatic pathways to form oxysterols, which in turn modulate the activities of neurotransmitter receptors (e.g., NMDA and adrenergic receptors), signaling molecules (nitric oxide synthases, protein kinase C, liver X receptors), and synaptic vesicle cycling involved in neurotransmitters release. 24-Hydroxycholesterol, produced by neurons in the brain, could directly affect neighboring synapses and change neurotransmission. 27-Hydroxycholesterol, which can cross the blood–brain barrier, can alter both synaptogenesis and synaptic plasticity. Increased generation of 25-hydroxycholesterol by activated microglia and macrophages could link inflammatory processes to learning and neuronal regulation. Amyloids and oxidative stress can lead to an increase in the levels of ring-oxidized sterols and some of these oxysterols (4-cholesten-3-one, 5α-cholestan-3-one, 7β-hydroxycholesterol, 7-ketocholesterol) have a high potency to disturb or modulate neurotransmission at both the presynaptic and postsynaptic levels. Overall, oxysterols could be used as "molecular prototypes" for therapeutic approaches. Analogs of 24-hydroxycholesterol (SGE-301, SGE-550, SAGE718) can be used for correction of NMDA receptor hypofunction-related states, whereas inhibitors of cholesterol 24-hydroxylase, cholestane-3β,5α,6β-triol, and cholest-4-en-3-one oxime (olesoxime) can be utilized as potential anti-epileptic drugs and (or) protectors from excitotoxicity.

Keywords

Amyloid beta peptide · Adrenergic receptor · Cholesterol · Epilepsy · Lipid raft · Liver X receptor · Neurodegeneration · Neuromuscular junction · Neurotransmitter release · NMDA receptor · Nitric oxide · Oxysterol · Reactive oxygen species · Synaptic transmission · Synaptic vesicle

Abbreviations

7β-HC	7β-Hydroxycholesterol
7-KC	7-Ketocholesterol

A. M. Petrov (✉)
Laboratory of Biophysics of Synaptic Processes, Kazan Institute of Biochemistry and Biophysics, Federal Research Center "Kazan Scientific Center of RAS", Kazan, RT, Russia

Kazan State Medial University, Kazan, RT, Russia

Kazan Federal University, Kazan, RT, Russia
e-mail: alexey.petrov@kazangmu.ru

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24-HC	24-Hydroxycholesterol
27-HC	27-Hydroxycholesterol
ALS	Amyotrophic lateral sclerosis
AMPA receptor	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CNS	Central nerve system
CSF	Cerebrospinal fluid
ER	Endoplasmic reticulum
GABA	Gamma aminobutyric acid
LTP	Long-term potentiation
LXR	Liver X receptor
NMDA receptor	*N*-Methyl-D-aspartate receptor
NMJ	Neuromuscular junction
NO	Nitric oxide
ROS	Reactive oxygen species
VDAC	Voltage-dependent anion channel

6.1 Oxysterols Connected to Synaptic Transmission

Cholesterol is a major component of synaptic membranes and its high levels are maintained by local synthesis, delivery of membrane materials as a part of transport vesicles from neuronal soma, and endocytotic uptake of cholesterol-rich apolipoprotein particles produced mainly by neighboring astrocytes (Pfrieger 2003; Martin et al. 2014; Krivoi and Petrov 2019). Higher concentrations of cholesterol are present in the synaptic vesicles (Binotti et al. 2021; Takamori et al. 2006), whose numbers vary from several tens to thousands in presynaptic nerve terminals. The majority of synaptic vesicles constitute a high-frequency mobilizable reserve pool and these vesicles are reluctant to be released in response to mild activity (Rizzoli and Betz 2005; Gafurova et al. 2022). Synaptic vesicles of the reserve pool via liquid–liquid phase separation can serve as a mobile depo ("buffer") of vital components (including cholesterol) that are crucially required for neurotransmission and formation of synapses (Denker et al. 2011; Sansevrino et al. 2023). Cholesterol-rich vesicles can be digested via autophagic mechanism locally or directed to the soma for further degradation (Gundelfinger et al. 2022; Kuijpers et al. 2021).

A continuous flux of cholesterol into the synaptic compartment is balanced by the active efflux of cholesterol in form of oxysterols and extracellular vesicles, which contain a high portion of lipid raft fraction that "peels" off from the plasma membranes (Petrov and Pikuleva 2019; Petrov et al. 2019a; Sodero et al. 2012; Mitroi et al. 2019; Kotti et al. 2006; Graykowski et al. 2020; Ouweneel et al. 2020). Finally, ABC transporters mediate cholesterol efflux to accept apolipoproteins, forming a core of lipoprotein particles (Chen et al. 2013; Petrov et al. 2016; Saadane et al. 2018). The main oxysterol that mediates synaptic (as well as whole brain) cholesterol elimination is 24-hydroxycholesterol (24-HC) (Bjorkhem et al. 1998; Lund et al. 1999). The latter is produced by cholesterol 24-hydroxylase (CYP46A1) located in the endoplasmic reticulum (ER) of some neurons (Fig. 6.1), mainly in the hippocampus, cortical layers, and Purkinje neurons of the cerebellum (Ramirez et al. 2008; Cataldi et al. 2023). Endoplasmic reticulum extends into the presynaptic nerve terminals and dendrites (Khan 2022), where excessive synaptic cholesterol is converted to 24-HC. Brain expression of CYP46A1 is highest in the striatum>cortex>hippocampus>cerebellum; and the levels of 24-HC are changed in the same order (Popiolek et al. 2020). There is evidence that production of 24-HC can be increased as a result of normal synaptic activity, inducing long-term potentiation (LTP), and overactivation, leading to glutamate excitotoxicity. Under these conditions, CYP46A1 is able to mobilize toward the synaptic plasmalemma (containing significantly more cholesterol than ER membranes) via a calcium-dependent mechanism probably linked with the formation of membrane contact site of the ER-plasmalemma (Sodero et al. 2012; Mitroi et al. 2019; Brachet et al. 2015). An increase in both neuronal cholesterol synthesis and lipoprotein particle uptake favors the formation of excessive cholesterol in the ER and hence its

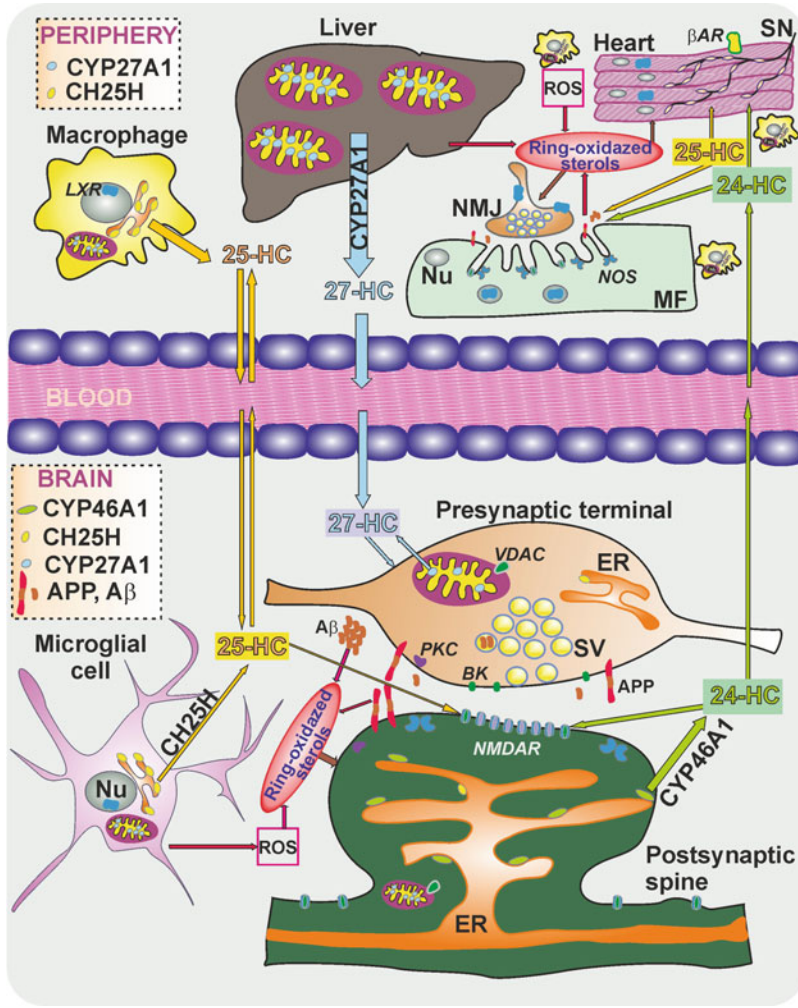

Fig. 6.1 Oxysterols in regulation of synaptic communication. 24-HC is produced by the neuron-specific enzyme CYP46A1 located in the ER. This enzyme can be mobilized to the postsynaptic plasma membrane (upon glutamate receptor activation) causing an increase in production of 24-HC. The latter can act locally on NMDA receptors and potassium BK channels as well as travel to the systemic circulation affecting neuromuscular transmission (via nuclear LXRs and NO synthases) and neurocardiac coupling (via β-adrenergic receptors). Synthesis of 25-HC in ER is increased substantially in microglia and macrophages during inflammatory reactions, and 25-HC can pass the vascular barriers in both directions based on the concentration gradient. In the CNS, 25-HC can influence synaptic transmission targeting NMDA receptors, BK channels, and possibly GABA receptors (not shown), whereas, in periphery, 25-HC is able to modulate neuromuscular transmission and neurocardiac interactions through membrane-bound LXRs and has a direct effect on membrane properties (e.g., lipid raft integrity). 27-HC is mainly generated by hepatocytes and can cross the blood–brain barrier to disturb neuronal communication in central synapses partially via activation of retinoid X receptors γ (not shown). Ring-oxidized sterols can be generated as a result of free radical attack (during oxidative stress), disturbance of cholesterol homeostasis (e.g., like in case of *Cerebrotendinous xanthomatosis*), action of APP or amyloid β peptide in complex with Cu^{2+}. The latter complexes are especially abundant in brain synapses in neurodegenerative diseases. Ring-oxidized sterols can interfere with exocytotic release of neurotransmitters and neurotransmitter reception. Many signaling proteins (including NMDA receptors, β-adrenergic receptors, serotonin 1A receptors, protein kinases, ion channels, and VDACs) as well as changes in lipid raft integrity can mediate the effects of ring-oxidized sterols on synaptic transmission. Abbreviations: βAR, β-adrenergic receptor; Aβ, amyloid β peptide; APP, amyloid β precursor protein; BK, large conductance calcium-activated potassium channel; CH25H, cholesterol 25-hydroxylase; CYP46A1, cholesterol 24-hydroxylase; CYP27A1, cholesterol 27-hydroxylase; ER, endoplasmic reticulum; 24-HC, 24-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; LXR, liver X receptor; MF, muscle fiber; NMJ, neuromuscular junction; NOS, nitric oxide synthase; Nu, nucleus; ROS, reactive oxygen species; PKC, protein kinase C; SN, sympathetic nerve; SV, synaptic vesicle; VDAC, voltage-gated anion channel

metabolism to 24HC. In turn, the resultant decrease in cholesterol levels triggers cholesterol synthesis and uptake of lipoprotein particles by neurons. Such cholesterol turnover can contribute to the maintenance of neuronal and brain metabolic status (Petrov and Pikuleva 2019; Lund et al. 2003). A higher brain metabolic activity can be accompanied by an increase in 24HC generation to speed up the cholesterol turnover, which in itself is a highly energy-consuming process (Russell et al. 2009). In addition, oxidative stress can enhance expression of CYP46A1 and, hence, production of 24-HC (Ohyama et al. 2006). Under pathological conditions, associated with oxidative stress, CYP46A1 expression appears in activated glial cells, particularly, astrocytes and (or) microglia (Smiljanic et al. 2010; Cartagena et al. 2008). As a result, plasma levels of 24-HC can be substantially altered in neurodegenerative disease and brain injuries (Sodero 2021; Leoni and Caccia 2013). For example, plasma 24-HC is a blood biomarker for the severity of neonatal hypoxia–ischemia brain damage and associated functional impairments (Lu et al. 2020).

Reactive oxygen species can directly modify cholesterol (mainly in the B-ring), which is susceptible to non-enzymatic oxidation (Vejux et al. 2020). These oxysterols are represented mainly by 7β-hydroxycholesterol (7β-HC), 7-ketocholesterol (7-KC), and cholestane-3β, 5α, 6β-triol in neuronal cells and brain (Hu et al. 2014; Vejux et al. 2020). Neurotransmission, especially during intense presynaptic nerve activation and even in instances of mild-moderate neuronal firing in pathological conditions, can be accompanied by a significant increase in the production of reactive oxygen species (ROS), whose levels increase in both intracellular and extracellular spaces. Membrane-bound enzymes (e.g., NADPH oxidases) and the mitochondria appear to be major sources of ROS (Tsentsevitsky et al. 2020; Giniatullin et al. 2015; Chen et al. 2001; Carriedo et al. 2000; Oswald et al. 2018). Accordingly, cholesterol molecules residing in the exofacial and cytoplasmic leaflets of the synaptic membranes can be oxidized. Furthermore, amyloid β peptide in complex with Cu^{2+} can serve as an endogenous cholesterol oxidase (Puglielli et al. 2005), metabolizing cholesterol to 4-cholesten-3-one that can be sequentially converted to 5α-cholestan-3-one and cholestanol (Ute et al. 2007). Amyloid β peptide is normally produced in low concentrations and can be released into the synaptic cleft or accumulated intracellularly. Clearance of amyloid β peptide can occur through its digestion by peptidases or transport by lipoprotein particles together with excessive cholesterol (Petrov et al. 2017; Burrinha et al. 2021; van der Kant et al. 2020). In certain pathological conditions (e.g., Alzheimer's disease), the levels of amyloid β peptide as well as cholesterol oxidation products (4-cholesten-3-one, 7-KC, and 7β-HC) greatly increase (Puglielli et al. 2005; Nury et al. 2020). Evidently, this enzymatic action of amyloid β peptide should be more clearly expressed if the peptide is internalized during endocytosis into the synaptic vesicles, which are cholesterol-rich and contain Cu^{2+}.

Mitochondria occupy a substantial volume in the presynaptic and postsynaptic compartments (Delgado et al. 2019). Their activity is very high due to the energetic cost of synaptic transmission (Justs et al. 2022; Faria-Pereira and Morais 2022). Mitochondrial enzyme CYP27A1 converts mitochondrial membrane cholesterol to 27-hydroxycholesterol (27-HC) (Pandak et al. 2002). Also, excess (active) plasmalemmal cholesterol can rapidly reach mitochondria, stimulating 27-HC biosynthesis (Lange et al. 2009). The activity of this enzyme is much less in the brain than in liver preparations (Pedersen et al. 1989), as 27-HC is an intermediate in bile acid synthesis. Mitochondrial cholesterol flux and efflux in the form of 27-HC could shape the mitochondrial electron transport chain activity, since an accumulation of cholesterol in the mitochondria can disrupt its overall performance and organization of the respiratory supercomplexes assembly (Solsona-Vilarrasa et al. 2019). Mitochondrial cholesterol accumulation sensitizes neurons to amyloid β peptide-induced toxicity and promotes cognitive decline in neurodegenerative disease models (Torres et al. 2019). Hypothetically, active neurotransmission fueled

by mitochondrial ATP production could be accompanied by 27-HC generation. In this scenario, 27-HC can serve as a potential modulator of the loop coupling the neurotransmission and mitochondria function (Fig. 6.1).

A specific ER-derived oxysterol that is produced in trace amounts in all cells for precise control of local cholesterol homeostasis is 25-HC (Odnoshivkina et al. 2022; Cyster et al. 2014). In the brain, expression of cholesterol-25-hydroxylase increases in activated microglial cells (Wong et al. 2020; Lee et al. 2022). Activation of microglial cells occurs as a component of neuroinflammation and can be a response to synaptic hyperactivity as well as induction of long-term depression (Henson et al. 2017; Dissing-Olesen et al. 2014; Wu et al. 2015). Microglia mediate synaptic pruning, causing a downscaling of connectivity between the pre- and postsynaptic neurons (Guedes et al. 2022; Ball et al. 2022). Hence, 25-HC might be a signaling avenue from microglia/macrophages to changes in neurotransmission (Fig. 6.1).

6.2 24-Hydroxycholesterol as Endogenous Modulator of Neurotransmission in Central and Peripheral Synapses: The Role of NMDA Receptors, Nitric Oxide, and Liver X Receptors

6.2.1 Effects and Targets of 24-Hydroxycholesterol in Synapses of CNS

6.2.1.1 Synaptic Effects of CYP46A1 Modulation

A possible role of 24-HC in synaptic transmission has been suggested by Tiina Kotti and colleagues (Kotti et al. 2006). It has been reported that mice lacking cholesterol 24-hydroxylase had defects in their spatial, associative, and motor learning as well as in LTP. The deficiency in LTP was reversed by acute treatment of hippocampal slices with geranylgeraniol or administration of geranylgeraniol via the recording electrode directly into the dendritic trees of CA1 hippocampal neurons. This suggested that an activation of the mevalonate pathway due to enhanced brain cholesterol turnover (increased neuronal cholesterol elimination in the form of 24-HC and a compensatory increase in cholesterol synthesis) maintains a constant production of geranylgeraniol required for postsynaptic mechanisms of LTP (Kotti et al. 2006, 2008). CYP46A1-driven increase in geranylgeraniol also induced dendritic outgrowth and synaptogenesis in cultured cortical neurons partially via enhancement of geranylgeranylation of signaling proteins, e.g., small GTPases (Moutinho et al. 2016). In addition, changes in CYP46A1 activity can lead to presynaptic changes. Indeed, inactivation and stimulation of 24-hydroxycholesterol production caused a decrease and increase in synaptosomal membrane ordering, respectively. Furthermore, activation of CYP46A1 enhanced sucrose-induced glutamate release but suppressed high K^+-depolarization-mediated neurotransmitter release from the synaptosomes. This can reflect an increase in exocytosis of the synaptic vesicles from ready releasable pool, but a decrease in the involvement of synaptic vesicles from reserve pool in neurotransmission (Petrov et al. 2020). The latter might be a protective mechanism against excitotoxicity. These data are consistent with an increase in the levels of key pre- and postsynaptic proteins (Munc13-1, PSD-95), changes in many synaptic protein phosphorylation (e.g., Piccolo, RIMS1, N-type Ca^{2+} channel, GAP43, DPYSL2, and MARCKS) as well as behavioral improvements upon chronical treatment of 5XFAD mice (Alzheimer's disease model) with a CYP46A1 activator (Petrov et al. 2019b, c). It seems that the enhanced conversion of cholesterol to 24-HC can decrease the overactivity of CDK5 and GSK3β kinases thereby improving the pattern of glutamate release in the synaptosomes from brain of 5XFAD mice (Petrov et al. 2020).

In another study by Alejandro Sodero and colleagues (Sodero et al. 2012) it was reported that glutamate *N*-methyl-D-aspartate (NMDA) receptor activation for 30 min caused a slight but significant cholesterol loss (accompanied by

24-HC upregulation) due to a calcium-dependent CYP46A1 mobilization toward the plasma membrane in hippocampal neurons. Similarly, LTP triggered NMDA receptor-dependent cholesterol loss that was required for GTPase activation and then delivery of the new glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors from the endosomes to the synaptic membranes in hippocampal slices (Brachet et al. 2015). During induction of LTP, sterol carrier protein NPC1 via an unidentified mechanism can increase the availability of cholesterol for CYP46A1, leading to 24-HC production and a decrease in cholesterol in the synaptic membranes. Pharmacological activation of CYP46A1 was able to decrease synaptic levels of cholesterol and improve LTP as well as cognitive abilities in $NPC1^{nmf164}$ mice, a model of Niemann–Pick disease type C (Mitroi et al. 2019). In neuronal cell studies, glutamate treatment (due to stimulation of NMDA receptors and metabotropic glutamate receptor type 5) increased CYP46A1 gene expression likely via the interaction of transcriptional factor CREB with CYP46A1 promoter (Zhang et al. 2020). Notably, stimulation of cholesterol conversion to 24-HC by pharmacological (efavirenz at low dose) or genetical means (adeno-associated vector encoding CYP46A1) had beneficial effects on synapses and behavioral aspects in models of neurodegenerative diseases, including Alzheimer, Huntington, Niemann–Pick, prion diseases, and spinocerebellar ataxia (Mitroi et al. 2019; Petrov et al. 2019b, 2020; Ali et al. 2021; Kacher et al. 2019; Hudry et al. 2010; Nobrega et al. 2019). The contribution of 24-HC itself in these therapeutic actions as well as synaptic plasticity is still unclear, since brain cholesterol turnover is also elevated under these conditions.

6.2.1.2 Discovery of Glutamate NMDA Receptors as a Target for 24-Hydroxycholesterol

A pure synaptic action of 24-HC has been described by Steven Paul and colleagues (Paul et al. 2013), where it was demonstrated that 24-HC is a potent and selective positive allosteric modulator of NMDA receptors, which acts via mechanisms different from those of other allosteric modulators. Indeed, 24-HC at sub-micromolar concentrations (10 nM–10 μM, with EC_{50} ~1.2 μM) potentiated NMDA-evoked currents, enhanced LTP (marginally at 0.1 μM and robustly at 1 μM) in hippocampal slices as well as reversed deficits of both LTP and behavioral performance induced by ketamine (an NMDA receptor blocker). Chimeric receptor study suggested that the 24-HC-mediated positive modulation of NMDA receptors requires solely the transmembrane domain of GluN2 subunits in combination with GluN1 subunits (Wilding et al. 2016). Probably, 24-HC inserted in the plasmalemma can interact with a unique site of the transmembrane domain of GluN2 subunits. A recent study using a site-directed mutagenesis revealed that the structural determinants of 24-HC action reside within pre-M1 linker and M3 transmembrane helix in both GluN1 and GluN2 subunits. In addition, the primary mechanism of the enhancement of NMDA receptor current is probably related to an increase in NMDA receptor channel open probability without altering conductance (Tang et al. 2023). In the dentate gyrus, it appeared that 24-HC (1 μM) specifically increased tonic currents via GluN2B containing NMDA receptors, but surprisingly 24-HC lost this effect in CYP46A1 knockout mice (Wei et al. 2019). GluN2B-NMDA receptors predominantly reside at the extra-synaptic sites and these receptors are targets for clinically important drugs, e.g., ketamine and memantine (Miller et al. 2014; Xia et al. 2010; Zanos and Gould 2018). Missense GRIN2B variations that decrease GluN2B activity are accompanied by intellectual disabilities, autism spectrum disorder, and development delays. In vitro 24-HC and its analogs can enhance the amplitude of NMDA receptor-mediated currents in the loss-of-function variants of GRIN2B (Tang et al. 2020, 2023).

To date, the question about the role of endogenous 24-HC remains valid. In CYP46A1 knockout mice, NMDA receptor "tone" (defined as a ratio of NMDA receptor to AMPA receptor postsynaptic currents) was reduced in hippocampal slices, leading to a decrease in NMDA receptor-driven spikes. Also, these slices had a higher

resistance to the persistent NMDA receptor-dependent depression of neurotransmission induced by oxygen-glucose deprivation (a model of pathophysiological overstimulation of NMDA receptors due to ischemic-like challenge). This effect on the depression of neurotransmission was restored by a 24-HC analog, specifically, 1 μM SGE-301. At the same time, spontaneous neurotransmission, neuronal intrinsic excitability, and LTP were indistinguishable from the hippocampal slices of wild-type mice (Sun et al. 2016). Accordingly, it was concluded that the decrease in NMDA receptor tone can protect against NMDA receptor-mediated dysfunction upon excessive glutamate release (Sun et al. 2016). Consistent with that finding, exogenous (2 μM) and endogenous 24-HC exacerbated NMDA receptor overactivation-induced excitotoxicity in primary neuron cultures following oxygen-glucose deprivation (Sun et al. 2017). Additionally, at higher concentrations (<10 μM) and prolonged applications (24 h), 24-HC can exert neurotoxic properties and provoke oxidative stress in neuronal cell lines (Noguchi et al. 2015). However, endogenous 24-HC can also have a protective action. In the retina, 24-HC was neuroprotective against pressure-mediated retinal degeneration in rat ex vivo glaucoma model, and its synthesis in ganglion cells increased in response to an increase in the pressure, implying the role of 24-HC in the compensatory mechanism (Ishikawa et al. 2016). Interestingly, the beneficial effects of 24-HC appeared at 1 μM in the presence of endogenous synthesis, and 30 μM in combination with inhibition of CYP46A1 that itself caused severe neuronal retina damage (Ishikawa et al. 2016). Note that 30 μM is in the physiological range in human brain homogenates (Lutjohann et al. 1996). The neuroprotective effect of 24-HC relied partially on the increase in allopregnanolone (a neurosteroid) synthesis which was downstream of elevation of NMDA receptor activity in the retina (Ishikawa et al. 2018). In the pressure-loaded retina, NMDA receptor-dependent increase in allopregnanolone production can diminish retinal degeneration via positive modulation of inhibitory gamma aminobutyric acid (GABA)-A receptors (Ishikawa et al. 2014).

6.2.1.3 Using 24-HC and Its Analogs for Correction of NMDA Receptor Hypofunction

Positive NMDA receptor modulators can be therapeutic to correct a hypofunction of NMDA receptors (Tang et al. 2020; Geoffroy et al. 2022), e.g., anti-NMDA receptor encephalitis (NMDARE), an autoimmune disease associated with GluN1 antibody-mediated NMDA receptor internalization. 24-HC mimetic molecules (SGE-301 or SGE-550) were able to restore NMDA receptor function in a model, where rat hippocampal neurons were incubated (48 h) with the cerebrospinal fluid (CSF) of NMDARE patients (Warikoo et al. 2018). Next, it was found that SGE-301 prevented NMDARE patient's antibody-mediated pathogenic effects (memory deficits, decrease in synaptic NMDA receptor clusters, impairment of LTP) in mouse models, despite that this 24-HC mimetic molecule did not antagonize the antibody binding to NMDA receptors. Neuronal cell experiments showed that SGE-301 prolonged an open time of the NMDA receptor channel and decreased internalization of antibody-bound NMDA receptors (Mannara et al. 2020). Similar results were obtained in another study evaluating the effect of SGE-301 on synaptic and memory defects induced by injection (via osmotic pump connected to the cerebroventricular system of mice) of CSF from NMDARE patients (Radosevic et al. 2022). Noticeably, SAGE718, a 24-HC derivative (Hill et al. 2022) is proposed to improve cognitive performance in healthy volunteers who were administered ketamine (Geoffroy et al. 2022) and it is now in Phase 2 trial in patients with Huntington disease (NCT05107128). 24-HC at the range of 0.1–10 μM as well as its analogs (10 μM SGE-301 and 10 μM SGE-550) potentiated glutamate-induced currents (in the presence of glycine) via NMDA receptors with disease-associated loss-of-function GRIN variants expressed in *Xenopus oocytes* (Tang et al. 2020,

2023). In addition, 24-HC (1 μM) and SGE-301 (1 μM) overcame the depressant effects of ethanol (60 mM) on NMDA receptor-mediated synaptic responses in hippocampal slices and SGE-301 rescued from ethanol-evoked defects in both LTP and one-trial inhibitory avoidance learning (Izumi et al. 2021a).

6.2.1.4 Inhibition of 24-Hydroxycholesterol Production as Strategy to Overcome Consequences of NMDA Receptor Hyperactivation

Excessive positive modulation of NMDA receptors, particularly containing GluN2B subunits, can increase the severity of epileptic activity (Loss et al. 2019; Okuda et al. 2017), since a decrease in 24-HC levels was assessed in several studies as an anticonvulsive approach. Furthermore, kainite treatment (a model of acute brain seizures) in vivo increased the concentration of 24-HC (>50 μM) up to toxic levels and lovastatin (an inhibitor of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis) was able to decrease both 24-HC content and neuronal loss in rat hippocampus (He et al. 2006). Soticlestat (TAK-935/OV935), a cholesterol 24-hydroxylase inhibitor, suppressed excessive glutamate release in response to high K^+ depolarization in the hippocampus of APP/PS1-Tg mice, characterized by excitatory/inhibitory imbalance and seizure-related sudden death (Nishi et al. 2020). The anticonvulsive properties of soticlestat were confirmed in different rodent models, where the anticonvulsive efficacy of soticlestat was correlated with 24-HC lowering in the brain and it required a decrease in 24-HC levels by 50–60% (Nishi et al. 2022).

Expression of CYP46A1 was higher in neurons in human temporal lobe epileptic *foci* and in neurons of the hippocampus in epileptic mice. Treatment with soticlestat decreased the loss of hippocampal CA1 neurons and hilar mossy cells without affecting astrocyte and microglia activation in epileptic mice (Salamone et al. 2022). Inhibition of 24-HC production was effective even in case of Dravet syndrome model (a severe developmental and epileptic encephalopathy due to de novo pathogenic variants in SCN1A). Indeed, soticlestat decreased seizure burden, protected against hyperthermia-induced seizures, and completely prevented the sudden unexplained death in epilepsy of these mice (Hawkins et al. 2021). A phase 2 study confirmed that soticlestat treatment caused reductions from baseline in median seizure frequency (combined cohort of Dravet syndrome and Lennox–Gastaut syndrome) and convulsive seizure frequency (Dravet syndrome cohort). During this treatment plasma 24HC levels decreased (by ~70%) over the first 2 weeks of treatment and then remained stable at the reduced levels (Hahn et al. 2022).

There are some possible caveats for use of 24-HC lowering therapy for treatment of epileptic seizures. One of them is the role of 24-HC in long-term depression (LTD), which hypothetically can counteract epileptic activity. Indeed, pharmacological inhibition of CYP46A1 causing a reduction of 24-HC in vivo led to ablation of LTD in hippocampal slices. Importantly, LTD was fully restored by the acute application of exogenous 24-HC at a concentration of 10 μM (Popiolek et al. 2020). As the authors discussed LTD could be more sensitive to the changes in NMDA receptor function triggered by locally produced 24-HC. However, electrical and *N*-methyl-D-aspartate-induced LTD did not alleviate low-Mg^{2+}-induced epileptiform activity in the entorhinal cortex (Solger et al. 2005). Also, CYP46A1 inhibition-induced changes in brain cholesterol levels can improve insulin signaling and restore insulin-evoked LTD hippocampus, which are impaired in aged mice (Martin-Segura et al. 2019).

Large conductance calcium-activated potassium channels (BK channels) are abundant in areas related to epilepsy, and paradoxically their blockers and openers demonstrated efficacy as potential anti-epileptic drugs. The BK channels are a potential target of 24-HC in synapses (Tajima et al. 1864). These channels are functionally coupled to voltage-gated Ca^{2+} channels in the active zone (sites of exocytosis) of presynaptic nerve terminals and activation of BK channels after Ca^{2+} influx quickly hyperpolarizes the

membranes, shutting the calcium channels and, hence, limiting neurotransmitter release (Griguoli et al. 2016). 24-HC (IC_{50} of ~2 μM) inhibited Slo1 BK channels expressed in HEK293T cells and sterol scavenger γ-cyclodextrin can rescue slo1 BK channels from the inhibition (Tajima et al. 1864). However, a relevance of this 24-HC action in synapses remains uninvestigated.

6.2.2 A Possible Neurohumoral Action of 24-Hydroxycholesterol on Peripheral Neurotransmission

6.2.2.1 The Role of NMDA Receptors and Nitric Oxide Synthases in Transduction of 24-Hydroxycholesterol Signal in Neuromuscular Junctions

The study of Steven Paul et al. (2013) demonstrated that NMDA receptors in hippocampal neurons are a target for 24-HC. These receptors are also expressed in peripheral synapses, particularly, in regions adjacent to the postsynaptic membrane in mammalian neuromuscular junctions (NMJs) (Mays et al. 2009; Malomouzh et al. 2011). Herein, these receptors play an essential role in the normal elimination of excessive innervation of each NMJ during muscle development and re-establishing functional connections (transition from polyneuronal to mono-innervation) after nerve crush in adult mice (Personius et al. 2016, 2022). In addition, activation of the NMDA receptor via a calcium-dependent mechanism can increase the activity of NO synthases, and NO might act as retrograde messenger (for regulation of neurotransmitter release) as well as secondary messenger in muscle fibers (for control protein synthesis and contraction) (Malomouzh et al. 2003; Pinard and Robitaille 2008; Kumar et al. 2022; Tyganov et al. 2021; Petrov et al. 2013). Endogenously, *N*-acetylaspartylglutamate can be released from synaptic vesicles and then be converted to glutamate and *N*-acetylasparate by enzymes present on the terminal Schwann cells in the cholinergic NMJs (Walder et al. 2013). In addition, high micromolar concentrations of glutamate and glycine are present in the plasma (Shimmura et al. 2011). These create the conditions for "facilitated" activation of peripheral NMDA receptors. Note that extracellular levels of glutamate could change during muscle activity and regeneration, and local macrophages can produce and utilize glutamate during metabolism (Shang et al. 2020; Yawata et al. 2008; Henriksson 1991). Hence, the influence of 24-HC on NMDA receptor of the NMJs can open the avenue for humoral connection of brain cholesterol metabolism with the regulation of skeletal muscles. Plasma levels of 24-HC are mostly dependent on its production in brain and there is no marked expression of CYP46A1 in skeletal muscle (Popiolek et al. 2020; Lutjohann et al. 1996).

It has been found that acute and relatively short (1/3 h) application of 24-HC (0.4–4 μM) increases neurotransmitter release, synaptic vesicle mobilization and decrease in NO production during synaptic activity, whereas glutamate (in the presence of glycine) has an opposite influence and prevents these effects of 24-HC in the NMJs of mice. Furthermore, selective antagonists of NMDA receptors potentiated the stimulatory action of 24-HC on neuromuscular transmission (Kasimov et al. 2017). The enhancement of presynaptic exocytosis by 24-HC was mediated by a decrease in NO production mainly by endothelial NO synthase (Kasimov et al. 2017). This enzyme is able to interact with lipid raft scaffold protein caveolin, which is also essential for anchoring NMDA receptors in lipid microdomains and has a strong postsynaptic residence at the NMJs (Head et al. 2008; Carlson et al. 2003; Hezel et al. 2010). Hence, 24-HC has an antagonistic function as compared to NMDA receptors in the NMJs. At the same time, there is a convergence of 24-HC and NMDA receptor signaling on NO synthase, which could occur due to an interaction of endothelial NO synthase and NMDA receptors within lipid microdomains. Indeed, lipid raft disrupting manipulations prevented 24-HC-mediated decrease in NO production during neuromuscular activity. Furthermore, 24-HC (0.4 μM) was able to increase the labeling of

synaptic membranes with lipid raft marker (Mukhutdinova et al. 2018). In this scenario, it is possible that 24-HC can stabilize lipid rafts, whose integrity contributes to inhibition of endothelial NO synthase (Garcia-Cardena et al. 1997; Odnoshivkina et al. 2017) and can affect synaptic vesicle mobilization (Tsentsevitsky et al. 2023). Hence, 24-HC-mediated decrease in NO production removes "brake" from synaptic vesicle exocytosis and mobilization. Note that the stimulatory action of 24-HC on neurotransmission can be blocked by intracellular acidification in both mice and frog NMJs (Zefirov et al. 2020).

6.2.2.2 A Possible Role of 24-Hydroxycholesterol/Nitric Oxide Link in Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, causing skeletal muscle denervation and progressive loss of motor neurons. In many cases, the pathology has a "dying back" character, starting with NMJ dysfunction that spreads to the motor neuron cell body (Verma et al. 2022; Coleman 2022; Mukhamedyarov et al. 2023). Enhanced NO synthase expression was found in synapses in the anterior horn neurons in sporadic ALS patients (Sasaki et al. 2001). Also, a much stronger NO production occurred during neurotransmission in NMJs of SODG93A mice, a model of ALS (Mukhutdinova et al. 2018). As a result, nitrosative stress can trigger aggregation of the RNA-binding protein TDP-43 (a major component of aberrant cytoplasmic inclusions) and cell-to-cell spread, leading to neuronal damage in ALS. S-nitrosylation of TDP-43 was observed in postmortem ALS brains of humans and hiPSC-derived motor neurons (Pirie et al. 2021). Exposure to NO leads to death of embryonic motor neurons from ALS model mice, but not from wild-type mice (Duplan et al. 2010). Chronical treatment with an inhibitor of NO synthases reduced the loss of inhibitory synapses on motor neuron somas in SODG93A mice (Sunico et al. 2011). 24-HC (0.4 μM) was able to strongly suppress the excessive NO production during synaptic activity at the NMJs of SODG93A mice (Mukhutdinova et al. 2018). Additional data demonstrated that intravenous injection of AAV-CYP46A1 can decrease the decline in skeletal muscle performance and motor function as well as preserve NMJs and motor neurons in SODG93A mice (patent US20220054597A1). Note that plasma and CSF levels of 24-HC were reduced in ALS patients, suggesting that a decrease in 24-HC can contribute to disease progression (La Marca et al. 2016; Abdel-Khalik et al. 2017; Di Natale et al. 2023).

6.2.2.3 Nuclear Liver X Receptor as Target of 24-Hydroxycholesterol in Neuromuscular Junctions

Nuclear liver X receptors (LXRs) are targets for endogenous oxysterols, including 24-HC (Janowski et al. 1999). However, the elevation of 24-HC production (4–7-fold) in transgenic mice expressing human CYP46A1 did not activate LXR target genes in the brain (Shafaati et al. 2011). Probably, this can suggest that synaptic sites located far from the neuronal soma are main sources of 24-HC which can be quickly eliminated from brain to bloodstream. Alternatively, brain LXRs might have a lower sensitivity to this oxysterol. At the same time, under pathological conditions pharmacological stimulation of 24-HC production was able to suppress glioblastoma growth partially via LXRs (Han et al. 2020).

Postsynaptic region of NMJs had a unique organization with infoldings and underlying subsynaptic nuclei (close to postsynaptic receptors), which are essential for stabilization of the end plate (postsynaptic) region and programming of the muscle fiber metabolic activity. Nerve-evoked electrical activity specifically regulates many genes in subsynaptic nuclei in the muscle fibers (Belotti and Schaeffer 2020; Ravel-Chapuis et al. 2007). LXRβ are pivotal regulators of skeletal muscle lipogenesis and cholesterol homeostasis (Archer et al. 2014). An increase in energy expenditure and a switch from glucose to lipid oxidation occurred in the skeletal muscles of $LXR\beta^{-/-}$ mice (Archer et al. 2014). In addition, $LXR\beta^{-/-}$ mice suffer ALS-like pathology accompanied by impaired motor coordination, decrease in NMJs as well as

inflammation, lipid (cholesterol) accumulation, and loss of motor neurons in the spinal cord (Andersson et al. 2005; Bigini et al. 2010). The genetic ablation of both LXR isoforms (α and β) also provoked locomotor defects that correlated with oxidative stress in the sciatic nerves of mice (Hichor et al. 2018). These data imply the importance of LXRs for peripheral nerves, NMJs, and motor neurons. It has been found that prolonged application (2½ h) of 24-HC (0.04–0.4 μM) leads to an increased expression of neuronal and endothelial isoforms of NO synthases, which was accompanied by an increase in NO synthesis (in NMJ region) during neurotransmission and, hence, depression of synaptic vesicle recruitment into neurotransmitter release at the NMJs. These effects of prolonged administration of 24-HC were completely blocked with selective LXR antagonist (Mukhutdinova et al. 2019). Furthermore, chronical injections of CYP46A1 inhibitor for 5 days (but not its action on *ex vitro* skeletal muscle for 2½ h) had an opposite effect than that of the prolonged application of 24-HC. Accordingly, there is a possibility of a negative control of neuromuscular transmission by brain-derived 24-HC via LXR- and NO-dependent genomic mechanisms. This can rescue NMJs from overexcitation during intense activity and be essential for the preservation of the synaptic vesicle pool capacity. Note that the ability of LXRs to increase NOS expression seems to be important for the attenuation of TNFα-evoked impairment of endothelium-dependent relaxation of aortic rings in response to the neurotransmitter acetylcholine (Spillmann et al. 2014).

6.2.2.4 Effects of 24-Hydroxycholesterol on β-Adrenergic Signaling: A Possible Implication in Neurocardiac Coupling

Blood vessel tone as well as heart rhythm and contractility are controlled via quasi-synaptic junctions, belonging to autonomic nerve system (Franzoso et al. 2016; Odnoshivkina and Petrov 2021; Di Natale et al. 2021). Muscarinic acetylcholine receptors and adrenergic receptors serve as main postsynaptic transducers. Acute application of 24-HC (4 nM–1 μM) markedly depressed the effects of β-adrenergic activation on contractility and intracellular Ca^{2+} transient in atrial cardiomyocytes. The underlying mechanism was related to an increase in stimulatory action of β2-adrenergic receptors on phosphodiesterase 4 activity (Odnoshivkina et al. 2019). The latter is key "break" for β1-adrenoceptor signaling (the main contributor to stimulatory action of catechol amines on cardiomyocytes) via hydrolysis of cAMP in cardiomyocytes (Bhogal et al. 2018; Shi et al. 2017; Xiang 2011). According to this scenario, 24-HC can serve as a brain-derived humoral factor in the plasma that can limit cardiomyocyte overactivation in response to β-adrenergic receptor stimulation due to a higher sympathetic drive. Overactivation of adrenergic receptors is associated with heart failure, hypertension, arrhythmias and increases the risk of sudden death (Odnoshivkina and Petrov 2021; Bardsley and Paterson 2020). Noticeably, β2-adrenergic receptors reside in lipid rafts, whereas phosphodiesterase 4 can be recruited to the lipid rafts (Bhogal et al. 2018). One possibility is that 24-HC can favor an interplay of β2-adrenergic receptors and phosphodiesterase 4 within the lipid microdomain. At least, 24-HC was able to increase the labeling of the cardiomyocyte membrane with a lipid raft marker (Odnoshivkina et al. 2019). Alternatively, 24-HC can interact directly with cholesterol-binding sites of β-adrenergic receptors. Cholesterol stabilizes various structural regions of β2-adrenergic receptors with the strongest effects in the regions important for signaling (Serdiuk et al. 2022).

It should be noted that in case of selective stimulation of β1-adrenergic receptors, 24-HC was able to potentiate β1-adrenergic receptor-mediated increase in contractility and intracellular Ca^{2+} transient in atrial cardiomyocytes due to suppression of NO production. However, this potentiating effect was reversed when β1 and β2-adrenergic receptors were coactivated (Odnoshivkina et al. 2019). Accordingly, the main action of 24-HC can be realized through β2-adrenergic receptor-dependent pathway in the heart.

6.3 The Role of 25-Hydroxycholesterol in Mediating the Effects of Inflammation on Synaptic Transmission

6.3.1 GABA and Glutamate NMDA Receptors as Targets of 25-Hydroxycholesterol in CNS

25-HC is one of the most potent regulators of cell cholesterol homeostasis, even in the CNS (Odnoshivkina et al. 2022; Wang et al. 2020). However, it seems that in normal conditions 25-HC does not display a meaningful role in intercellular communication in the brain, since there are only trace concentrations in extracellular fluids. Indeed, estimation of 25-HC in human brains revealed that its concentration was always <3% of that of 24-HC (Lutjohann et al. 1996). However, production of 25-HC greatly increased in numerous pathological conditions related to inflammation, including neuroinflammation. 25-HC influence on inflammatory regulation is evident (Cyster et al. 2014). However, few studies concerning the synaptic effects of 25-HC are currently available. At the same time, 25-HC could be a "bridge" between inflammatory reactions and alterations in neurotransmission and, hence, neuronal network activity. It was revealed that 25-HC (5 μM) can increase the peak amplitude of the GABA-A receptor-mediated inhibitory postsynaptic potentials in some neurons tested (within 5–20 min of the application), but during prolonged perfusion (up to 5 h) a reduction of the GABA-B receptor-dependent inhibitory postsynaptic potentials occurred in some neurons in the brain slices of the rat lateral septum (Phelan and Mahler 1997). However, it is not clear why these effects did not manifest in all cells and how common they are in other brain regions. In hippocampal neurons, 25-HC had no effects on GABA-A receptor-mediated currents (Linsenbardt et al. 2014). In HEK293T cells 25-HC (like 24-HC, but less potent) inhibited BK channels (Tajima et al. 1864), which limit neurotransmitter release in response to calcium influx (Griguoli et al. 2016).

The discovery of 24-HC-mediated positive allosteric modulation of NMDA receptors (Paul et al. 2013) prompted attention to the role of 25-HC in glutamatergic transmission. 25-HC (at 0.1–10 μM; during seconds) caused a slight increase in response to NMDA in cultured hippocampal neurons. This effect of 25-HC was not enhanced by the prolongation of its application till several minutes. In this study effects of 25-HC on evoked transmission at AMPA receptors and GABA-A receptors were not revealed, suggesting no a presynaptic action on voltage-gated ion channels and neurotransmitter release. Thus, 25-HC is a mild selective positive modulator of NMDA receptors, but it can act in sub-micromolar concentrations (Linsenbardt et al. 2014). At the same time, 25-HC (with EC_{50} of ~0.1 μM and at 10 μM completely) inhibited NMDA receptor potentiation caused by 24-HC (1 μM) and its analog SGE201 (0.2 μM). The inhibitory effect of 25-HC on the oxysterol-dependent enhancement of NMDA receptor currents had rather a non-competitive character (Linsenbardt et al. 2014). Along the same line, 25-HC at 10 μM antagonized NMDA receptor-dependent excitotoxicity following oxygen-glucose deprivation and suppressed the exacerbation of this toxicity upon 24-HC (2 μM) in primary neuron culture. The mechanism of 25-HC action was only partially NMDA receptor dependent (Sun et al. 2017). It should be noted that at extremally high concentrations (50 μM) and (or) prolonged incubations (days), 25-HC can exert neurotoxic properties in neuronal cell lines (PC-12, cultured rat sympathetic neurons) (Chang and Liu 1997; Chang et al. 1998) and organotypic brain slices of the basal nucleus of Meynert (Ullrich et al. 2010).

The study of Yukitoshi Izumi and colleagues (Izumi et al. 2021b) points to the pathophysiological relevance of endogenous 25-HC for hippocampal synaptic plasticity and learning in mouse models. Activation of microglia with lipopolysaccharide caused an increase in the expression of cholesterol 25-hydroxylase and

levels of 25-HC. As a result, 25-HC disrupted LTP in hippocampal slices and suppressed one-trial learning. The induction of LTP was inhibited due to a slowly developing enhancement of NMDA receptor-mediated synaptic responses (i.e., metaplastic mechanism (Zorumski and Izumi 2012)) upon the elevation of 25-HC. This effect was prevented by inhibition of NMDA receptors (with selective antagonist d-APV) during lipopolysaccharide application (Izumi et al. 2021b).

6.3.2 Role of 25-Hydroxycholesterol in Modulation of Structural and Functional Properties of Neuromuscular Junctions: A Contribution of Lipid Rafts and Membrane-Bound Liver X Receptors

The first evidence suggesting that 25-HC may affect synaptic processes outside the brain came from studies denoted ALS pathogenesis. The serum levels of 25-HC were increased in ALS patients during the first year of disease manifestation. Brain and spinal cord expressions of cholesterol 25-hydroxylase, as well as 25-HC levels in the lumbar spinal cord (but not in plasma), were unregulated in model SOD1G93A mice at the early stages (Kim et al. 2017; Dodge et al. 2021). At the later stages, expression of cholesterol 25-hydroxylase declined in brain (but not spinal cord) of SOD1G93A mice and the levels of 25-HC metabolite (7α,25-dihydroxycholest-4-en-3-one) were lower in CSF, but higher in plasma of ALS patients as compared to the healthy controls (Abdel-Khalik et al. 2017; Kim et al. 2017). These data point to a possible contribution of 25-HC to ALS-related changes in NMJ structure and function. Furthermore, macrophages, a main producer of 25-HC, can directly come into contact with NMJs, promoting their regeneration in cases of nerve injury, ALS, and spinal muscular atrophy (Rios et al. 2021).

It has been found that 25-HC is able to recover some of the membrane and functional changes at the NMJs of SOD1G93A mice at the early pre-onset stage. Indeed, acute application of 25-HC (1 μM, 1½ h) restored the membrane order, which was partially disrupted in the NMJs of the model mice. Intraperitoneal injections of 25-HC (0.4 mg/kg, once *per* 4 days during 1 month) alleviated early NMJ defects in SOD1G93A mice, namely, lipid peroxidation, ceramide accumulation, fragmentation of nicotinic acetylcholine receptor clusters in postsynaptic regions of the NMJs and enhancement of non-quantal acetylcholine "leakage" (Zakyrjanova et al. 2021a). Note that in wild-type mice, acute application of 25-HC at the same concentration did not affect the synaptic membrane properties. This discrepancy can be explained by a higher ability of "the defected" NMJs (from ALS model mice) to bind 25-HC. Indeed, uptake of 25-(C4 TopFluor®) 25-OH cholesterol was markedly increased in NMJs of SOD1G93A mice (Zakyrjanova et al. 2021a).

The question about the protective or toxic properties of 25-HC in the case of ALS remains unresolved. As often happens with oxysterols, the final effect can be dependent on concentration, duration, and conditions of 25-HC action. Administration of 25-HC (2.5–40 μM) for 24 hours decreased the viability of motor neuron-like cell line expressing mutant G93A superoxide dismutase 1 gene (Kim et al. 2017). Also, exposure to 25-HC at high concentration (40 μM; 4 days) disrupted primary motor axon and muscle morphology in zebrafish larvae, but these effects appeared only after 25-HC treatment at early stage of development (Jamadagni and Patten 2019). Intraperitoneal injections of 25-HC at a high dose (~2 mg/kg/day) for 7 days induced muscle wasting accompanied by an increase in markers of apoptotic and ubiquitin-proteasome pathways (Shen et al. 2017). At the same time, 25-HC was identified as a potent senolytic in mice skeletal muscles (Limbad et al. 2022). In vitro 25-HC at low concentration (3 μM) increased viability of human iPSC-derived motor neurons and only at higher concentrations (30 and 100 μM) 25-HC had opposite effect, disrupting the membrane permeability (Dodge et al. 2021). Also, 25-HC (10 μM) can bidirectionally affect myelin gene expression

in cultured oligodendrocytes and Schwann cells due to complex interactions between LXR and Wnt/beta-catenin pathways (Meffre et al. 2015; Shackleford et al. 2013). Overall, it is not clear whether the elevation of 25-HC during ALS can reflect an inflammatory response exacerbating the pathology or be a compensatory response slowing down motor neuron loss and denervation of skeletal muscles (Odnoshivkina et al. 2022). Probably, low vs. high concentrations or(and) short vs. long-term administrations of 25-HC exert opposite effects on neuromuscular and motor neuron functions.

In NMJs, short-term (1/3 h) application of 25-HC at low (0.01–0.1 μM) and higher (1–10 μM) concentrations decreased and increased mobilization of synaptic vesicles during rhythmic activity, respectively. The stimulatory action of 25-HC seems to be mediated by membrane lipid raft-bound LXRs (in synaptic zone), which stimulated the signaling pathway of estrogen receptor α/G_i-protein/$G_{\beta\gamma}$/phospholipase C/inositol 1,4,5-trisphosphate and 1,2-diacylglycerol/Ca^{2+} release from ER and protein kinase C activation (Zakyrjanova et al. 2021b). In addition, 25-HC (1 μM) transiently increased ROS production (within 5–7 min of the application; without a sign of lipid peroxidation) in an $[Ca^{2+}]_{in}$ dependent manner in the NMJs. The elevation of ROS partially contributed to the enhancement of synaptic vesicle recruitment for neurotransmission (Zakyrjanova et al. 2021b). Note that membrane-bound LXRs were also found in endothelial cells and platelets (Ishikawa et al. 2013; Unsworth et al. 2018). Hence, membrane LXRs can mediate some acute effects of 25-HC, engaging signaling cascades that are convenient for G-protein coupled receptors (Zakyrjanova et al. 2021b; Ishikawa et al. 2013; Odnoshivkina and Petrov 2023). In atrial tissue, positive inotropy of β-adrenergic receptor stimulation was markedly blunted by acute application of 25-HC (0.01–1 μM) and selective LXR modulators. This effect of 25-HC was dependent on LXRs and lipid raft integrity. Furthermore, 25-HC itself increased lipid packing in the atrial membranes (Odnoshivkina and Petrov 2023). Given the key role of β-adrenergic receptors in neurocardiac coupling (Franzoso et al. 2016; Odnoshivkina and Petrov 2021; Zaglia and Mongillo 2017), 25-HC could be a potent modulator of transmission in the autonomic nervous system.

6.4 27-Hydroxycholesterol, a "Message" from Periphery to Brain

Expression of CYP27A1 is the highest in the liver. Higher levels of 27-HC were found in brain and CSF in patients with Alzheimer's and Parkinson's diseases (Kim et al. 2022). A major portion of 27-HC penetrates into the brain through the brain–blood barrier from circulation, i.e., has an extracerebral origin in CNS (Heverin et al. 2005). Cholesterol-fed rabbits had an increase in 27-HC levels in plasma and brain as well as neurodegeneration, associated with upregulation of estrogen receptors β and decrease in mitochondrial protein content in hippocampus (Brooks et al. 2017).

The increase in 27-HC levels in brain is considered a negative factor that can (1) deteriorate learning and memory (Zhang et al. 2015, 2018); (2) increase the formation of amyloid plaques in the hippocampus and expression of proteins, involved in amyloid β peptide synthesis (e.g., APP, BACE1, and RAGE) (Zhang et al. 2019); (3) decrease brain glucose uptake and Glut4 expression in hippocampus (Ismail et al. 2017) as well as insulin-like growth factor-1, leptin and p-Akt levels in organotypic hippocampal slices from adult rabbits (Sharma et al. 2008; Marwarha et al. 2010). Also, 27-HC (25 μM, 72 h) caused endoplasmic reticulum stress associated with increased amyloid β peptide production, tau phosphorylation, oxidative stress, and iron dyshomeostasis in the organotypic slices from rabbit hippocampus (Prasanthi et al. 2011). Activity of angiotensin converting enzyme positively correlated with 27-HC concentration in both plasma and CSF of patients with mild cognitive impairment and Alzheimer's disease (Mateos et al. 2011). In clinical trial, a reduction of plasma 27-HC level was associated with improvement of

memory in older individuals (60–77 years) with increased risk for dementia (Sandebring-Matton et al. 2021). These data point to the ability of 27-HC to affect synaptic processes in CNS and suggest that 27-HC can influence brain function.

27-HC (1 μM, 24 h) decreased levels of Arc (a key molecule for maintenance of synaptic potentiation), active Src kinase (a positive regulator of NMDA receptor function via phosphorylation of the receptor GluN2A subunits), NR1 subunit of NMDA receptors and tyrosine 1325 phosphorylation of GluN2A subunits in rat primary hippocampal neurons (Mateos et al. 2009). In primary hippocampal neurons, treatment with 27-HC (for 24 h; 1–30 μM) caused cell death, reduction of neurite number and length, downregulation of synaptic proteins (synaptophysin, SNAP-25, PSD-95, GAP-43, MAP2), and decrease in the thickness of postsynaptic density (Wang et al. 2019). Also, daily treatment (for several days) of hippocampal neurons with 1 μM of 27-HC led to the appearance of widespread pathological morphology in neurons with extremely short neurites, reduction of a number of postsynaptic spines and PSD95-immunopositive signals. The decrease in PSD95 levels was evoked by a triggering of REST–miR124a–PTBP1 axis. Under these conditions, 27-HC acts on this axis via retinoid X receptors γ, but not via LXRs or estrogen receptors (Merino-Serrais et al. 2019).

REST is a transcriptional repressor of many neuronal genes, including neuron-specific miR-124a. In turn, miR-124a regulates neurite outgrowth (by targeting oxysterol-binding protein) and inhibits PTBP1 (a negative regulator of PSD-95 abundance at the RNA level) (Merino-Serrais et al. 2019; Gu et al. 2016; Zheng et al. 2012). 27-HC increased expression of REST and PTBP1 in hippocampal primary neurons (Merino-Serrais et al. 2019). Interestingly, REST upregulation has a protective role and occurs in human cortical and hippocampal neurons during normal aging and in response to neuronal insults (Zhao et al. 2017).

Analysis of dendritic spine morphology revealed a reduction in dendritic arborization and spine density in CA1 pyramidal neurons of young transgenic Cyp27Tg mice with elevated levels of 27-HC. Also, a decrease in synaptic proteins (PSD95 and SNAP25) and miR124a, accompanied by increase in expression of REST and PTBP1 were found in the hippocampus in Cyp27Tg mice (Merino-Serrais et al. 2019). However, Cyp27Tg mice displayed enhanced LTP in the Schaffer collateral-CA1 synapses. This was associated with a higher density of synaptopodin (actin-binding protein involved in the spine apparatus formation)-positive puncta in CA1 region and abnormally large dendritic spines in the *stratum radiatum*. These abnormalities can disturb the fine-tuned processing of information in the hippocampal circuits contributing to 27-HC-mediated cognitive impairment (Loera-Valencia et al. 2021). Accordingly, excessive 27-HC via genomic mechanism (probably, retinoid X receptors γ) can disturb the normal maintenance of axo-spinal synapses and alter long-term synaptic plasticity. However, the relevance of these synaptic changes upon elevation of 27-HC is yet to be determined.

6.5 Synaptic Action of Products of Cholesterol Oxidase Activity and Free Radical-Derived Oxysterols

6.5.1 Amyloid β Peptide as Endogenous Cholesterol Oxidase

Cholesterol, apolipoprotein E, and amyloid β peptide were found to be co-localized in thioflavin S-positive (fibrillar) plaques (Burns et al. 2003). In addition, Cu^{2+} is enriched in the plaques (Dong et al. 2003). Next, it was found that amyloid β peptide forms a stable complex with Cu^{2+}, and the complex catalyzes the conversion of cholesterol to 4-cholesten-3-one. The levels of this oxysterol were greatly elevated in the brains of Alzheimer's disease subjects and the concentration of 4-cholesten-3-one correlated with amyloid β peptide neurotoxicity (Puglielli et al. 2005). In studies, exogenous cholesterol oxidase that generates 4-cholesten-3-one is frequently used as lipid raft

disrupting agent together with (or instead of) methyl-β-cyclodextrin, a membrane cholesterol removing agent. In general, the effects of cholesterol oxidase and methyl-β-cyclodextrin can be similar or can have specific features (Brachet et al. 2015; Korinek et al. 2015; Yue and Xu 2015; Cuddy et al. 2014; Mercer et al. 2012; Scanlon et al. 2001; Petrov et al. 2010, 2014a, b, 2015; Kravtsova et al. 2015). Like a cholesterol depletion, its enzymatic oxidation: (1) attenuated glutamate NMDA receptor responses as a result of the reduction in the probability of channel opening in cultured rat cerebellar granule cells (Korinek et al. 2015); (2) inhibited fast and slow types of synaptic vesicle endocytosis in brainstem slices containing the medial nucleus of the trapezoid body (Yue and Xu 2015); (3) decreased choline uptake by presynaptic high-affinity choline transporters, abundant in SH-SY5Y cells and nerve terminals from the forebrain of mice (Cuddy et al. 2014); (4) suppressed serotonin reuptake by serotonin transporters due to both the loss of affinity to a substrate and the reduction in the maximal transport rate (Scanlon et al. 2001); (5) caused an expansion in the voltage-gated Ca^{2+} channel confinement domains, leading to a reduction in both the peak efficiency of the neurotransmitter release and in the number of vesicle fusion events accompanying the opening of each Ca^{2+} channel in the cone ribbon synapses (Mercer et al. 2012); (6) markedly reduced neuropeptide galanin binding to galanin GalR2 receptors (their activation has neuroprotective effects (Elliott-Hunt et al. 2007)) expressed in Chinese hamster ovary cells (Pang et al. 1999). In addition, cholesterol oxidation decreased the activity of Ca^{2+}-ATPase which was accompanied by an increase in membrane fluidity of isolated brain synaptic plasma membranes (Wood et al. 1995). Binding of the agonist and antagonist to serotonin 1A receptors of isolated hippocampal membranes were inhibited after cholesterol oxidase application (Pucadyil et al. 2005), but in the ovary cells of Chinese hamster, the agonist binding and Gi-protein coupling to the serotonin 1A receptors were enhanced by cholesterol oxidase (Jafurulla et al. 2017). Evidently, the collective effects of cholesterol oxidation can lead to the attenuation of synaptic vesicle exo-endocytotic cycling and release of neurotransmitters (especially, acetylcholine and serotonin) as well as disturbances in neurotransmitter reception. Hence, synaptic transmission should be markedly suppressed by cholesterol oxidation in both presynaptic and postsynaptic sides.

At the same time, in certain cases cholesterol oxidation can augment glutamatergic transmission. Indeed, in organotypic hippocampal slice cultures treatments with either cholesterol oxidase or methyl-β-cyclodextrin (at relatively high concentration) increased AMPA-receptor-mediated synaptic transmission as well as LTP via the Cdc42 and Rab11-dependent mechanism (Brachet et al. 2015). The product of cholesterol oxidase can have some beneficial effects. Indeed, cholest-4-en-3-one was able to inhibit human β-site amyloid cleaving enzyme, BACE1 (Zhu et al. 2018). Also, cholest-4-en-3-one can act via LXRs, having anti-cancer properties (Elia et al. 2019), but it promotes the differentiation of neural stem cells into dopaminergic neurons (Ye et al. 2020). Interestingly, cholest-4-en-3-one oxime (TRO19622 or olesoxime) had neuroprotective action and increased the neurite outgrowth in models of ALS, neuropathy, Parkinson, Huntington, and Alzheimer's diseases, via targeting two components of mitochondrial permeability transition pore, the voltage-dependent anion channel (VDAC) and the translocator protein 18 kDa (Bordet et al. 2007, 2008; Eckmann et al. 2014; Rovini et al. 2019; Eckert et al. 2020). Hence, endogenous cholest-4-en-3-one can have some physiological relevance and (or) pathophysiological meaning and its production may be one of the essential "activities" of Cu^{2+}/amyloid β peptide.

6.5.2 Effects of Non-enzymatically Generated Oxysterols on Neurotransmission-Related Targets

7β-HC and other B-ring-oxidized oxysterols (7-KC, epoxy cholesterols, cholestane-3β, 5α, 6β-triol) are mainly produced as a result of free

radical attack and can contribute to neurodegenerative diseases (Vejux et al. 2020). In addition, amyloid precursor protein (APP) and amyloid β peptide can oxidize cholesterol to 7β-HC. This reaction requires Cu^{2+} and is accompanied by the formation of an equivalent amount of H_2O_2. It was suggested that the synthesis of 7β-HC at low levels can be a "function" of synaptic APP. 7β-HC had neurotoxicity at high nanomolar concentrations (100 nM, 96 h), but was able to inhibit soluble APP secretion and block protein kinase Cα (K_i ~0.2 nM) at lower concentrations (Nelson and Alkon 2005). Elevated activity of protein kinase Cα can lead to neurite degeneration, enhanced amyloid β peptide-induced synaptic depression, and cognitive decline in mouse models (Lorden et al. 2022). Hence, there is a possibility that 7β-HC at lower concentrations might limit amyloid β peptide-mediated synaptic defects and be neuroprotective. Indeed, liposomes containing a derivative of 7β-HC (7-β-hydroxycholesteryl-3-oleate, 15 μg/μl) can reduce astrocytic reactions following spinal cord injuries, promoting the serotonergic reinnervation of denervated territories (Giménez y Ribotta et al. 1995). Also, liposomes with 7β-HC reduced astrogliosis after an injection of iron into the hippocampus, but did not affect iron-induced seizures (Yao et al. 2006).

Levels of 7-KC appear to be increased in many pathologies, including neurodegenerative diseases (Pajares et al. 2015). Neuronal overactivity can provoke oxidation of cholesterol to 7-KC. Indeed, stimulation of glutamate kainite receptors by intracerebroventricular injection of kainite (a model of excitotoxic injury) was accompanied by an increase in 7-KC in the hippocampus. This elevation of 7-KC as well as a loss of the hippocampal neurons were inhibited by an intraperitoneal injection of lovastatin to block cholesterol synthesis (He et al. 2006). Neurotoxicity of 7-KC and 7β-HC is related to an increase in mitochondrial permeability and oxidative stress (Kim et al. 2006; Miguet-Alfonsi et al. 2002; Yammine et al. 2020). These alterations can be aroused as a result of calcium dysregulation and hyperstimulation of calmodulin-dependent pathways upon 7-KC (Han et al. 2007). In addition, 7-KC at a relatively high concentration (50 μM) decreased lipid raft stability in cultured neurons, thereby leading to an alteration in the function of voltage-gated ion channels (particularly, $Na_V1.8$) residing in raft fraction and, hence, impaired neuronal excitability (Pristera et al. 2012). In the Langmuir monolayer study, 7-KC together with 25-HC were characterized by the strongest myelin/oxysterol interactions, causing an increase in the membrane fluidity (Chachaj-Brekiesz et al. 2020).

Hypothetically, 7-KC and 7β-HC may affect presynaptic processes, ensuring and regulating neurotransmitter release. Injection of 7β-HC and 7-KC into the prefrontal cortex of rats caused a downregulation of numerous genes the majority of which encode G-protein coupled receptors, including oxytocin receptor 1. The downregulation of oxytocin receptor 1, expressed on the axon nerve terminals in the prefrontal cortex, was confirmed at the protein level (Loke et al. 2013). Activity of these receptors in the prefrontal cortex is essential for maternal care and reduction of anxiety (Jurek and Neumann 2018). 7-KC (2 μM) enhanced exocytosis and intracellular calcium in PC12 cells. These effects of 7-KC were inhibited by the lipid raft disruptor (methyl-β-cyclodextrin) as well as depletion of intracellular Ca^{2+} stores and blockage of Ca^{2+} channels (Ma et al. 2010). Also, 7-KC is a potential inhibitor of endocytosis, since it can prevent activation of phospholipases Cγ and β, and, hence, phosphatidylinositol 4,5-bisphosphate turnover (Lu and Fairn 2018), required for synaptic vesicle recycling (Micheva et al. 2001; Saheki and De Camilli 2012).

Cholestane-3β,5α,6β-triol is one of the major oxysterols in the brain. Its concentration ranges from 0.32 to 1.07 μM (spinal cord > liver > brain ≥ kidney > plasma) in rat tissues (Hu et al. 2014). It can protect neurons against glutamate-mediated excitotoxicity in vitro (at 5–15 μM) and ischemia-induced neuronal injury in vivo. The mechanism of the protective effects of cholestane-3β,5α,6-β-triol relies on the negative modulation of NMDA receptor-mediated Ca^{2+} currents (Hu et al. 2014). Overstimulation of the NMDA

receptors leads to Ca^{2+} overload in the postsynaptic compartment and triggers cell death (Arundine and Tymianski 2003). In addition, excessive glutamate promotes glycolysis and inhibits mitochondrial oxidative phosphorylation, leading to lactate accumulation, which contributes to neuronal injury and cytoplasmic acidification. 5α-androst-3β,5α,6β-triol, a synthetic neuroactive derivative of cholestane-3β, 5α, 6β-triol, remarkably reversed glutamate-induced intracellular acidification and alleviated the neuronal injury through the inhibition of AMP kinase signaling in primary cultured neurons as well as reduced the infarct volume and neurologic defects in acute ischemic stroke models of middle cerebral artery occlusion (Xue et al. 2022). Additionally, cholestane-3β,5α,6β-triol (10 μM) markedly reduced the density of Na^+ currents in hippocampal neurons, shifting steady-state/fast/slow inactivation curves of voltage-gated sodium channels toward hyperpolarization (Tang et al. 2015). Molecular docking and molecular dynamics simulation showed that this triol binds to the indole ring of Trp122 of the Na_v channel in silico with high biological affinity (Tang et al. 2018). The inhibition of Na_v channels can explain the ability of cholestane-3β,5α,6β-triol to attenuate the action potentials bursts of hippocampal neurons, low Mg^{2+}-induced hyperexcitability in vitro, pentylenetetrazole-induced convulsive form behavioral deficits and severity of kainic acid-induced seizures in mouse models (Tang et al. 2015, 2018). Accordingly, cholestane-3β,5α,6β-triol, and its derivatives can be used for the development of therapies for diseases associated with excitotoxicity and epileptic activity. Interestingly, plasma levels of cholestane-3β,5α,6β-triol were increased in some neurodegenerative diseases, particularly Niemann-Pick type C and *Cerebrotendinous xanthomatosis* (Pajares et al. 2015). This elevation of cholestane-3β,5α,6β-triol can reflect a compensatory response or be a sign of disrupted cholesterol homeostasis.

6.5.3 The Peripheral Synapses Under the Influence of Cholesterol Oxidase and Non-enzymatically Generated Oxysterols

Outside the brain, cholesterol oxidation to 4-cholesten-3-one can also be driven by amyloid β peptide complexed with Cu^{2+}(Puglielli et al. 2005). Since soluble amyloid β peptide is present in plasma lipoproteins (D'Alonzo et al. 2023) as well as APP and amyloid β peptide aggregates are present in peripheral tissues, including NMJs and heart (Akaaboune et al. 2000; Troncone et al. 2016; Caldwell et al. 2013). Bacterial cholesterol oxidase is an essential virulence factor (Bednarska et al. 2014), that affects peripheral tissues. Treatment with an exogenous cholesterol oxidase suppressed neurotransmitter release and mobilization of synaptic vesicles during intense activity in the frog NMJs. Additionally, it caused a dispersal of synaptic vesicle clusters and affected the mode of synaptic vesicle exocytosis (Petrov et al. 2014a). Cholesterol oxidase via disordering membrane led to a gain of function of nicotinic acetylcholine receptor from the electric organ of *T. californica* (Fabiani et al. 2022), but there were no changes in the amplitude-time parameters of nicotinic acetylcholine receptor-mediated currents in response to a single quantum in the frog NMJs (Petrov et al. 2014a).

The product of cholesterol oxidase, 4-cholesten-3-one can be converted to 5-α-cholestan-3-one (Ute et al. 2007), an intermediate in cholestanol synthesis in liver and extrahepatic tissues and its plasma levels can reach sub-micromolar ranges (~0.2 μM) in case of *Cerebrotendinous xanthomatosis* (DeBarber et al. 2011; Salen and Polito 1972). This disease is accompanied by both neurological dysfunction and myopathy (Nie et al. 2014). 5α-Cholestan-3-one (0.2 μM) reduced neurotransmitter release from motor nerve terminals during both low- and higher-frequency activities. The depressant action of 5α-cholestan-3-one was accompanied

by a decrease in the synaptic vesicle pool engaged in exo- and endocytosis during intense activity in frog and mice NMJs (Kasimov et al. 2015, 2016). In addition, 5α-cholestan-3-one decreased lipid ordering in synaptic membranes (Kasimov et al. 2015, 2016), and pretreatment with methyl-β-cyclodextrin at a low concentration (but not its application after oxysterol treatment) was able to attenuate the effects of the oxysterol on synaptic vesicle mobilization and lipid ordering at the NMJs (Kasimov et al. 2015). The latter suggests the presence of a potential receptor to 5α-cholestan-3-one in lipid raft fraction.

Neuroprotective cholest-4-en-3-one-like compound, olesoxime screened during identification of a potential small molecule therapeutics (Bordet et al. 2007) increased lipid ordering in synaptic membranes, as well as potentiated neurotransmitter release, enhancing the participation of synaptic vesicle in exocytosis and their recycling in the frog NMJs (Kasimov et al. 2016). At the same time, in mice NMJs olesoxime limited exocytosis of synaptic vesicles, especially those belonging to mobilizing (recycling) pool. The mechanism of olesoxime action relied on the increase in Cl^- influx into the presynaptic nerve terminals via plasmalemmal VDAC-like channels (Zakyrjanova et al. 2020), sensitive to 4,4′--diisothiocyanatostilbene-2,2′-disulfonate (a membrane-permeable chloride channel blocker) and S-18 phosphorothioate randomer oligonucleotide, G3139 (a membrane-impermeable inhibitor of VDACs (Stein and Colombini 2008)). Probably, this pathway of presynaptic inhibition in mammalian synapses can be activated by some endogenous oxysterols and neurosteroids or lipids (e.g., ceramide and 15-deoxy-Δ12,14-prostaglandin J2), which may bind to VDAC (Darbandi-Tonkabon et al. 2004; Dadsena et al. 2019; Koma et al. 2020; Sabirov and Merzlyak 2012).

Amyloid β peptide and APP which can serve as endogenous cholesterol oxidases, were found in atherosclerotic lesions and vessels (Li et al. 2003; d'Uscio et al. 2017). Cholesterol oxidation as well as 5α-cholestan-3-one had a marked influence on cardiac β-adrenergic signaling, responsible for translation of commands from sympathetic nerves to the heart (Sytchev et al. 2017; Ursan et al. 2019). Indeed, after cholesterol oxidase pretreatment, β1-adrenoceptor-mediated increase in atrial contractility, intracellular Ca^{2+} transient, and NO production were largely suppressed due to an elevation of β2-adrenoceptor-dependent ROS production. Note that under these conditions, cholesterol oxidation did not lead to cholesterol depletion, suggesting that the generated 4-cholesten-3-one remained in the plasmalemma and was responsible for the alterations in β-adrenergic signaling (Ursan et al. 2019). Accordingly, cholesterol oxidation can provoke oxidative stress, especially in stressful conditions accompanied by increased levels of catechol amines. Furthermore, membrane cholesterol oxidation increased depolarization-induced exocytosis in the sympathetic terminals in mice atria, which should elevate the concentration of catechol amines near cardiomyocytes (Odnoshivkina et al. 2023).

Another oxysterol, 5α-cholestan-3-one (20 nM–1 μM) suppressed β2-adrenergic-dependent contractility in atrial cardiomyocytes by increasing signaling via β2-adrenoceptor/Gi-protein/Akt kinase/NO/protein kinase G pathway (Sytchev et al. 2017), which limited the sensitivity of myofilament to cytosolic Ca^{2+} (Pfitzer et al. 1982). At the same time, under inhibition of the Gi-protein/Akt kinase/NO/protein kinase G pathway, 5α-cholestan-3-one increased the β2-adrenoceptor-driven contractile responses due to an increase in cytosolic Ca^{2+} transient (Sytchev et al. 2017). Such dual action of 5α-cholestan-3-one indicates its specific influence on coupling β2-adrenergic receptors with the downstream signaling pathways. Hence, oxidized cholesterols, 4-cholesten-3-one, and 5α-cholestan-3-one, can be powerful modulators of cardiac β-adrenergic signaling, thereby affecting neurocardiac transmission (Odnoshivkina and Petrov 2021).

Oxidized forms of cholesterol can interfere with vasomotor response to neurotransmitters of autonomic nerve system. Indeed, the acetylcholine-induced, endothelial-dependent relaxation of rat aortic segments was significantly impaired by oxysterols (7-ketocholesterol, 7-β-hydroxycholesterol) present in oxidized light

density lipoprotein (Mougenot et al. 1997; Deckert et al. 1997). 7-KC can block acetylcholine-evoked release of NO by vascular endothelial cells through the ability of the oxysterol to activate protein kinase C (Deckert et al. 2002). This action of oxysterols can enhance peripheral vessel resistance and untimely blood pressure, contributing to hypertension, as a part of metabolic syndrome.

6.6 Conclusion

Cholesterol is a major component of membranes in synapses which are metabolically active compartments of the neurons. Oxidation of cholesterol produces a number of oxysterols. Side-chain oxysterols (24-HC, 25-HC and 27-HC) as well as ring-oxidized sterols (7β-HC, 7-KC, cholestane-3β,5α,6β-triol, 4-cholesten-3-one, 5-α-cholestan-3-one) have numerous biological activities that engage receptors (NMDA receptors, β-adrenoceptors, LXRs, retinoid X receptors γ) as well as signaling molecules and ion channels (protein kinase C, nitric oxide syntheses, VDACs, BK channels) present in the nervous system. These oxysterols can serve as potent modulators of neurotransmission at the presynaptic and postsynaptic levels in both the central and the peripheral synapses. They may also play physiological roles at low concentrations and/or short-term action, whereas excessive amounts of oxysterols can disturb normal synaptic communication. Hypothetically, side chain oxysterols can participate in the formation of humoral regulatory axes for additional connections: brain to peripheral (neuromuscular and "neurocardiac" junctions) synapses/organs (24-HC), inflammatory processes mediated by macrophages and microglia to synaptic transmission (25-HC), liver cholesterol metabolism to synaptic activity in CNS (27-HC). Adverse effects accompanying pathological conditions, oxidative stress, chronic inflammation, metabolic alterations and accumulation of amyloid β peptide greatly enhance the generation of some oxysterols (e.g., 25-HC, 27-HC, 7β-HC, 7-KC, 4-cholesten-3-one, and 5α-cholestan-3-one), which can contribute to the cognitive and motor deficits. These negative effects of oxysterols can be attributed to abnormal synaptic transmission. At the same time, oxysterols and their analogs can be used for the development of new drugs targeting neurotransmission. 24-Hydroxycholesterol mimetics (SGE-301, SGE-550 and SAGE718) as well as 24-HC-elevating compounds can be used for the correction of NMDA receptor hypofunction-related states, whereas cholestane-3β,5α,6β-triol, cholest-4-en-3-one oxime (olesoxime) and antagonists of 24-HC synthesis are potential anti-epileptic drugs and/or protectors of excitotoxicity. Overall, oxysterol signaling network can be a component of a complex adaptive mechanism that fine-tunes synaptic communication to changes in metabolism, immune status, and activities of both neighboring and remote neuronal centers.

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Oxysterols in Infectious Diseases

7

Cheng X. Foo, Michael B. Fessler, and Katharina Ronacher

Abstract

Oxysterols have emerged as important bioactive lipids in the immune response to infectious diseases. This chapter discusses our current knowledge of oxysterols and their receptors in bacterial and viral infections of the respiratory and gastrointestinal tracts. Oxysterols are produced in response to infections and have multiple roles including chemotaxis of immune cells to the site of infection and regulation of inflammation. Some oxysterols have been shown to possess antiviral or antibacterial activity.

Lastly, we delve into the emerging mechanisms of action of oxysterols. Oxysterols can enhance host cell resistance via reduction of membrane accessible cholesterol, modulate membrane immune signalling, and impact inflammasome activation and efferocytosis.

Keywords

Oxysterol · 25-hydroxycholesterol · 7α,25-hydroxycholesterol · GPR183 · LXR · Infection · Tuberculosis · SARS-CoV-2

Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
4β-OHC	4β-hydroxycholesterol
7α-OHC	7α,hydroxycholesterol
7α,25-OHC	7α,25-hydroxycholesterol
7-KC	7-ketocholesterol
25-OHC	25-hydroxycholesterol
27-OHC	27-hydroxycholesterol
ACAT1	Acetyl-CoA acetyltransferase
AIM2	Absent in melanoma 2
AP-1	Activator protein 1
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BCG	*Mycobacterium bovis* Bacillus Calmette-Guerin
BMDM	Bone marrow-derived macrophages
CDC	Cytolysin
CGT	Cholesterol-α-glucosyl transferase
CH25H	Cholesterol 25-hydroxylase

C. X. Foo
Mater Research Institute – The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia

M. B. Fessler
Immunity, Inflammation and Disease Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

K. Ronacher (✉)
Mater Research Institute – The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia

Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, QLD, Australia
e-mail: katharina.ronacher@mater.uq.edu.au

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COVID-19	Coronavirus disease-19
CXCR2	CXC chemokine receptor 2
CYPs	Cytochromes P450
DUSP6	Dual specificity phosphatase 6
EBER	Epstein-Barr virus small RNA
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
GAS6	Growth arrest-specific 6
GCN2	General control nondepressible 2
GPR183	G protein-coupled receptor 183
hACE2	Human angiotensin-converting enzyme 2
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HRV	Human rhinovirus
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cells
IAV	Influenza A virus
IFN	Interferon
IL-36	Interleukin 36
ILC3	Innate lymphoid cells 3
INSIG	Insulin-induced gene
IRF	Interferon regulatory factor
KSHV	Kaposi sarcoma herpes virus
LASV	Lassa Virus
LMP-1	Latent membrane protein 1
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAVS	Mitochondrial antiviral-signalling protein
MCMV	Mouse cytomegalovirus
MDA5	Melanoma differentiation-associated gene 5
MERS-CoV	Middle East respiratory syndrome coronavirus
MERTK	MER Proto-oncogene, tyrosine kinase
MHV	Murine gamma herpes virus
Mtb	*Mycobacterium tuberculosis*
NLRC4	NLR family CARD domain containing 4
NLRP3	NOD-like receptor protein 3
OSBP	Oxysterol-binding protein
PACP	Pauci-asymptomatic SARS-CoV-2-positive individuals
PM	Plasma membrane
Rab7	RAS-associated protein 7
SARS-CoV-2	Severe acute respiratory syndrome-coronavirus-2
SCAP	SREBP Cleavage activating protein
SREBP	Sterol regulatory element binding protein
STING	Stimulator of interferon genes
TB	Tuberculosis
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VAP-A	Vesicle-associated membrane protein-associated protein A
VZV	Varicella Zoster virus

7.1 Introduction

Oxidised cholesterols or oxysterols are a class of cholesterol derivatives that are formed through the hydroxylation of cholesterol by enzymatic reaction or through autooxidation. Over the past decade, studies have identified oxysterols as bioactive molecules in the immune system. However, the effects and potential roles of these oxysterols in regulating the immune response to infections are largely underexplored. Here we discuss the currently available knowledge of the contribution of oxysterols in the immune response to bacterial and viral infections and emerging cellular mechanisms of oxysterols in host defence.

7.2 The Role of Oxysterols in Respiratory Infections

The respiratory tract is a common site of infection by both bacterial and viral pathogens. In this section, we focus on two respiratory infections that have been shown to increase local oxysterol production in the lung, namely tuberculosis and severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection.

7.2.1 Oxysterols in the Immune Response to Tuberculosis

Tuberculosis (TB) is a respiratory infectious disease caused by *Mycobacterium tuberculosis* (Mtb). TB is a significant global health threat with over 10.6 million cases and 1.4 million deaths annually (World Health Organization 2022). Over the past 3 years, we and others have demonstrated that oxysterols and their receptors are novel regulators of the host immune response to Mtb infection.

When mice were infected with Mtb, the expression of the oxysterol-producing enzymes CH25H and CYP7B1 in the lung was increased compared to uninfected animals. This coincided with increased concentrations of the oxysterol 25-hydroxycholesterol (25-OHC) (Ngo et al. 2022). CH25H is an enzyme that metabolises cholesterol to 25-OHC, which is further metabolised by CYP7B1 to 7α,25-dihydroxycholesterol (7α,25-OHC) (Foo et al. 2022). In Mtb-infected animals, the expression of CH25H and CYP7B1 in the lung was largely restricted to alveolar and infiltrating macrophages, and CYP7B1 expression was highest in the macrophage core of TB granulomas (Ngo et al. 2022).

Both 7α,25-OHC and 25-OHC are ligands for the oxysterol-sensing receptor GPR183, with 7α,25-OHC being the most potent endogenous agonist (Hannedouche et al. 2011). GPR183 is expressed on immune cells including dendritic cells, eosinophils, macrophages, innate lymphoid cells (ILCs), and T and B cells (Foo et al. 2022; Bohrer et al. 2022). In murine TB, the increase in local oxysterol production in the lung was associated with reduced expression of GPR183 in blood but increased GPR183 expression in the lung suggesting migration of GPR183^{+} immune cells to the site of infection (Ngo et al. 2022). This was consistent with observations in TB patients where reduced GPR183 expression correlated with TB disease severity on chest X-ray (Bartlett et al. 2020). Furthermore, GPR183 downregulation was found to precede the diagnosis of active TB by 6 months in a longitudinal household contact study (Bohrer et al. 2022). These findings indicate that during subclinical TB GPR183^{+} immune cells migrate to the lung in response to oxysterol production and suggest that GPR183 and oxysterols may be useful biomarkers for early TB diagnosis and TB disease severity (Bohrer et al. 2022; Bartlett et al. 2020).

In mice, Mtb-induced production of these oxysterols facilitated the recruitment of GPR183-expressing macrophages and eosinophils towards the lung (Ngo et al. 2022; Bohrer et al. 2022). However, it cannot be excluded that other immune cell subsets that express GPR183 like T or B cells similarly migrate towards an oxysterol gradient. Mice deficient in GPR183 (*Gpr183*$^{-/-}$) displayed impaired recruitment of macrophages and eosinophils during the early stages of Mtb infection (Ngo et al. 2022; Bohrer et al. 2022), but GPR183 was not required for migration of T cells to the lung (Hoft et al. 2019). Delayed recruitment of macrophages to the Mtb-infected lung was associated with an increased mycobacterial burden and dysregulated type I interferon (IFN) response in the *Gpr183*$^{-/-}$ mouse during early infection (Bartlett et al. 2020). In addition, in a murine model of pre-diabetes and TB, hyperglycaemia blunted Mtb-induced expression of CYP7B1, but not CH25H in the lung. The blunted CYP7B1 expression was associated with a delay in macrophage infiltration similar to what was observed in *Gpr183*$^{-/-}$ mice. These studies indicate that both GPR183 and its high-affinity agonist 7α,25-OHC, which is produced by CYP7B1, are required for rapid macrophage infiltration into the lung upon Mtb infection.

In *ex vivo* and in vitro studies, the mechanism by which the GPR183/oxysterol axis regulates the immune response to Mtb infection was further explored. In primary human monocytes, 7α,25-OHC-mediated activation of GPR183 resulted in intracellular restriction of both Mtb and *Mycobacterium bovis* BCG. This effect was abolished by the addition of a GPR183 antagonist, suggesting that GPR183 plays a role in regulating intracellular growth of mycobacteria and potentially other bacteria (Bartlett et al. 2020). The observed inhibition of mycobacterial growth, which was

associated with induction of autophagy and downregulation of type I IFNs, was specific to primary human monocytes and was not observed in a murine macrophage cell line (Tang et al. 2020).

Apart from the GPR183/oxysterol axis, another study demonstrated oxysterol regulation by the IL-36/liver X receptor (LXR) axis. The study conducted in THP-1-derived macrophages and primary human monocyte-derived macrophages demonstrated that IL-36 is produced in response to Mtb infection, leading to the synthesis of the LXR ligands 25-OHC and 27-hydroxycholesterol (27-OHC). LXR activation drives the production of antimicrobial peptides such as cathelicidin and defensins, which contribute to better mycobacterial control. In addition, knockdown of the oxysterol synthesising enzymes *CH25H* and *CYP27A1* resulted in increased mycobacterial growth, further demonstrating the importance of oxysterols in macrophage-mediated host defence against Mtb infection (Ahsan et al. 2018).

Given the role of oxysterols in the innate immune response and host defence against Mtb, the pathogen has developed mechanisms for inhibiting their activity. A recent study provided evidence that enzymes produced by Mtb can metabolise host oxysterols, which may be a host immune evasion strategy by Mtb. For instance, the mycobacterial enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) can metabolise 25-OHC and 7α,25-OHC into inactive forms (Varaksa et al. 2021). As these oxysterols have immunomodulatory and antimycobacterial activity against Mtb, a possible hypothesis is that the Mtb enzymes 3β-HSD and possibly CYP124, CYP125, and CYP142 target oxysterols to evade the host immune response and persist in host macrophages (Varaksa et al. 2021). Another possible strategy employed by Mtb to counteract the oxysterol response could be the production of antagonists to oxysterol receptors, such as GPR183. Studies conducted on *Eubacterium rectale* revealed that this bacterium produces lauroyl tryptamine, which is GPR183 antagonist with a half-maximal inhibitory concentration of 0.98 μM (Chang et al. 2021).

7.2.2 Oxysterols in the Immune Response to SARS-CoV-2 and Influenza Infection

Evidence for the involvement of oxysterols in the immune response to severe viral respiratory infections has been described in COVID-19 and influenza (Conlon and Yildirim 2023). Several studies have provided evidence that oxysterol concentrations change upon infection with SARS-CoV-2 with some oxysterols increasing and others decreasing (summarised in Table 7.1).

A study monitored the kinetics of serum 25-OHC over time in a single female COVID-19 patient (Zu et al. 2020) and found that 25-OHC levels were relatively unchanged compared to healthy controls during the patient's initial admission to the hospital. However, 25-OHC levels were markedly increased later in the course of the infection when the patient's clinical condition deteriorated significantly and peaked 2 days prior to the patient's death. This individual had underlying comorbidities including diabetes and heart disease (Zu et al. 2020). In contrast, in a different study of severe COVID-19 patients without reported comorbidities, several side chain oxysterols, including 25-OHC, 24-hydroxycholesterol (24-OHC), and 27-hydroxycholesterol (27-OHC) were reduced, while 7-ketocholesterol (7-KC) and 7-β-hydroxycholesterol (7β-OHC) were increased compared to healthy matched controls. In this study, 27-OHC serum concentrations were inversely correlated with disease severity (Marcello et al. 2020). Conversely, another study of COVID-19 patients with varying degrees of metabolic comorbidities found that the oxysterols, 25-OHC, 24S-OHC, and 27-OHC, were increased, while 7-KC was decreased compared to healthy controls. No difference was noted in the concentrations of 4β--hydroxycholesterol (4β-OHC) and 7-α-hydroxycholesterol (7α-OHC) (Asano et al. 2023). In addition, the study also discovered that COVID-19 patients treated with dexamethasone had lower 25-OHC serum concentrations compared to patients who did not receive

Table 7.1 Serum oxysterols in response to SARS-CoV-2 infection

Oxysterol	Underlying comorbidities/clinical intervention involved	Disease severity stage	Change relative to healthy controls	Ref.
25-OHC	Not reported	Pauci-asymptomatic SARS-CoV-2-positive individuals (PACP; $n = 27$)	Increased	Marcello et al. (2020)
		Moderate ($n = 36$)	Unchanged	Marcello et al. (2020)
		Severe ($n = 81$)	Decreased	Marcello et al. (2020)
	12 patients obese, 6 hypertension, 3 diabetes, 1 dyslipidaemia; sample collected after dexamethasone therapy	Severe ($n = 17$)	Increased	Asano et al. (2023)
	Hypertension, coronary heart disease, diabetes, and sicca syndrome	Severe ($n = 1$)	Increased	Zu et al. (2020)
24(S)-OHC	Not reported	PACP ($n = 27$)	Decreased	Marcello et al. (2020)
		Moderate ($n = 36$)	Decreased	Marcello et al. (2020)
		Severe ($n = 81$)	Decreased	Marcello et al. (2020)
	12 patients obese, 6 hypertension, 3 diabetes, 1 dyslipidaemia; sample collected after dexamethasone therapy	Severe ($n = 17$)	Increased	Asano et al. (2023)
27-OHC	Not reported	PACP ($n = 27$)	Decreased	Marcello et al. (2020)
		Moderate ($n = 36$)	Decreased	Marcello et al. (2020)
		Severe ($n = 81$)	Decreased	Marcello et al. (2020)
	12 patients obese, 6 hypertension, 3 diabetes, 1 dyslipidaemia; sample collected after dexamethasone therapy	Severe ($n = 17$)	Increased	Asano et al. (2023)
7-KC	Not reported	PACP ($n = 27$)	Unchanged	Marcello et al. (2020)
		Moderate ($n = 36$)	Increased	Marcello et al. (2020)
		Severe ($n = 81$)	Increased	Marcello et al. (2020)
	12 patients obese, 6 hypertension, 3 diabetes, 1 dyslipidaemia; sample collected after dexamethasone therapy	Severe ($n = 17$)	Decreased	Asano et al. (2023)

(continued)

Table 7.1 (continued)

Oxysterol	Underlying comorbidities/clinical intervention involved	Disease severity stage	Change relative to healthy controls	Ref.
7β-OHC	Not reported	PACP ($n = 27$)	Unchanged	Marcello et al. (2020)
		Moderate ($n = 36$)	Unchanged	Marcello et al. (2020)
		Severe ($n = 81$)	Increased	Marcello et al. (2020)
4β-OHC	12 patients obese, 6 hypertension, 3 diabetes, 1 dyslipidaemia; sample collected after dexamethasone therapy	Severe ($n = 17$)	Unchanged	Asano et al. (2023)
7α-OHC	12 patients obese, 6 hypertension, 3 diabetes, 1 dyslipidaemia; sample collected after dexamethasone therapy	Severe ($n = 17$)	Unchanged	Asano et al. (2023)

glucocorticoids. Dexamethasone is known to inhibit IFN signalling, which explains the reduced expression of CH25H, an IFN-inducible gene (Liu et al. 2013), and the reduced production of 25-OHC. The authors further show that dexamethasone reduces the expression of CH25H in untreated, IFN-, and LPS-stimulated primary human macrophages (Asano et al. 2023). Apart from serum-based oxysterol analyses, a study focused on oxysterols extracted from extracellular vesicles in plasma samples from COVID-19 patients demonstrated temporal production of oxysterols in response to infection (Lam et al. 2021). The study revealed that the majority of oxysterols were reduced during the very early phase of infection (pre-symptomatic phase, SARS-CoV-2 positive). These oxysterols were subsequently elevated during the later symptomatic phase, which is associated with the hyperinflammatory response induced by the host (Lam et al. 2021).

In COVID-19 patients with moderate and severe disease, single-cell sequencing of bronchoalveolar lavage samples showed that the oxysterol-producing enzymes CH25H and CYP7B1 are increased in lung macrophages and myeloid dendritic cells compared to those of healthy controls and that their increased expression is associated with COVID-19 severity. In addition, the expression of GPR183 on macrophages increased with COVID-19, while GPR183 expression on other immune cell types such as T and B cells was unaffected by infection (Foo et al. 2023). These findings suggest that macrophages are the main producers of the oxysterols 25-OHC and 7α,25-OHC in the lung and at the same time increase their responsiveness to these oxysterols by upregulating GPR183.

These clinical studies show that serum oxysterol concentrations are altered by SARS-CoV-2 infection and likely other viral respiratory infections but indicate that they can also be modified by metabolic comorbidities. There is some evidence that 7KC and 4β-OHC are elevated in serum of diabetes patients without any infections (Endo et al. 2008; Ferderbar et al. 2007). This can explain the conflicting findings observed in the increase vs. decrease of specific oxysterols in the COVID-19 patient cohorts and highlights the complexity of patient-centred oxysterol research. Since the above-mentioned studies were not powered to stratify patients by metabolic phenotype, further cohort studies with larger sample sizes and well-described metabolic phenotypes should aim to identify whether metabolic diseases affect the kinetics of oxysterol production in the presence and absence of infectious diseases.

In a murine model of COVID-19 using a mouse-adapted SARS-CoV-2 strain, we recently showed that SARS-CoV-2 leads to upregulation of CH25H and CYP7B1 in the lung resulting in increased production of both 25-OHC and 7α,25-OHC (Foo et al. 2023). Similar to what was described in the murine model of TB, 25-OHC and 7α,25-OHC dictated macrophage influx via the oxysterol-sensing receptor GPR183. Abolishing GPR183 activity, either through genetic deletion (*Gpr183*$^{-/-}$ mice) or pharmacological targeting of GPR183 with the GPR183-specific antagonist NIBR189, led to delayed recruitment of macrophages to the lungs upon infection. This reduction in macrophages was associated with reduced pro-inflammatory cytokine production, without impairing the early type I and III IFN response. We made similar observations with mice infected with influenza A virus (IAV) (Foo et al. 2023). Interestingly, in SARS-CoV-2 infected mice, GPR183 antagonism was associated with reduced viral loads and better disease outcomes, suggesting the therapeutic benefits of inhibiting GPR183 to improve SARS-CoV-2 infection outcomes (Foo et al. 2023). Whether NIBR189 has direct antiviral activity remains to be elucidated. A different study did not observe any benefit of administering NIBR189; however, this study used a lower dose of this antagonist, and it is likely that not all receptors were antagonised at that dose (Fessler et al. 2022). They did not observe differences between SARS-CoV-2-infected wild-type (WT) and *Gpr183*$^{-/-}$ mice.

Others also reported the upregulation of CH25H in K18-hACE2 mice and mice infected with a mouse-adapted strain (Fessler et al. 2022). 25-OHC levels were increased in the serum of these mice, further demonstrating that 25-OHC is produced in response to SARS-CoV-2 infection. Intragastrical pre-treatment of these animals with 25-OHC resulted in reduced viral loads in both the lung and trachea (Zu et al. 2020). In contrast, a different study did not observe any benefit of intraperitoneal administration of 25-OHC in SARS-CoV-2-infected mice, and it is possible that the dose and mode of administration of 25-OHC impact the different outcomes of these seemingly conflicting studies (Fessler et al. 2022).

In influenza A virus (IAV) infection, a study demonstrated that *Ch25h*$^{-/-}$ mice were more resistant to infection compared to WT mice (Gold et al. 2014). Despite similar viral loads in the lungs of these mice, *Ch25h*$^{-/-}$ mice had fewer histopathological changes in the lungs and reduced pro-inflammatory response compared to WT mice. This is similar to observations we made in *Gpr183*$^{-/-}$ mice infected with IAV. Interestingly, immortalised airway epithelial (LET1a) cells from *Ch25h*$^{-/-}$ mice were more susceptible to IAV infection and pre-treatment of 25-OHC in WT LET1a cells displayed potent antiviral activity (Gold et al. 2014). The study found that CH25H amplifies pro-inflammatory responses in bone marrow-derived macrophages (BMDMs) in an LXR-independent manner following Poly I:C (a Toll-like receptor 3; TLR3 agonist) stimulation. They further found that this pro-inflammatory response is mediated through the activation of activator protein-1 (AP-1) transcription factor. CH25H-dependent 25-OHC also sustains pro-inflammatory cytokine expression in the lung in vivo and may compromise pulmonary microvascular integrity during inflammation (Madenspacher et al. 2023). The growing recognition of the complex roles that oxysterols play in lung disease and their potential connection to common metabolic disorders such as obesity has recently led to increased attention by the field (Fessler 2017; Suratt et al. 2017).

A visual summary of the role of oxysterols during respiratory infections is shown in Fig. 7.1.

7.2.3 Antiviral Activity of Oxysterols in Cell Systems

To complement the antiviral activities of oxysterols described in the murine models, in vitro experiments revealed several insights into the mechanisms of action of oxysterols in inhibiting viral replication across several viruses. In SARS-CoV-2 infection, the antiviral activities of 25-OHC and 27-OHC have been described by some (Zu et al. 2020; Marcello et al. 2020; Zang

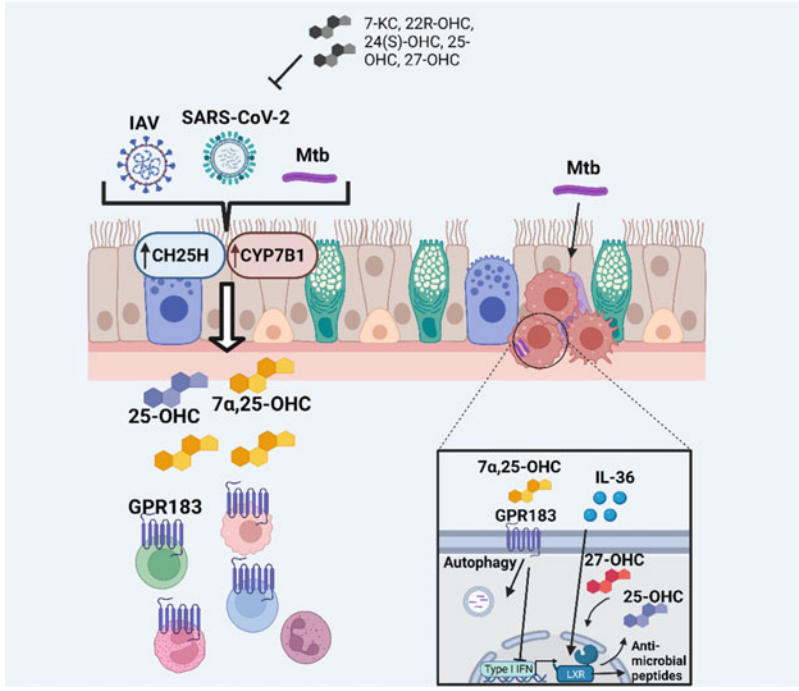

Fig. 7.1 Oxysterols regulate the host response to respiratory pathogens. Infection with *Mycobacterium tuberculosis* (Mtb), SARS-CoV-2, and influenza A virus (left) upregulates the oxysterol synthesising enzymes CH25H and CYP7B1 in the lung which in turn leads to the local production of the oxysterols, 25-OHC and 7α,25-OHC. During Mtb infection, the production of oxysterols facilitates the migration for GPR183-expressing macrophages and eosinophils to the lung. During SARS-CoV-2 and IAV infection, the production of oxysterols facilitates the migration for pro-inflammatory, GPR183-expressing macrophages to the lungs that contribute to immunopathology. In cell systems, exogenous addition of 7-KC, 22R-OHC, 24(S)-OHC, 25-OHC, and 27-OHC had antiviral effects. In human macrophage models (right), the activation of GPR183 by 7α,25-OHC restricts intracellular growth of Mtb by inducing autophagy and limiting type I IFN responses. In addition, IL-36, 27-OHC, and 25-OHC production by macrophages activates LXR signalling, resulting in the production of antimicrobial peptides which contribute to the intracellular inhibition of Mtb growth

et al. 2020; Wang et al. 2020), but not observed by others (Fessler et al. 2022). Majority of these studies suggest that these oxysterols act by preventing viral entry into the cell, rather than having a direct antiviral effect against the pathogen.

Furthermore, conjugating 25-OHC with a peptide-based viral inhibitor with a different mode of action (EK1P4HC) demonstrated a synergistic antiviral effect against a range of human coronaviruses (Lan et al. 2021). Mechanistically, the peptide EK1 inhibits viral entry by disrupting viral six-helical bundle (6-HB) formation by targeting the Heptad repeat 1 domain in the spike protein, whereas 25-OHC remodels the cell surface cholesterol to inhibit membrane fusion, thereby achieving a synergistic effect. In addition to triggering cholesterol remodelling on cell surfaces, a study found that 25-OHC can also localise in late endosomes where it inhibits endosomal cholesterol export, blocking viral fusion (Zang et al. 2020). Utilising a chimeric vesicular stomatitis virus with full-length SARS-CoV-2 S protein in place of the native glycoprotein, the authors found that *Ch25h*$^{-/-}$ hACE2- expressing HEK293 cells resulted in increased replication of the chimeric virus. They further found that the antiviral effects of 25-OHC can be attributed to its localisation within late

endosomes, preventing cholesterol export thereby blocking viral fusion (Zang et al. 2020).

Apart from inhibiting viral entry, a cell-based screen assay found that oxysterols but not cholesterol have antiviral activity against SARS-CoV-2 by limiting viral replication in TMPRSS2-overexpressed VeroE6 cells (Ohashi et al. 2021). Co-incubation of the oxysterols, 7-KC, 22R-hydroxycholesterol, 24S-hydroxycholesterol, and 27-OHC, with the virus inhibits viral replication without causing cytotoxic effects. In addition, semi-synthetic oxysterol derivatives Oxy210 and Oxy232 displayed improved antiviral activity compared to natural oxysterols. Using time-of-addition assays, the authors further showed that Oxy210 prevents viral replication by inhibiting the formation of double-membrane vesicles that SARS-CoV-2 depends on for replication.

In rhinovirus infections, a study found that the oxysterols 25-OHC and 27-OHC have antiviral activity against group A and B human rhinoviruses (HRV A1 and HRVB48, respectively) (Civra et al. 2022). Administration of these oxysterols to HeLa cells reduced viral replication in a dose-dependent manner. In addition, serial passaging of the virus in the presence of these oxysterols did not induce selection pressure for the virus as compared to conventional antivirals (rupintrivir and pleconaril) that resulted in the generation of drug-resistant variants. Administration of the oxysterols to the antiviral resistant variants resulted in similar antiviral activity comparable to the parental strain. Furthermore, the antiviral activity of 27-OHC was explored in 3D models of human nasal and bronchial epithelia derived from cystic fibrosis patients. Treatment of 27-OHC in these 3D structures resulted in reduced production of infectious HRV A1 and prevented virus-induced histological damages (Civra et al. 2022).

The broad antiviral roles of oxysterols do not extend to adenoviruses. 25-OHC does not have antiviral effect against adenovirus 5 and 19a (Blanc et al. 2013). In addition, other oxysterols, 27-OHC, 7α-OHC, 7β-OHC, and 7-KC, have no antiviral affect against adenovirus 5 (Civra et al. 2014).

Collectively, these findings demonstrate that oxysterols are produced both in humans and in mice in response to bacterial and viral respiratory infections in the lung and are detectable in serum. The host oxysterols appear to have a dual role of firstly attracting immune cells to the site of infection via oxysterol-sensing receptors such as GPR183 and potentially CXCR2 and secondly by eliciting an antiviral activity (Mutemberezi et al. 2016). The emerging mechanisms of oxysterol action in the host immune response are discussed in more detail in Sect. 7.5.

7.3 The Role of Oxysterols in Infections of the Gastrointestinal Tract and Liver

7.3.1 Oxysterols and Viral Pathogens of the Gastrointestinal Tract

The antiviral role of oxysterols has been described for non-enveloped virus rotavirus and reovirus. A study initially screened the antiviral capacity of several physiological oxysterols present in the blood among non-enveloped viruses, rotavirus, rhinovirus, and human papillomavirus-16 (Civra et al. 2014). Dose response studies revealed broad antiviral activities of 25-OHC and 27-OHC against these three viruses. Following up with rotavirus and rhinovirus, administration of 25-OHC and 27-OHC prior to infection (but not after infection) reduces the productive replication of the viruses. In addition, a modest but significant antiviral activity of 7α-OHC, 7β-OHC, and 7-KC against these viruses was observed (Civra et al. 2014). In a follow-up study focused on rotavirus (Civra et al. 2018), the authors demonstrated that the antiviral activity of 25-OHC and 27-OHC is not strain specific or cell type specific. They further demonstrated that these oxysterols affect virus entry into the cell, with no effect observed on viral attachment and replication. Mechanistically, in MA104 cells, administration of these oxysterols displaces oxysterol-binding protein (OSBP) from the endoplasmic reticulum (ER), preventing its

subsequent interaction with vesicle-associated membrane protein-associated protein-A (VAP-A) interaction. The displacement and disruption of the OSBP/VAP-A complex prevented cholesterol recycling between the ER and late endosomes. The cholesterol loading in the late endosomes in turn prevented rotavirus entry into the cytoplasm (Civra et al. 2018).

In addition, the disruption of endosomal dynamics induced by 25-OHC is extended to another non-enveloped virus, reovirus, albeit through a different mechanism (Doms et al. 2018). In HeLa cells, the authors found that 25-OHC disrupts reovirus uncoating in the late endosomes by preventing viral co-localisation with Rab7 found in late endosomes. This results in reduced viral entry into the host cytoplasm, affecting infection efficiency and accounting for the antiviral effects observed upon 25-OHC administration (Doms et al. 2018).

The antiviral role of 25-OHC has also been shown for norovirus, using murine norovirus as a model system (Shawli et al. 2019). The authors demonstrated that 25-OHC but not 22(S)-hydroxycholesterol (22(S)-OHC) had antiviral activity against murine noroviruses. In addition, they further discovered that 25-OHC accelerated caspase 3/7 induction in RAW264.7 cells, leading to enhanced cell death in the infected cells, preventing virion production (Shawli et al. 2019).

7.3.2 Role of Oxysterols in Hepatitis Virus Infection

The antiviral role of oxysterols in the liver has also been investigated in in vitro models of hepatitis C and B infections (HCV and HBV, respectively). In hepatocyte Huh7 cells, CH25H expression is found to be upregulated upon infection, as part of the host response to HCV infection (Xiang et al. 2015). In Huh7 cells expressing a MAVS mutant that is resistant to NS3/4A protease cleavage (Huh7-MAVSR cells) CH25H knockdown resulted in increased susceptibility to early HCV infection. In addition, upregulation of CH25H was mediated by several antiviral host factors such as melanoma differentiation-associated gene 5 (MDA5), mitochondrial antiviral-signalling protein (MAVS), interferon regulatory factor 3 (IRF3), and nuclear factor-kB (NF-kB), with MDA5 being a key viral sensor to trigger CH25H induction. Mechanistic studies demonstrated that the antiviral roles of 25-OHC occurred post-entry and were associated with CH25H-mediated disruption of sterol regulatory element binding protein (SREBP) function (Xiang et al. 2015). In a separate study, a semi-synthetic oxysterol Oxy210 inhibited double-membrane vesicles-dependent viral replication in HCV but not HDV (Ohashi et al. 2021). In addition, a chemical-based screen in HepG2 cells transfected with human sodium taurocholate co-transporting polypeptide (HepG2-hNTCP-C4) identified that oxysterols are potential candidates as HBV entry inhibitors. Their study demonstrated that pre-treatment with 22(S)-OHC, 25-OHC, 20α-OHC, and 7β-OHC had anti-HBV effects, suggesting that these oxysterols mediate their antiviral response through blocking HBV entry.

7.3.3 Oxysterols and Bacterial Pathogens of the Gastrointestinal Tract

In addition to the antiviral activity of some oxysterols against a broad range of viruses, the antibacterial activities of oxysterols have also been proposed for intracellular bacteria of the gastrointestinal tract.

The GPR183/oxysterol axis was found to play a protective role against *Citrobacter rodentium* infection through the effective accumulation of GPR183-expressing ILC3s within its cellular niches (Chu et al. 2018). In a haemostatic state, GPR183 regulates the accumulation but not the proliferation of GPR183-expressing ILC3s within their cellular niches in the intestine and mesenteric lymph nodes through 7α,25-OHC production by intestinal stromal cells. In response to *C. rodentium* infection, the lack of GPR183 as demonstrated by $Gpr183^{-/-}$ $Rag1^{-/-}$ double knockout mice resulted in reduced numbers of IL-22-producing GPR183-expressing ILC3s,

more severe disease, and higher mortality rates compared to control animals (*Rag1*$^{-/-}$ mice) (Chu et al. 2018).

CH25H was identified as a key enzyme in the host response against *Listeria monocytogenes* (Abrams et al. 2020) with 25-OHC having antibacterial activity against *L. monocytogenes*. Interestingly, the antibacterial effect was only observed on epithelial cells (HEK293A, Caco-2, and huh7 among others) but not in BMDMs where 25-OHC administration led to increased susceptibility to infection. In mice, intraperitoneal administration of 25-OHC resulted in a >10-fold increase in circulating serum 25-OHC (from 31.7 to 457 nM). Additionally, 25-OHC treatment led to reduced spleen bacterial burden, suggesting reduced tissue dissemination. This effect was also observed in *Ch25h*$^{-/-}$ mice infected with *L. monocytogenes*, which showed increased spleen bacterial burden. The antibacterial activity of 25-OHC is thought to prevent intercellular dissemination, with little effect on bacterial growth, cell death, virulence factors, or the initial stages of infection. Mechanistic studies conducted on *L. monocytogenes* and *Shigella flexneri* revealed that 25-OHC activates acetyl-CoA acetyltransferase 1 (ACAT1) signalling, triggering cholesterol remodelling on the plasma membrane (Abrams et al. 2020). Given that 25-OHC is also a ligand for LXR, these findings may possibly explain an early observation where mice deficient in LXR had increased susceptibility to *L. monocytogenes infections* (Joseph et al. 2004).

25-OHC has also been shown to be protective against cholesterol-dependent cytolysins (CDCs) secreted by pathogenic bacteria (Zhou et al. 2020). The CH25H/25-OHC axis reduces cholesterol availability on the plasma membrane, preventing CDC binding and conferring resistance to CDC-induced pore damage. In vivo, murine CDC models demonstrated that *Ch25h*$^{-/-}$ mice had more severe disease progression characterised by ulcerative lesions and larger lesion sizes. In addition, pre-injection of 25-OHC to the site of CDC damage prevented CDC-mediated tissue damage (Zhou et al. 2020).

In the context of *Helicobacter pylori* infection, 7-dehydrocholesterol was shown to have direct antimicrobial activity against the bacteria (Shimomura et al. 2013). This antibacterial activity is inhibited by 2,6-di-O-methyl-β-cyclodextrin (dMβCD), a sterol solubiliser. When 7-dehydrocholesterol and dMβCD are co-incubated, *H. pylori* could detoxify 7-dehydrocholesterol by glycosylation, causing the oxysterol to lose its toxicity. In addition, a *H. pylori* mutant deficient in cholesterol-α-glucosyltransferase (CGT) activity was found to be more sensitive to 7-dehydrocholesterol-induced toxicity compared to WT *H. pylori*. Interestingly, no difference in bactericidal activity was observed when the mutant strain was treated with dMβCD compared to WT (Shimomura et al. 2013).

7.4 Implications of Oxysterols for Other Viruses and Cell Types

7.4.1 Herpesvirus Family

Herpesviruses are DNA viruses that infect a wide range of host cells and tissues (Adler et al. 2017; Cohen 2020). Belonging to the *herpesviridae* family, Herpesviruses can be further categorised based on their cellular and tissue tropism, including alpha (α), beta (β), and gamma (γ) herpesviruses (Adler et al. 2017). α-Herpesviruses such as herpes simplex virus (HSV) or varicella zoster virus (VZV) primarily infect cells of the nervous system. β-Herpesviruses like human cytomegalovirus and human herpesvirus 6 and 7 (HHV6; HHV7) have a broader range of cell tropism, while γ-herpesviruses such as Epstein-Barr virus (EBV) and Kaposi sarcoma herpesvirus (KSHV) have a more restricted cell tropism. The viral life cycle of herpesviruses has 2 distinct phases, namely lytic infection and latent infection (Cohen 2020). Lytic infection is characterised by active viral replication, resulting in the production of new infectious virions. However, the host's immune response eventually restricts this

replication, leading to the persistence of latently infected cells. During latency, the virus remains dormant and only a limited set of viral genes are expressed. Despite this, the virus retains the ability to reactivate and re-enter the lytic cycle (Cohen 2020). Over the past decade, multiple studies have elucidated that oxysterols have an antiviral effect against a board range of herpesviruses across a variety of cell types.

7.4.1.1 Herpes Simplex Virus 1

In HSV-1, the antiviral role for 25-OHC was shown to extend to a broad range of cells, including HeLa, A549, HEK293T, Vero, and RAW264.7 cells (Blanc et al. 2013; Shawli et al. 2019; Cagno et al. 2017). In addition, other oxysterols such as 22(S)-OHC and 27-OHC had antiviral activity against HSV-1 (Shawli et al. 2019; Cagno et al. 2017). Mechanistic studies in Vero cells revealed that 25-OHC and 27-OHC enhance NF-kB activation, resulting in increased IL-6 production in these cells, contributing to reduced viral replication (Cagno et al. 2017).

7.4.1.2 Varicella Zoster Virus

25-OHC was shown to have a potent antiviral role against VZV in MeWo cells (Blanc et al. 2013). Although the mechanisms by which 25-OHC mediates its antiviral activity during VZV have not been described yet, it is highly likely that 25-OHC prevents VZV entry into the cell by cholesterol remodelling, as VZV entry into the cell is highly dependent on cholesterol availability on cell membranes (Hambleton et al. 2007).

7.4.1.3 Murine Gamma Herpes Virus

In the context of murine gamma herpes virus 68 (MHV-68), antiviral activities of CH25H/25-OHC were demonstrated across several cell types, namely hamster kidney fibroblasts (BHK cells), mouse embryo fibroblasts (NIH/3T3), human embryonic kidney cells (HEK293T), and macrophages (Liu et al. 2013; Mboko et al. 2014). In MHV-68-infected *Ch25h*$^{-/-}$ BMDMs, enhanced viral gene replication and viral DNA synthesis was observed (Mboko et al. 2014). The authors further showed that the CH25H/25-OHC axis did not inhibit MHV68 entry or the initial replication stage of MHV68, but rather restricted the later viral cycle of MHV68 by reducing viral gene expression and DNA synthesis in BMDMs. In addition, the authors showed that IRF1 is required for the optimal expression of CH25H and production of 25-OHC in MHV68-infected BMDMs (Mboko et al. 2014). In murine models, *Ch25h*$^{-/-}$ mice displayed increased susceptibility to MHV-68 infection characterised by a greater and more persistent lytic replication activity and greater genomic DNA load in the spleen compared to WT mice (Liu et al. 2013).

7.4.1.4 Mouse Cytomegalovirus

In BMDMs infected with mouse cytomegalovirus (MCMV), 25-OHC is rapidly produced both intracellularly and extracellularly in the media (Blanc et al. 2013). Other oxysterols, 24(S),25-epoxycholesterol, 3β,7α-dihydroxycholestenoic acid, and 7α,25-OHC were also detected, at very low concentrations. In addition, 25-OHC impeded viral replication post-entry (Blanc et al. 2013). This was consistent with a different study showing that 25-OHC upregulates the integrated stress response pathway and inhibits MCMV replication post-entry (Shibata et al. 2013). The production of 25-OHC led to the activation of general control nonderepressible 2 (GCN2), which in turn led to the downstream transcription of stress response genes (*Chop, Trib3*) independent of LXRs and SREBP activation. The activation of the integrated stress response inhibits protein synthesis which could impede viral replication (Shibata et al. 2013).

7.4.1.5 Kaposi Sarcoma Herpesvirus

A study demonstrated that KSHV possesses strategies to inhibit cholesterol synthesis and oxysterol production (Serquina et al. 2017). The authors found that CH25H expression is upregulated in primary human endothelial cells (human umbilical vein endothelial cells; HUVECs), upon infection with KSHV, but its expression is repressed in latently infected cell lines (MC116.219 and iSLK cell lines). They further discovered in HUVECs, KSHV viral miRNA miR-K63p and miR-K10b repress

CH25H expression. This suggests that KSHV developed viral factors that manipulate the CH25H/25-OHC axis post-infection to establish latency. Pre-treatment but not post-treatment with 25-OHC had antiviral effects in KSHV infection, but 25-OHC did not block viral entry, suggesting a different mechanism of action (Serquina et al. 2017). In a follow-up study, RNA-sequencing data from KSHV-infected HUVECs treated with 25-OHC revealed upregulation of pro-inflammatory pathways which contributed to the antiviral response to KSHV (Serquina et al. 2021).

7.4.1.6 Epstein-Barr Virus

An antiviral role of 25-OHC has also been described in EBV infection (Serquina et al. 2021). The authors found that treatment of PBMCs with 25-OHC both pre- and post-EBV infection induced apoptosis of EBV-infected B cells and prevented the formation of lymphoblastoid cell lines. RNA-sequencing data from EBV-infected B cells treated with 25-OHC revealed a subset of EBV latency genes (EBER1, EBER2, RPMS1, LMP-1) that were downregulated upon 25-OHC treatment. As LMP-1 functions as a critical gene for EBV-mediated B cell transformation by downregulation of DUSP6, they suggest that 25-OHC-induced downregulation of LMP-1 led to the upregulation of DUSP6, which promotes apoptosis of EBV-infected B cells (Serquina et al. 2021).

7.4.2 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a single-stranded RNA virus belonging to the *retroviridae* family. The primary cell type targeted by HIV are $CD4^+$ T cells. Untreated HIV infection results in progressive loss of $CD4^+$ T cells, leading to HIV-associated immunodeficiency (acquired immunodeficiency syndrome; AIDS) (Deeks et al. 2015).

A study demonstrated the antiviral role of CH25H/25-OHC by impeding HIV entry into the cell (Liu et al. 2013). The authors demonstrated that pre-stimulating PBMCs with CH25H-conditioned media prior to HIV NL4-3 infection led to reduced viral p24 expression. This was also observed with pre-treatment of 25-OHC but not 22S-OHC. Subsequent mechanistic studies on the HIV life cycle revealed that CH25H/25-OHC prevents HIV membrane fusion crucial for viral entry. The later stages of HIV life cycle (transcription, translation, and budding) were unaffected by 25-OHC treatment. In addition, intraperitoneal administration of 25-OHC to HIV-infected humanised mice led to reduced serum viral loads compared to vehicle-treated animals. 25-OHC treatment prevented HIV-induced depletion of $CD3^+CD4^+$ T cells, and this was further associated with reduced $CD4^+$ T cells co-expressing viral p24 (Liu et al. 2013).

7.4.3 Zika Virus

Belonging to the *flaviviridae* family, zika virus is a mosquito-transmitted flavivirus that infects a range of cells, including keratinocytes, immune cells, and cells of the nervous system, among others (Sirohi and Kuhn 2017). The virus primarily infects neural progenitor cells, resulting in neurological symptoms such as Guillain-Barré syndrome in adults and children and congenital malformations following infection during pregnancy (Pierson and Diamond 2018). A compound screening for autophagy modulators in Vero and C6/36 cells infected with zika virus revealed the role of 7-KC in regulating the antiviral response towards zika virus (Willard et al. 2018). Time-to-addition experiments conducted in Vero cells demonstrated that 7-KC affects the budding efficiency and productive virion production, the later stages of the viral life cycle (Willard et al. 2018).

7.4.4 Lassa Virus

Lassa virus (LASV) belongs to the *arenaviridae* family of enveloped RNA viruses that causes Lassa fever, an acute viral haemorrhagic fever

(Garry 2023). LASV primarily infects endothelial cells and myeloid immune cells, in particular myeloid dendritic cells and macrophages (Prescott et al. 2017). 25-OHC was shown to limit the production of infectious virus by disrupting viral glycosylation before budding (Shrivastava-Ranjan et al. 2016). In Huh-7 cells, 25-OHC disrupts the glycosylation of glycoprotein 1, resulting in an aberrant form of glycoprotein 1 with an excess of immature N-glycans, resulting in reduced infectivity of the virus. Additionally, CH25H overexpression causes similar impaired glycosylation to that induced by 25-OHC, while knocking down CH25H leads to increased production of infectious virus (Shrivastava-Ranjan et al. 2016).

7.5 Emerging Cellular Mechanisms of Oxysterols in Host Defence

7.5.1 Oxysterols Enhance Host Cell Resistance via Reduction of Membrane Accessible Cholesterol

Oxysterols augment host cell resistance to infection through several mechanisms, including inhibiting cholesterol-dependent cytolysins, reducing transcellular spread of bacteria, and suppressing virus–host cell fusion. A visual summary of the mechanisms described below is shown in Fig. 7.2. A recently identified unifying mechanism for these various properties of oxysterols is their reduction of a minor pool of host cell plasma membrane (PM) cholesterol, so-called accessible cholesterol.

7.5.1.1 A Brief Primer on Intracellular Cholesterol Trafficking and Accessible Cholesterol

Cholesterol is a vital determinant of membrane integrity, and thus cellular cholesterol levels are tightly regulated by a balance of cellular uptake (mediated by the low-density lipoprotein receptor and scavenger receptors), cellular efflux (mediated by the ATP binding cassette [ABC] transporters A1 and G1), storage in lipid droplets as cholesterol esters (mediated by ACAT1), and biosynthesis (orchestrated by the SREBP transcription factors) (Fessler 2016). Up to 90% of cholesterol in mammalian cells resides in the PM, comprising ~30–40 mol% of PM-resident lipids (Lange et al. 1989; van Meer et al. 2008). By contrast, the endoplasmic reticulum (ER) contains ~1% of cellular cholesterol (Lange and Steck 1997), endowing it with high sensitivity to subtle swings in cholesterol. When ER cholesterol falls below ~5 mol%, the cholesterol-sensing ER-resident protein SREBP cleavage-activating protein (SCAP) facilitates activation of the lipogenic transcription factor SREBP-2, driving compensatory cholesterol biosynthesis. By contrast, when ER cholesterol rises above this threshold, SCAP interacts with the ER-retention protein, insulin-induced gene (INSIG), thereby preventing SREBP-2 activation and cholesterol biosynthesis (Radhakrishnan et al. 2008).

Oxysterols, in particular, side-chain oxysterols (22(R), 24(S)-, 25-, 27-OH-cholesterol), serve as dynamic indicators and effectors of cholesterol balance (Cyster et al. 2014). 25-OHC is synthesised from cholesterol by the ER-resident enzyme CH25H when ER cholesterol rises (Lund et al. 1998), thereby signalling cholesterol excess. This and several other oxysterols in turn correct cholesterol excess by co-ordinately blocking SREBP-2 activation, inducing degradation of the rate-limiting cholesterol-synthetic enzyme HMG-CoA reductase, activating the cholesterol efflux-promoting LXR transcription factors, and promoting activation of ACAT1 (Fessler 2016). As oxysterols are more hydrophilic and membrane-permeant than cholesterol, they can also be released to the extracellular milieu and serve as paracrine mediators. Among natural oxysterols, 25-OHC is unique in being secreted by macrophages upon exposure to interferons (IFN) or virus, thus also defining it as an immune-responsive lipid (Blanc et al. 2013).

Given the central importance of the ER in sensing and regulation of cellular cholesterol, a great deal of study has been devoted to identifying the mechanisms by which PM cholesterol equilibrates with the ER. A minor fraction of

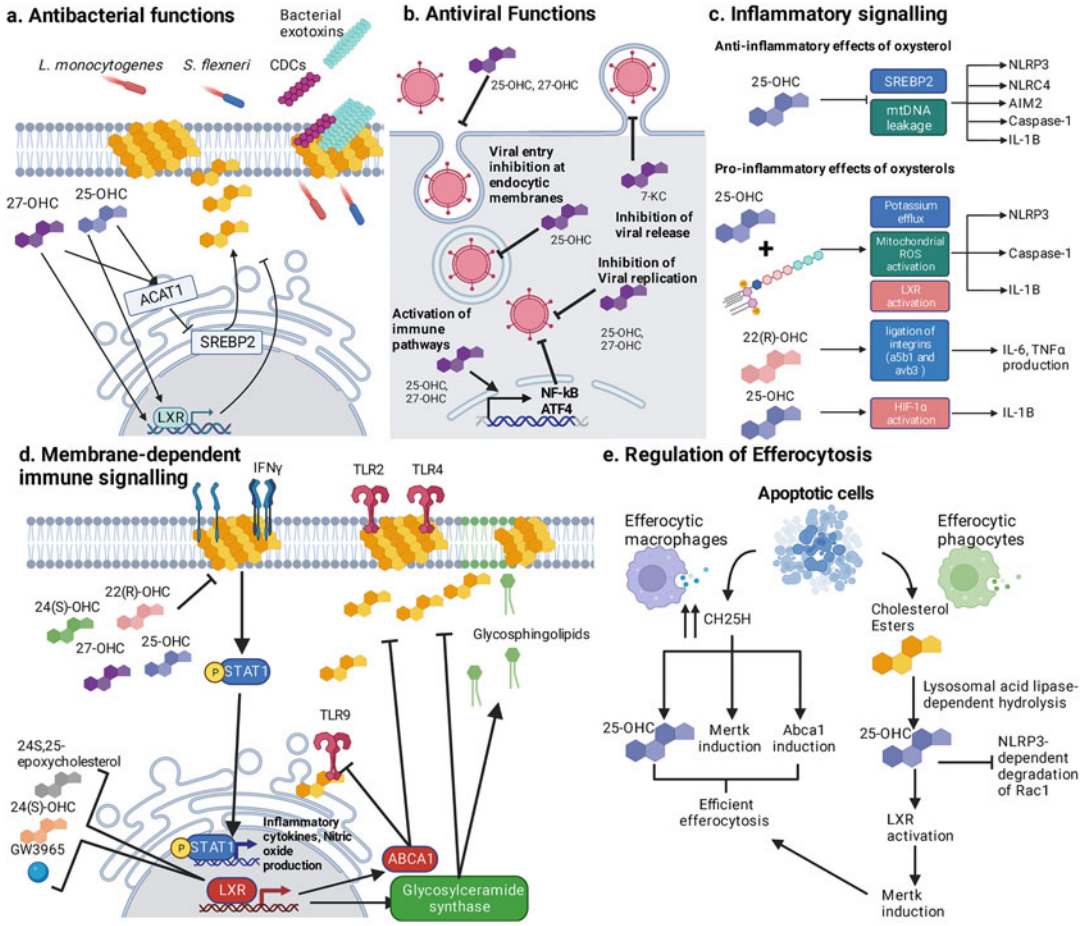

Fig. 7.2 Emerging mechanisms of oxysterol action in regulating host-defence pathways. Oxysterols regulate host-defence pathways through several mechanisms. (**a**) 25-OHC and 27-OHC have antibacterial activity against intracellular pathogens (*Listeria monocytogenes* and *Shigella flexneri*) and bacterial toxins (cholesterol-dependent cytolysins and bacterial exotoxins) by modifying cholesterol content on cellular membranes through ACAT activation or LXR signalling. (**b**) Oxysterols regulate antiviral mechanism of the host cells by (1) impeding viral entry through the modification of cholesterol contents on plasma and endocytic membranes (25-OHC and 27-OHC). (2) Activation of immune signalling pathways such as ATF4 and NF-kB (25-OHC and 27-OHC). (3) Direct antiviral replication (25-OHC and 27-OHC) and (4) inhibition of viral release (7-KC). (**c**) Oxysterols regulate the inflammatory signalling within the host cells. (1) The anti-inflammatory role of 25-OHC has been described by SREBP2 inhibition and inhibition of mitochondrial DNA leakage. (2) Pro-inflammatory effects of oxysterols have been described for 25-OHC with LPS stimulation, activation of NLRP3, caspase-1 and IL-1B production; 22(R)-OHC is triggering IL-6 and TNF production and 25-OHC is triggering IL-1B production. (**d**) Oxysterols also regulate receptor signalling on cholesterol-rich membranes. (1) The oxysterols 24(S)-OHC, 22(R)-OHC, 27-OHC, and 25-OHC inhibit IFNγ receptor trafficking to membrane rafts, thereby suppressing induction of cytokines and nitric oxide. LXR activation (2) attenuates pro-inflammatory signalling by TLR2, TLR4, and TLR9 in macrophages through ABCA1-dependent reduction of lipid raft cholesterol (3) increasing glycosphingolipids and reducing cholesterol in the PM altering proximal T cell receptor signalling. (**e**) Oxysterols also play a role in efferocytosis where cholesterol esters from apoptotic cells induce production of 25-OHC in efferocytic phagocytes, leading to LXR activation and inhibition of NLRP3-dependent degradation of Rac1, all of which contributes to efferocytic activity. In efferocytic macrophages, apoptotic cells trigger the upregulation of CH25H, which in turn leads to the production of 25-OHC and the induction of Mertk and Abca1 leading to efficient efferocytosis

PM cholesterol has long been thought to become 'activated' (i.e. more freely mobile and accessible to extra-membrane acceptor molecules) (Radhakrishnan and McConnell 2000), when sequestration by membrane phospholipids and sphingomyelin is saturated, a transition that has been proposed to occur when PM cholesterol exceeds ~35 mol% (Das et al. 2013; Lange et al. 1980). Recent studies have shown that the size of this pool of 'accessible cholesterol' in the PM is regulated by the ASTER/GRAMD1 cholesterol-transfer proteins, which ensure its rapid equilibration with the ER (Ferrari et al. 2020; Naito et al. 2019; Sandhu et al. 2018). Of interest, side-chain oxysterols enhance the lability of accessible cholesterol, likely by thinning and disordering membranes (Bielska et al. 2014; Olsen et al. 2013). Oxysterol-induced movement of PM cholesterol to the ER is thought to cause sustained reduction of PM accessible cholesterol by promoting ACAT1-dependent cholesterol esterification and inhibiting SREBP-2-dependent cholesterol biosynthesis. Thus, 25-OHC but not 7α-HC severely depletes PM accessible cholesterol within one hour of application (Abrams et al. 2020).

7.5.1.2 Antagonism of Cholesterol-Dependent Cytolysins and Other Bacterial Exotoxins

The same feature of accessible cholesterol that allows it to serve as a rapidly transferable signal—namely, its lability and increased exposure to the environment—has been exploited by pathogens. Cholesterol-dependent cytolysin (CDC) exotoxins, including those expressed by *L. monocytogenes* (listeriolysin O), *Streptococcus pneumoniae* (pneumolysin O), *Bacillus anthracis* (anthrolysin O), *Streptococcus pyogenes* (streptolysin O), and *Clostridium perfringens* (perfringolysin O) all dock via a cholesterol-binding domain on accessible cholesterol in the mammalian PM, and then oligomerise to execute lytic pore formation (Johnstone et al. 2022). Indeed, the cholesterol-binding domains of anthrolysin O and perfringolysin O have been exploited experimentally as reporters for PM accessible cholesterol by fusion to or labelling by fluors (e.g. GFP, Alexa Fluors), epitope tags, or radioisotopes (Abrams et al. 2020; Zhou et al. 2020; Das et al. 2013).

Several groups have recently reported that 25-OHC and other side-chain oxysterols are protective against CDC-expressing bacteria through their ability to reduce PM accessible cholesterol. Thus, exogenous 25-OHC inhibits *L. monocytogenes* infection of epithelial cells by reducing PM accessible cholesterol; indeed, suggesting importance for endogenous 25-OHC, media from IFNγ-treated WT macrophages but not from IFNγ-treated *Ch25h*$^{-/-}$ macrophages protect epithelial cells from *L. monocytogenes* in trans (Abrams et al. 2020). Evidence suggests that 25-OHC and other side-chain oxysterols reduce accessible cholesterol rapidly via allosteric activation of ACAT1, and in a more delayed and sustained fashion through SREBP-2 inhibition (blocking cholesterol synthesis) (Abrams et al. 2020). Type I and II IFNs protect macrophages against several CDCs by decreasing toxin binding to a small pool of cholesterol in the PM, a response that is mediated by native CH25H-dependent production of 25-OHC and effected in part via ACAT-dependent esterification and in part by inhibition of new cholesterol synthesis (Zhou et al. 2020). Suggesting therapeutic applications, exogenous 25-OHC was found to protect the skin of mice from CDC-induced tissue damage (Zhou et al. 2020). Similarly, 27-OHC and 25-OHC reportedly protect epithelial cells from *T. pyogenes* pyolysin-induced cytolysis, and 27-OHC protects against streptolysin O and *S. aureus* hemolysin, partly due to ACAT-dependent reduction of PM accessible cholesterol and partly due to activation of LXRs (Ormsby et al. 2022; Ormsby et al. 2021). Although they are unrelated to the CDCs, *C. difficile* toxins A and B also translocate into host cells through the endosomal membrane in a fashion that is dependent on membrane cholesterol, and it has been shown that this can be attenuated by 25-OHC through SREBP-2 inhibition (Giesemann et al. 2006; Papatheodorou et al. 2019).

7.5.1.3 Inhibition of Transcellular Pathogen Spread

Although 25-OHC does not inhibit cellular invasion of *L. monocytogenes*, including listeriolysin O-dependent vacuolar escape, it reportedly inhibits contact-dependent cell-to-cell spread of the pathogen through host cell protrusions in a membrane cholesterol-dependent fashion (Abrams et al. 2020). This likely explains the higher pathogen burden observed in *L. monocytogenes*-infected *Ch25h*$^{-/-}$ mice and the capacity of supplemental 25-OHC to reduce *Listerial* burden in WT mice (Abrams et al. 2020). *S. flexneri*, the Gram-negative bacterial cause of the diarrheal disease shigellosis, spreads in vivo in a similar contact-dependent transcellular fashion and is similarly impeded in its dissemination by 25-OHC (Abrams et al. 2020). In the case of both pathogens, inhibition of dissemination by 25-OHC is reversed by loading host cells with exogenous cholesterol (Abrams et al. 2020), suggesting that 25-OHC operates through reduction of PM accessible cholesterol. In an analogous fashion, 25-OHC inhibits syncytia formation by virus (Liu et al. 2013).

7.5.1.4 Inhibition of Viral Fusion

The recently discovered broad-spanning antiviral activity of oxysterols has been the topic of comprehensive reviews (Fessler 2016; Lembo et al. 2016) and is discussed at length elsewhere in this chapter. One of the central mechanisms by which 25HC impedes virus infection—its direct actions on host cell membranes to reduce virus fusion (Liu et al. 2013; Zang et al. 2020; Wang et al. 2020)—has recently been appreciated to derive from depletion of accessible cholesterol. Thus, 25HC but not 7α-HC restricts entry of SARS-CoV-2 and Middle East respiratory syndrome-coronavirus (MERS-CoV) into epithelial cell lines by inhibiting viral spike protein-mediated fusion, and does so by mobilising accessible cholesterol through an ACAT-dependent mechanism (Wang et al. 2020). Other studies suggest that 25-OHC anti-fusion activity may alternatively derive from cholesterol accumulation in the endolysosomal compartment of infected cells (Zang et al. 2020). Future studies will be required to resolve the relative contribution of these divergent mechanisms.

7.5.2 Oxysterols Modulate Membrane-Dependent Immune Signalling

Signal transduction by immune receptors (e.g. Toll-like receptors, T cell receptor) during host defence is critically sensitive to PM cholesterol content, in part because many receptors are assembled and activated in cholesterol-rich lipid raft membrane microdomains (Fessler and Parks 2011). In recent years, studies have clarified that oxysterols can impact immune cell signalling through effects on PM lipids. Thus, 22(R)-, 24(S)-, 25-, and 27-OHC reportedly attenuate IFNγ signalling in microglia by reducing PM accessible cholesterol and disrupting IFNγ receptor recruitment to rafts, thereby suppressing induction of cytokines and nitric oxide (Lee et al. 2022). This effect was found to persist in LXRα- and LXRβ-silenced cells, but an LXR-dependent mechanism was not excluded as dually LXRα/LXRβ-deficient cells were not tested (Lee et al. 2022). Alternatively, it has been reported that synthetic LXR agonists (oxysterol analogues) attenuate pro-inflammatory signalling by TLR2, TLR4, and TLR9 in macrophages through ABCA1-dependent reduction of lipid raft cholesterol (Ito et al. 2015); although natural oxysterol LXR ligands were not tested in this report, it is intriguing to consider that autocrine CH25H-dependent 25-OHC could contribute to LPS tolerance. Finally, in human $CD4^+$ T cells, both synthetic LXR ligands (GW3965) and natural oxysterols increase glycosphingolipids and reduce cholesterol in the PM in part through induction of the LXR target glycosylceramide synthase, thereby reducing lipid order at the immune synapse and altering proximal T cell receptor signalling (Waddington et al. 2021). Taken together, these reports indicate that oxysterols not only augment host cell resistance

to pathogens through remodelling of PM lipids, but that they also modify the host cell signalling response to infection through transcriptional and non-transcriptional effects on PM lipid composition.

7.5.3 Mixed Effects of Oxysterols on Inflammasome Activation and IL-1β Induction

The NLRP3, NLRC4, and AIM2 inflammasomes, cytosolic complexes that activate caspase-1, play important roles during infection both through induction of pyroptotic death of infected cells and through processing of mature IL-1β, a cytokine critical to host defence (Nozaki et al. 2022; Swanson et al. 2019). Of interest, both enhancing and suppressive effects of oxysterols on inflammasome activation and IL-1β induction have been reported in different model systems. The extent to which these divergent effects derive from differences in oxysterol concentration, cell type, and cholesterol loading status of the cells studied remains unclear.

In a landmark paper, it was reported that 25-OHC reduces *Il1b* transcription through SREBP-2 inhibition and also broadly represses multiple inflammasomes (NLRP3, NLRC4, AIM2) and that, as a consequence, *Ch25h*-null mice overproduce IL-1 family cytokines and exhibit hypersensitivity to septic shock but enhanced ability to repress *Listerial* growth (Reboldi et al. 2014). It was subsequently determined that 25-OHC inhibits activation of AIM2 and NLRP3 at least in part by suppressing SREBP-2-dependent cholesterol synthesis, mitochondrial cholesterol overload, and consequent cytosolic release of mitochondrial DNA (Dang et al. 2017). Thus, endogenous 25-OHC, induced in response to TLR ligands, acts in an autocrine fashion to preserve mitochondrial integrity against cholesterol overload and thereby to guard against aberrant inflammasome activation. Of interest, suggesting direct coupling between cholesterol biosynthesis and NLRP3 activation, it was recently reported that NLRP3 associates with SREBP-2 and its chaperone SCAP in a ternary complex and relies on translocation of this complex to a mitochondrial-adjacent location in the Golgi apparatus for optimal inflammasome assembly (Guo et al. 2018). As a consequence, cholesterol depletion of cells promotes NLRP3 inflammasome activation by inducing SCAP-mediated SREBP-2 trafficking to the Golgi, whereas 25-OHC suppresses NLRP3 activation and attendant caspase-1 activation and IL-1β maturation by inhibiting this translocation (Guo et al. 2018). SCAP has also been shown to interact with stimulator of interferon genes (STING) and to be required for STING-induced IFNβ and host defence against virus and *L. monocytogenes*, but this reportedly does not derive from SCAP-dependent translocation of STING (Chen et al. 2016).

Contrary to these publications that 25-OHC suppresses inflammasome activation and IL-1β processing, it was recently reported that *Ch25h*-null murine microglia exhibit deficient induction of IL-1β in response to LPS and that supplemental 25-OHC dose-dependently augments LPS induction of mature IL-1β at concentrations as low as ~2.5 μM and in an enantioselective fashion (Wong et al. 2020). Another group found that exogenous 25-OHC augments LPS-induced IL-1β release, caspase-1 activation, and NLRP3 aggregation in macrophages at concentrations ≥50 μM, effects that were proposed to derive from potassium efflux, mitochondrial reactive oxygen species induction, and LXR activation (Jang et al. 2016). Synthetic LXR agonists as well as 22(R)-OHC have also been shown to induce *Il1b* mRNA in human but not murine macrophages through a mechanism involving TLR-independent activation of HIF-1α (Menegaut et al. 2020). Extending beyond the inflammasome and IL-1β, it was also recently reported that 25-OHC at high concentrations (50 μM) induces IL-6 and TNF in murine and human macrophages through direct ligation of integrins α5β1 and αvβ3 and consequent activation of focal adhesion kinase (Pokharel et al. 2019). In support of the physiological relevance of this pathway, it was found that, in response to infection with respiratory syncytial virus, *Ch25h*-null, α5β1-inhibited, and β3-null macrophages all

exhibit reduced expression of TNF (Pokharel et al. 2019).

7.5.4 Emerging Roles for 25-OHC in Efferocytosis

Efferocytosis, the phagocytic clearance of cell corpses by macrophages, dendritic cells, and parenchymal cells, is thought to play a key homeostatic role by promoting resolution of inflammation during infection and tissue injury (Boada-Romero et al. 2020). Over a decade ago, it was reported that LXR expression by phagocytes is required for anti-inflammatory efferocytic clearance of cell corpses at least in part through its direct induction of *Mertk*, an efferocytosis receptor (A-Gonzalez et al. 2009). Although this initial report provided evidence that induction of efferocytic cell *Mertk* by apoptotic cells could be blocked by the non-specific sterol-depleting agent, methyl-β-cyclodextrin, the identity of the putative LXR-activating sterols that accumulate in efferocytic phagocytes has remained uncertain.

Recently, two independent groups have provided evidence that 25-OHC plays a central role in ensuring successful efferocytosis. In the first report, it was shown that lysosomal acid lipase-dependent hydrolysis of cholesterol esters derived from internalised apoptotic cells is required for induction of 25-OHC in efferocytic phagocytes and that 25-OHC preserves efferocytosis by (1) protecting mitochondria and thereby preventing NLRP3-dependent degradation of Rac1 and (2) activating LXR and consequent *Mertk* induction (Viaud et al. 2018). The authors proposed that delivery of cell corpse-derived free cholesterol to the ER induces CH25H-dependent generation of 25-OHC, which then inhibits SREBP-2-dependent cholesterol biosynthesis (Viaud et al. 2018). Although not directly tested in this report, it is intriguing to speculate that 25-OHC may preserve mitochondria and thereby prevent inflammasome activation during efferocytosis through the same mechanism reported for it in LPS-stimulated macrophages, namely through prevention of mitochondrial cholesterol overload (Dang et al. 2017). In the second report, it was found that apoptotic cells upregulate *Ch25h* in efferocytic macrophages and that CH25H is required for efferocytic cell induction of *Mertk* and *Abca1* and for efferocytic clearance of apoptotic cells (Madenspacher et al. 2020). Thus, *Ch25h*-null mice were found to have defective efferocytic clearance of apoptotic neutrophils and reduced induction of LXR target genes and anti-inflammatory TGFβ in their lungs, whereas supplemental treatment of wild-type mice with 25-OHC accelerated clearance of lung neutrophils in an LXR-dependent manner. Suggesting that internalisation of cell corpses may not be required for the response, *Ch25h* was found to be inducible in macrophages by the MERTK ligand growth arrest-specific 6 (GAS6), and phosphatidylserine-containing liposomes were sufficient for *Mertk* and *Abca1* induction in macrophages (Madenspacher et al. 2020).

7.6 Conclusions

Oxysterols are produced in the immune response to both bacterial and viral infections. This suggests that oxysterols could serve as biomarkers of infectious disease severity and could be useful in predicting treatment outcomes.

The mechanisms of action of these bioactive cholesterols are broad ranging from chemotaxis of immune cells and regulation of inflammation to some oxysterols showing antiviral/antibacterial properties. Further research is required to fully elucidate the mechanisms of action of different oxysterol species and their receptors in the immune response to infections.

However, the immunomodulatory action and antimicrobial/antiviral properties of these oxysterols suggest that oxysterol receptors are promising targets for host-directed therapies to reduce inflammation and pathogen load and improve infectious disease outcomes.

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The Cholesterol-5,6-Epoxide Hydrolase: A Metabolic Checkpoint in Several Diseases

8

Philippe de Medina, Silia Ayadi, Khadijetou Diallo, Julio Buñay, Laly Pucheu, Regis Soulès, Michel Record, Severine Brillouet, Lavinia Vija, Frederic Courbon, Sandrine Silvente-Poirot, and Marc Poirot

Abstract

Cholesterol-5,6-epoxides (5,6-ECs) are oxysterols (OS) that have been linked to several pathologies including cancers and neurodegenerative diseases. 5,6-ECs can be produced from cholesterol by several mechanisms including reactive oxygen species, lipoperoxidation, and cytochrome P450 enzymes. 5,6-ECs exist as two different diastereoisomers: 5,6α-EC and 5,6β-EC with different metabolic fates. They can be produced as a mixture or as single products of epoxidation. The epoxide ring of 5,6α-EC and 5,6β-EC is very stable and 5,6-ECs are prone to hydration by the cholesterol-5,6-epoxide hydrolase (ChEH) to give cholestane-3β,5α,6β-triol, which can be further oxidized into oncosterone. 5,6α-EC is prone to chemical and enzymatic conjugation reactions leading to bioactive compounds such as dendrogenins, highlighting the existence of a new metabolic branch on the cholesterol pathway centered on 5,6α-EC. We will summarize in this chapter current knowledge on this pathway which is controlled by the ChEH.

Philippe de Medina, Sandrine Silvente-Poirot and Marc Poirot contributed equally with all other contributors.

P. de Medina · S. Ayadi · K. Diallo · J. Buñay · L. Pucheu · R. Soulès · M. Record · S. Silvente-Poirot · M. Poirot (✉)
Cancer Research Center of Toulouse (CRCT), Inserm, CNRS, University of Toulouse, Team INOV: "Cholesterol Metabolism and Therapeutic Innovations", Toulouse, France

Equipe labellisée par la Ligue Nationale contre le Cancer, Toulouse, France

French Network for Nutrition Physical Activity and Cancer Research (NACRe Network), Jouy-en-Josas, France
e-mail: marc.poirot@inserm.fr

S. Brillouet
Cancer Research Center of Toulouse (CRCT), Inserm, CNRS, University of Toulouse, Team INOV: "Cholesterol Metabolism and Therapeutic Innovations", Toulouse, France

Equipe labellisée par la Ligue Nationale contre le Cancer, Toulouse, France

French Network for Nutrition Physical Activity and Cancer Research (NACRe Network), Jouy-en-Josas, France

Department of Radiopharmacy, Institut Universitaire du Cancer Toulouse – Oncopole, Toulouse, France

L. Vija · F. Courbon
Cancer Research Center of Toulouse (CRCT), Inserm, CNRS, University of Toulouse, Team INOV: "Cholesterol Metabolism and Therapeutic Innovations", Toulouse, France

Equipe labellisée par la Ligue Nationale contre le Cancer, Toulouse, France

French Network for Nutrition Physical Activity and Cancer Research (NACRe Network), Jouy-en-Josas, France

Department of Medical Imaging, Institut Universitaire du Cancer Toulouse – Oncopole, Toulouse, France

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Keywords

Cholesterol · Sterols · Oxysterol · Oncosterone · Dendrogenins · Cancer · Cell differentiation · Autophagy · Exosomes

8.1 Introduction

The epoxide hydrolases (EHs) constitute a family of enzymes present in all organisms, which transform epoxide containing lipids by the addition of water to give a *trans*-diol. An epoxide (or oxirane) is a three-membered cyclic ether. Five EHs have been described in vertebrates which are: soluble EH (sEH), microsomal EH (mEH), cholesterol EH (Cholesterol-5,6-epoxide hydrolase or ChEH), hepoxilin hydrolase, and leukotriene A4 (LTA4) hydrolase (Morisseau 2013; Newman et al. 2005). ChEH (EC 3.3.2.11) represents a distinct subset among EHs with respect to its substrate specificity, activity, and molecular identity. ChEH is very selective for the cholesterol-5,6-epoxide (5,6-EC) diastereoisomers: cholesterol-5α,6α-epoxide (5,6α-EC) and cholesterol-5β,6β-epoxide (5,6β-EC) and catalyzes their stereoselective hydration into cholestane-3β,5α,6β-triol (CT) (Silvente-Poirot and Poirot 2012; Sevanian and Mcleod 1986; Nashed et al. 1985) (Fig. 8.1). ChEH has stimulated the interest of researchers when 5,6-EC were suspected of being involved in skin carcinogenesis (Chan and Black 1974; Lo and Black 1973; Black and Lo 1971). Because of the presence of the epoxide group, it was supposed that 5,6-EC could react spontaneously with nucleophiles and behave like alkylating agents with direct carcinogenic properties. However, contradictory results were published concerning the potential carcinogenic and mutagenic effects of 5,6-ECs. This was reviewed in (Poirot and Silvente-Poirot 2013). In addition, the potential alkylating activity of 5,6-ECs were recently ruled out by showing that 5,6-ECs are stable and un-reactive toward nucleophiles under non-catalytic conditions (Paillasse et al. 2012). The present review is focused on ChEH and its relationship with cholesterol biosynthesis in connection with cancer and neurodegenerative diseases.

8.2 The ChEH Enzyme

ChEH was characterized at the molecular level as being a pharmacological target of tamoxifen, a drug widely used and approved by the FDA for the treatment and the prevention of breast cancers (BC) expressing the estrogen receptor alpha (ESR1). ChEH is a hetero-oligomerous proteinaceous complex made mainly of two different enzymes involved on the post-lanosterol cholesterol biosynthesis: (1) the 3β-hydroxysteroid-Δ8,Δ7-isomerase also named as the emopamil-binding protein (EBP) or delta8-delta7-isomerase (D8D7I), which catalyzes the isomerization of zymostenol (5α-cholest-8-en-3β-ol) and zymosterol (5α-cholestadien-8,24-en-3β-ol) into

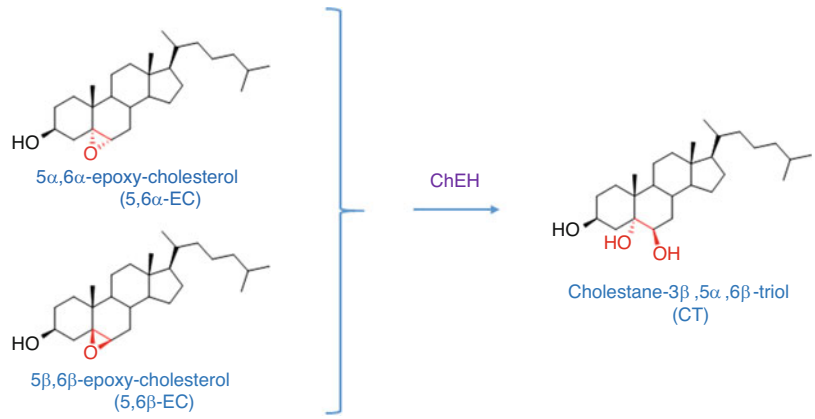

Fig. 8.1 The cholesterol-5,6-epoxide hydrolase (ChEH) catalyzes the trans hydration of 5,6α- and 5,6β-epoxycholesterol to give cholestane-3β,5α,6β-triol

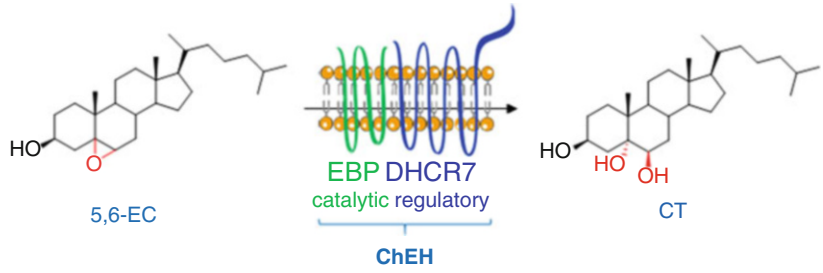

Fig. 8.2 ChEH is a heterodimer of EBP and DHCR7. EBP is the catalytic subunit and DHCR7 is the regulatory subunit

lathosterol (5α-cholest-7-en-3β-ol) and 24-dehydrolathosterol (5α-cholestadien-7,24-en-3β-ol), respectively; (2) the 3β-hydroxysteroid-Δ7-reductase (DHCR7) that converts 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) and 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol) into cholesterol and desmosterol. EBP and DHCR7 were found necessary and sufficient to reconstitute the ChEH (de Medina et al. 2010; Kedjouar et al. 2004). EBP was shown to carry out the catalytic activity of ChEH, while DHCR7 was the regulatory subunit of ChEH (Fig. 8.2). The crystal structure of EBP was published in the presence of Tamoxifen (PDB: 6OHU) (Long et al. 2019). Tamoxifen was shown to be a competitive inhibitor of ChEH (de Medina et al. 2010), and docking experiments of EBP showed that 5,6-EC fits well within the tamoxifen binding site of EBP (Fig. 8.3). Our team reported that the ChEH was identical to the microsomal anti-estrogen binding site (AEBS), a high affinity microsomal binding site for Tamoxifen and related compounds (Leignadier et al. 2017; de Medina et al. 2010; Kedjouar et al. 2004). All the AEBS ligands were found to be ChEH inhibitors and oxysterols known as substrates or inhibitors of ChEH were found ligands of the AEBS(de Medina et al. 2010; Sevanian and Mcleod 1986). It was also found that their affinity for the AEBS correlated positively with their potency to inhibit the ChEH (de Medina et al. 2010). Other proteins such as the microsomal epoxide hydrolase (mEH) and the 3β-hydroxysteroid-Δ24-reductase (DHCR24) were found to affect ChEH activity and the AEBS pharmacological profile when co-expressed with EBP and DHCR7 (M Poirot, unpublished results). ChEH is a promiscuous enzyme that binds drugs belonging to different pharmacological classes including selective estrogen receptors modulators such as Tamoxifen, diphenylmethane compounds such as tesmilifene, phenothiazines, and amiodarone (Silvente-Poirot and Poirot 2012). It includes also natural compounds such as B-ring oxysterols (CT, OCDO, 7-hydroxy- and 7-ketocholesterol) (Silvente-Poirot and Poirot 2012), dendrogenin A (de Medina et al. 2013), and polyunsaturated fatty acids including oleic, arachidonic, and docosahexaenoic acids (de Medina et al. 2010). Recent inhibitors of EBP such as TASIN inhibitors developed for the treatment of colorectal cancers (Theodoropoulos et al. 2020; Wang et al. 2019; Zhang et al. 2018; Zhang et al. 2016) or for the remyelination of dendrocytes (Sax et al. 2022; Caprariello and Adams 2022; Han and Zhou 2019; Hubler et al. 2018) have been reported. These compounds are likely to be ChEH inhibitors and their evaluation deserves further investigations.

8.3 5,6-ECs Formation and Stability

ChEH activity requires 5,6-ECs for producing their hydration product CT. The conditions that are required for 5,6-ECs production have been reviewed before (Poirot and Silvente-Poirot 2013) (Fig. 8.4). These include some reactive oxygen species, lipoperoxidation, and cytochrome p450 for the stereoselective production of 5,6α-EC. The existence of a cytochrome p450

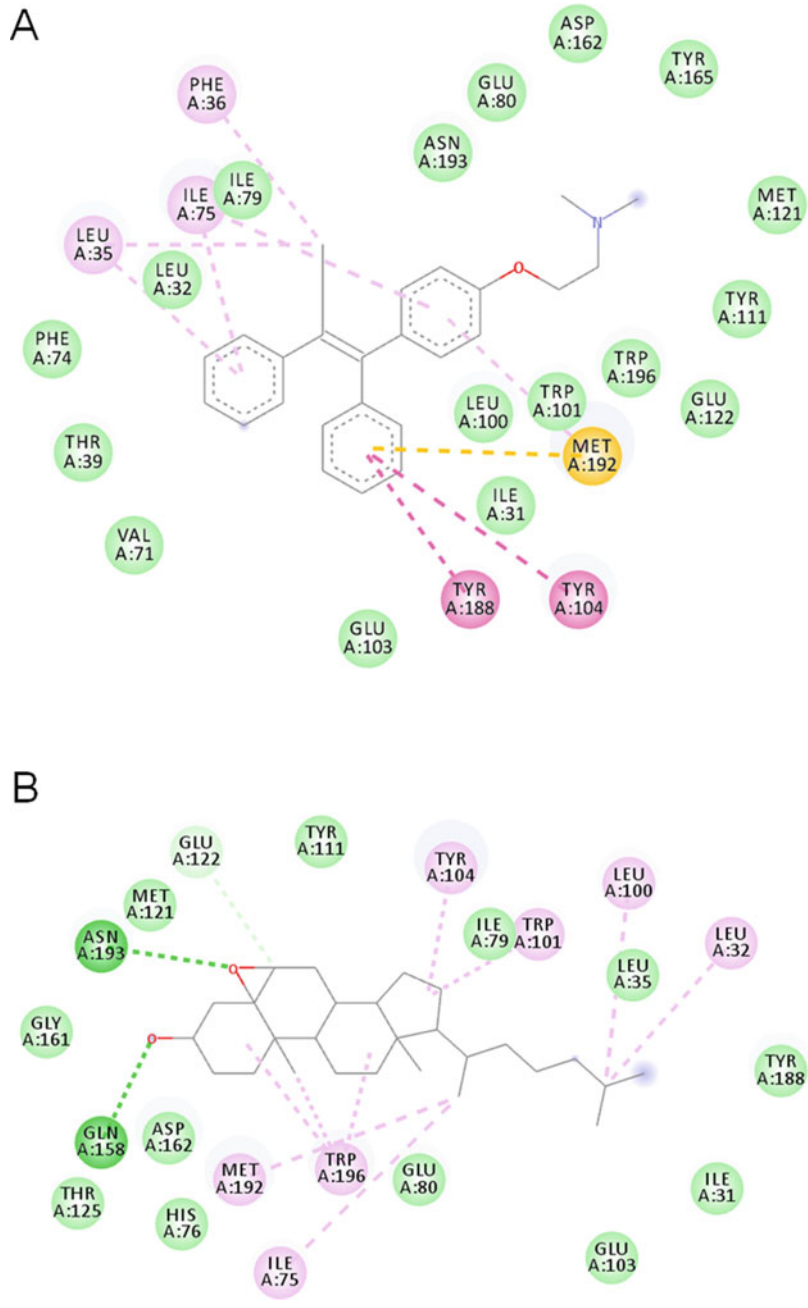

Fig. 8.3 Molecular modeling of the putative catalytic site of ChEH after docking of 5,6β-EC into the EBP. (**a**) Two-dimensional topography of the tamoxifen binding site on EBP (obtained using Discovery Studio v 2021). (**b**) Two-dimensional topography of the 5,6β-EC binding site on EBP highlighting that ASN193 could act as a proton donor that catalyzes the opening of the epoxide ring. *Interactions:* very light green: carbon Hydrogen bond; light green: van der Waals; dark green: conventional Hydrogen bond; orange: pi-sulfur; dark pink: pi-pi T-shaped; light pink: alkyl, pi-alkyl

involved in the stereoselective production of 5,6α-EC (Watabe and Sawahata 1979) supports the existence of specific metabolic branch based on 5,6α-EC transformation. Interestingly, recent studies from Zielinski et al showed that cholesterol epoxidation with a peroxide can give variable ratios of both diastereoisomers depending on the presence of a proton donor at proximity of the reaction (Zielinski and Pratt 2019). This suggests that even lipoperoxidation could give one or the other isomer as a main product according to the biochemical context in which the reaction takes place.

It was postulated that 5,6-ECs could be potent alkylating substances, like other chemicals bearing epoxide groups such as styrene oxides, but

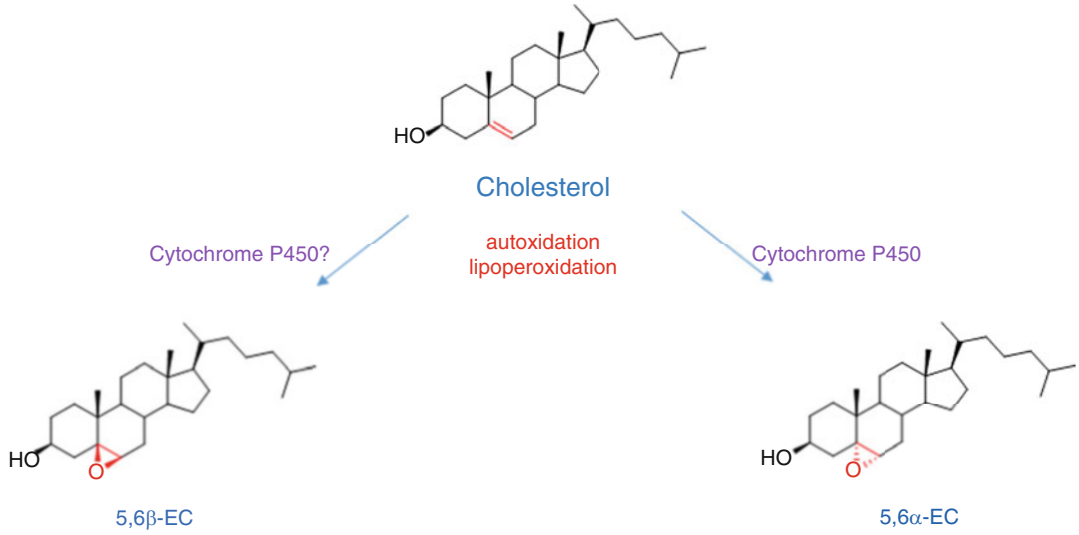

Fig. 8.4 5,6-EC formation and biosynthesis. 5,6-EC can be produced from cholesterol by different nonenzymatic and enzymatic mechanisms

5,6-ECs have been shown not to be carcinogenic when injected on rat nipples (El-Bayoumy et al. 1996). While 5,6-EC is known since a very long time (Schroepfer 2000), it is only recently that their reactivity toward nucleophilic substances including a nucleic acid base was tested. 5,6-ECs were shown to be exceptionally stable and totally un-reactive toward nucleophiles including guanine, at ambient and physiological temperature, as opposed to the carcinogen styrene-oxide (Paillasse et al. 2012). Importantly, 5,6-ECs are stable for several days in the presence of extremely high concentrations of nucleophiles, ruling out that 5,6-ECs are spontaneously reactive and behave like direct carcinogenic or alkylating agents. Thus, the unreactivity of 5,6-ECs diastereoisomers toward nucleophiles suggests that the biological function of ChEH is not to detoxify cells from 5,6-ECs by metabolizing them into a more soluble CT as opposed to what was first suggested (Morin et al. 1991).

8.4 ChEH Substrates

ChEH is very specific to the hydrolysis of 5,6-ECs into CT. It was shown to hydrolyze 5,6-epoxy-β-sitosterol, one of the major phytosterol (Aringer and Eneroth 1974). 7-dehydrocholesterol-5,6β-epoxide was reported to be an irreversible inhibitor of ChEH (Nashed et al. 1986) (Fig. 8.5). Other steroidal epoxides were not reported to date to be substrates or inhibitors of ChEH. Fatty acid and sulfate esters of cholesterol are not inhibitors of ChEH and thus are not substrates of the ChEH. This shows that esterification provides a protection against the hydration of ECs by ChEH (de Medina et al. 2010). Fatty acid and sulfate esters of 5,6-ECs are not inhibitors of ChEH (Fig. 8.5) (de Medina et al. 2010). Epoxides of polyunsaturated fatty acids (PUFAs) that were reported to be inhibitors of ChEH (de Medina et al. 2010) have not yet being tested as substrates of ChEH.

8.5 Subcellular Localization, Tissue Distribution and Regulation of ChEH

EBP and DHCR7 co-localized in the endoplasmic reticulum of cells (Koczok et al. 2019) where cholesterol biosynthesis takes place (Dietschy and Turley 2004). These enzymes are expressed in most mammalian tissues (liver, kidney, lung, testes, spleen, brain, intestinal epithelium, and

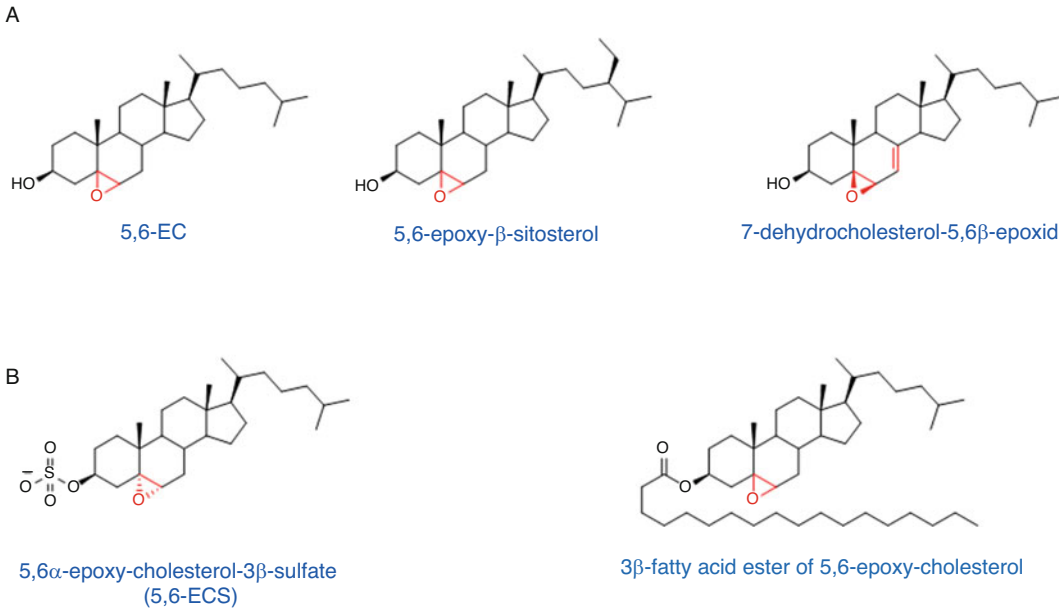

Fig. 8.5 Chemical structure of (**a**) ChEH substrates and (**b**) nonsubstrates

skin), with the liver being the richest source (see Human Protein Atlas, https://www.proteinatlas.org/). EBP and DHCR7 together with DHCR24 have been shown to colocalize in the nuclear envelope (Koczok et al. 2019). Consistently, ChEH is mainly present in the microsomes (subcellular fractions that contain the reticulum endoplasmic) of various tissues including the liver. ChEH is also found in tumor cells of different tissue origins ((Silvente-Poirot and Poirot 2012) and (M. Poirot and S. Silvente-Poirot, personal communication)). Since EBP and DHCR7 have been reported to be upregulated in several cancers (Kuzu et al. 2016), it is still to be defined what would be the impact of various EBP/DHCR7 ratio on ChEH activity.

8.6 Biological Functions of the ChEH

ChEH enzymatic function is to produce CT and to control 5,6-ECs levels. So we must consider the metabolism and the biological properties of CT, 5,6α-EC and 5,6β-EC and their metabolites.

8.6.1 Biological Properties of CT and Its Metabolites

CT was reported to be one of the most potent cytotoxic oxysterol (Schroepfer 2000), with little effect on cholesterol biosynthesis downregulation (Morin and Peng 1989). It was reported to inhibit the osteoblastic differentiation and to induce apoptosis suggesting it is a common factor underlying the pathogenesis of atherosclerosis and osteoporosis (Liu et al. 2005). CT was shown to inhibit prostate cancer cell proliferation, migration and invasion via a modulation of Liver-X-Receptor α (LXRα) (Lin et al. 2013). CT has been shown to be a neuroprotective endogenous compound that protects against neuronal injury by direct binding and by inducing a negative modulation on N-methyl-D-aspartate (NMDA) receptors (Hu et al. 2014). CT suppresses neuronal hyperexcitability through a direct interaction with the voltage-gated sodium channel (Tang et al. 2018). CT was shown to induce vascular smooth cells calcification, which may be a mechanism through which CT favors the formation of the atherosclerotic plaque (Liu et al. 2004, 2007).

On the other side, CT has been shown to be a precursor of secondary metabolites involved in the control of carcinogenic programs. CT is genotoxic through reactive oxygen species production (Cheng et al. 2005) and can be metabolized into oncosterone by the 11-β-hydroxysteroid dehydrogenase of type 2. Oncosterone was shown to be a tumor promoter in breast cancer and is a ligand of glucocorticoid receptor α (GRα) and LXRs (Voisin et al. 2017). Oncosterone is a biased agonist on GR that drives cellular proliferative programs (de Medina et al. 2021; Silvente-Poirot et al. 2018a; Poirot et al. 2018; Voisin et al. 2017).

CT is probably also prone to sulfation by the sulfotransferase SULT2B1b, since B-ring oxysterols are substrates of the enzyme but this deserves further evaluation (Fuda et al. 2007) and should give cholestane-5α,6β-diol-3β-*O*-sulfate (CDS) which could serve as a modulator of LXR (Song et al. 2001). However, assessment of the biological properties of CDS is difficult because it can be hydrolyzed by the steroid sulfatase (STS) which is widely expressed in human tissues (https://www.proteinatlas.org/ENSG00000101846-STS/tissue) to give back CT from CDS. So recently de Medina et al. have developed a non-hydrolyzable analog of CDS to test if CDS could be a biologically active metabolite (de Medina et al., 2023 Manuscript submitted for publication). CT is also known as a biomarker of several pathologies (Zanjani et al. 2023; Unluturk et al. 2023; Cooper et al. 2020; Vonica et al. 2019; Reddy et al. 1977) and inherited diseases such as Niemann–Pick C1 disease (Porter et al. 2010).

8.6.2 Biological Properties of 5,6β-EC and Its Metabolites

5,6β-EC has not been shown to be a ligand of LXRs and it was reported to display cell-specific LXR modulatory activities (Segala et al. 2013; Berrodin et al. 2010). 5,6β-EC is a good substrate for ChEH on cell lysates and a better substrate on whole cell assays (Voisin et al. 2017; de Medina et al. 2010) showing that it may have a major contribution as a preferred substrate of ChEH for the production of CT and its metabolites such as oncosterone. 5,6β-EC does not activate the acyl-coA:cholesterol acyl transferase 1 (ACAT1/SOAT1) and is a weak substrate of ACAT1 (Zhang et al. 2003). It is a substrate of the human plasma lecithin-cholesterol acyltransferase (LCAT) (Szedlacsek et al. 1995). 5,6β-EC was shown to be a weak substrate of SULT2B1b (Fuda et al. 2007). High concentrations in 5,6β-EC was shown to induce cell death through the impairment of the mitochondrial activity (Segala et al. 2013; Vejux et al. 2007; Lordan et al. 2007; O'Callaghan et al. 2001).

8.6.3 Biological Properties of 5,6α-EC and Its Metabolites

5,6α-EC has been shown to be a ligand and modulator of LXRs with cell-specific activities (Segala et al. 2013, Berrodin et al. 2010). 5,6α-EC is one of the best substrates of the sulfotransferase SULT2B1b (Fuda et al. 2007) to give 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS). 5,6-ECS was shown to act as the signaling molecule responsible for the induction of breast cancers cells redifferentiation by AEBS ligands (Segala et al. 2013) and to be a LXR modulator (Segala et al. 2013; Song et al. 2001). 5,6α-EC is a potent activator and substrate of the acyl-coA:cholesterol acyl transferase 1 (ACAT1/SOAT1) expressed on the endoplasmic reticulum of cells to produce 5,6α-EC- and cholesteryl-fatty acid esters (Zhang et al. 2003) while it was reported that plasmatic lecithin-cholesterol-acyltransferase (LCAT) contributes little for fatty acid esterification of 5,6α-EC (Yamamuro et al. 2020). Cholesteryl esters display tumor promoter properties (Websdale et al. 2022; Khallouki et al. 2018; Paillasse et al. 2009) but the eventual tumor promoter properties of 5,6α-EC-fatty acid esters have yet not been investigated and deserve further investigations. More interestingly, 5,6α-EC is the precursor of dendrogenin A (DDA), dendrogenin B (DDB), C17 compound that were all found as metabolites present in

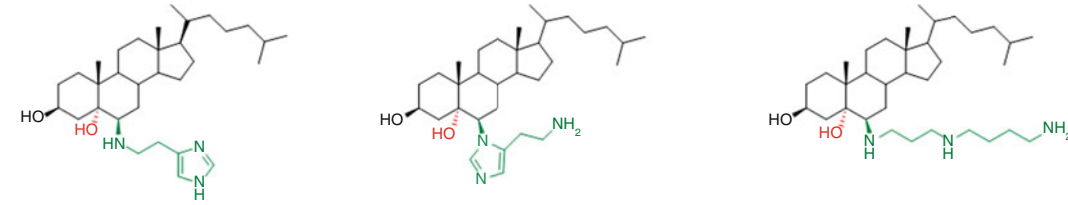

Fig. 8.6 Chemical structure of DDA, C17, and DDB. These compounds are 5,6α-EC natural mammalian metabolites

mammalian tissues (Fig. 8.6) (Soules et al. 2019; de Medina et al. 2013). DDA and C17 are two regioisomers that result from the conjugation of 5,6α-EC with histamine by its primary amine and by its imidazole ring, respectively, and DDB is a 5,6α-EC conjugate with a primary amine of spermidine (Soules et al. 2019; Noguer et al. 2017; de Medina et al. 2009) (Fig. 8.6). DDA is a bioactive conjugated oxysterol that has been proved to be a tumor suppressor metabolite that induces cancer cell redifferentiation, production of antitumor exosomes and at sub micromolar concentrations it induces a lethal autophagy, while the DDA regio-isomer C17 was found inactive on these effects (de Medina et al. 2009, 2013, 2021, 2023; Record et al. 2022; Serhan et al. 2020; Mouchel et al. 2020; Bauriaud-Mallet et al. 2019; Silvente-Poirot et al. 2016, 2018b; Poirot and Silvente-Poirot 2016, 2018; Segala et al. 2017; Dalenc et al. 2015; Silvente-Poirot and Poirot 2014). While it was reported that the receptor responsible for its tumor suppressive effects is the LXRβ, it is a ligand of both LXRα and LXRβ (Segala et al. 2017), so it is yet unknown what could be the biological effects of DDA mediated by LXRα if any. DDA is not an agonist of LXR but a biased agonist. As opposed to canonical LXR ligands, it is a weak antagonist on some LXR-dependent genes such as ABCA1 and an agonist on low density lipoprotein receptor (LDLR) expression and on the control of genes involved in cell differentiation, some lipids biosynthesis enzymes, and the control of lysosomes formation and autophagy processes (de Medina et al. 2023; Record et al. 2022; Serhan et al. 2020; Mouchel et al. 2020; Bauriaud-Mallet et al. 2019; Silvente-Poirot et al. 2018a, b; Poirot and Silvente-Poirot 2018; Segala et al. 2017). DDA is also a very potent inhibitor of the ChEH and of oncosterone formation highlighting the existence of a regulation loop at the ChEH level (de Medina et al. 2021; Poirot et al. 2018; Poirot and Silvente-Poirot 2018; Voisin et al. 2017; de Medina et al. 2013).

The C17 compound, which is the inactive regio-isomer of DDA on the induction of cell death and differentiation (Segala et al. 2017; de Medina et al. 2009, 2013) was shown to be, on a LXR-dependent luciferase cell system, a selective LXRα agonist (Segala et al. 2017).

DDB was shown to be a potent inductor of the redifferentiation of glioma and neuroblastoma cell lines into cells with morphological and phenotypical features of glutaminergic neurons (de Medina et al. 2009). A similar effect was observed with DDA, but DDA redifferentiation effect was not restricted to these cell lines (Fig. 8.7) (de Medina et al. 2009). DDA and DDB induce the proliferation, the formation of neurospheres, and the differentiation of adult mice neural stem cells. DDA and DDB restore neuronal excitability after injury, proving that they can compensate neuronal loss (Fransson et al. 2015; Khalifa et al. 2014).

Together these data showed that dendrogenin A and B constitute a new class of bioactive oxysterols. These conjugates are cationic alkylamino-oxysterols.

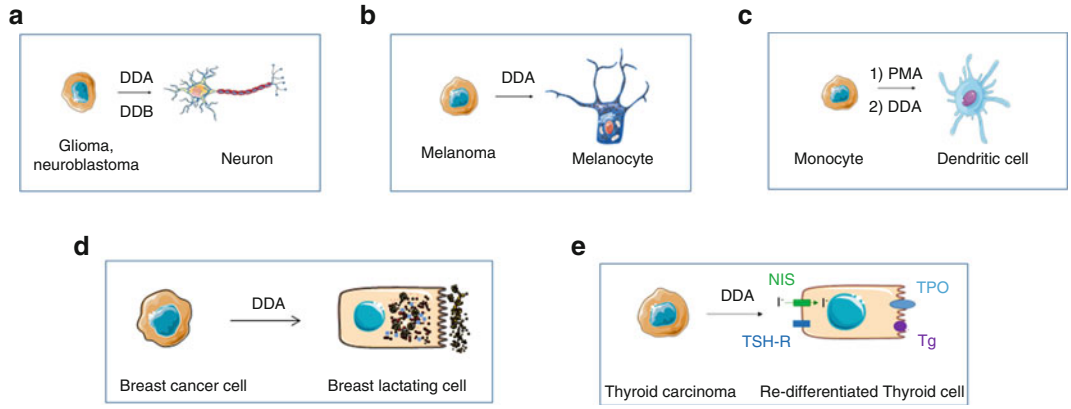

Fig. 8.7 Dendrogenins are bioactive metabolites of 5,6α-EC. (**a**) DDA and DDB induce the differentiation of glioma, neuroblastoma, and pluripotent progenitor cells into glutaminergic neurons. (**b**–**e**) pluripotency of DDA to differentiate cancer cells. (**b**) DDA induces the differentiation of melanoma cells into melanocyte, (**c**) of monocyte into dendritic cells, (**d**) of breast cancer cells into lactogenic cells, and (**e**) of thyroid carcinoma cells into functional thyrocytes. NIS, sodium iodide symporter; TPO, thyroperoxidase; Tg, thyroglobulin; TSH-R, thyroid stimulating hormone receptor

8.7 Regulation of ChEH

Little is known on that topic. We noticed a work from Faye et al. showing that AEBS (also known as ABS at that time) from rat uterus increased during estrus (Faye et al. 1980) suggesting that female sex steroid hormones could regulate ChEH activity. This is supported by more recent data showing that the overexpression of ERα increased cholesterogenesis through the upregulation of gene encoding cholesterogenesis enzymes under the transcriptional control of SREBP2 in mice (Wang et al. 2006). In addition, it is reported that both EBP (Misawa et al. 2003) and DHCR7 (Prabhu et al. 2014) are under the transcriptional control of SREBP2. Together these data suggest that the ChEH activity can be modulated by female sex steroid hormones. The fact that DHCR7 can be regulated by AMP kinase and protein kinase A (Prabhu et al. 2017) suggests that ChEH activity could be controlled by cell surface signaling (Patel and Smith 2023; London and Stratakis 2022) and nutrient sensing (Gonzalez et al. 2020).

8.8 Conclusion

We report on this chapter our current knowledge on the ChEH enzyme and propose future research directions. We show that ChEH constitutes a metabolic checkpoint controlling the production of bioactive metabolites such as dendrogenins, CT, oncosterone and probably other yet unknown metabolites. This illustrates the existence of a new fascinating metabolic branch on the cholesterol pathway. This new branch deserves further investigations that will led to a better understanding of fine processes involved on mammalian development, physiology and that may give new clues to improve our understanding of several degenerative diseases and aging processes.

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Impact of Oxysterols in Age-Related Disorders and Strategies to Alleviate Adverse Effects

9

Shubhrima Ghosh, Imen Ghzaiel, Anne Vejux, Steve Meaney, Sagnik Nag, Gérard Lizard, Garima Tripathi, Falal Naez, and Srijita Paul

Abstract

Oxysterols or cholesterol oxidation products are a class of molecules with the sterol moiety, derived from oxidative reaction of cholesterol through enzymatic and non-enzymatic processes. They are widely reported in animal-origin foods and prove significant involvement in the regulation of cholesterol homeostasis, lipid transport, cellular signaling, and other physiological processes. Reports of oxysterol-mediated cytotoxicity are in abundance and thus consequently implicated in several age-related and lifestyle disorders such as cardiovascular diseases, bone disorders, pancreatic disorders, age-related macular degeneration, cataract, neurodegenerative disorders such as Alzheimer's and Parkinson's disease, and some types of cancers. In this chapter, we attempt to review a selection of physiologically relevant oxysterols, with a focus on their formation, properties, and roles in health and disease, while also delving into the potential of natural and synthetic molecules along with bacterial enzymes for mitigating oxysterol-mediated cell damage.

Keywords

Oxysterols · Cholesterol oxidation products · Cholesterol metabolism · Signaling pathways · Age-related disorders

S. Ghosh (✉)
Trinity Translational Medicine Institute, School of Medicine, Trinity College Dublin, Dublin 8, Ireland

I. Ghzaiel
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Faculty of Medicine, Laboratory 'Nutrition, Functional Food and Vascular Health' (LR12ES05), University of Monastir, Monastir, Tunisia

A. Vejux · G. Lizard
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

S. Meaney
School of Biological, Health and Sports Sciences, Technological University Dublin, Dublin 7, Ireland

S. Nag · G. Tripathi
Department of Bio-Sciences, School of Biosciences & Technology, Vellore Institute of Technology (VIT), Vellore, Tamil Nadu, India

F. Naez
Department of Microbiology, Vijaygarh Jyotish Ray College, University of Calcutta, Kolkata, India

S. Paul
Department of Microbiology, Gurudas College, Kolkata, West Bengal, India

Abbreviations

5,6β-EC	5, 6β-Epoxycholesterol
5α,6α-EC	5α, 6α-Epoxycholesterol
5β,6β-EC	5β, 6β-Epoxycholesterol
7-α,25-OHC	7-α,25-Dihydroxycholesterol
7α-OHC	7-α Hydroxycholesterol
7β-OHC	7-β Hydroxycholesterol

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7KC	7-Ketocholesterol
11β-HSD1	11β-Hydroxysteroid dehydrogenase type 1
20S-OHC	20(S)-Hydroxycholesterol
22R-OHC	22(R)-Hydroxycholesterol
22S-OHC	22(S)-Hydroxycholesterol
24,25-EC	24S,25-Epoxycholesterol
24S-OHC	24(S)-Hydroxycholesterol
25-OHC	25-Hydroxycholesterol
27HC	27-Hydroxycholesterol
AD	Alzheimer's disease
AMD	Age-related macular degeneration
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
CAT	Catalase
CDs	Carbonylated dienes
CH25H	Cholesterol 25-hydroxylase
ChEH	Cholesterol epoxide hydrolase
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CPs	Carbonylated proteins
CSF	Cerebrospinal fluid
CYP27A1	Cytochrome P450 27A1
CYP7A1	7α-Hydroxylase
D8D7I	3β-Hydroxysterol-Δ(8)-Δ(7)-isomerase
DDA	Dendrogenin A
DHCR7	3β-Hydroxysterol-Δ(7)-reductase
DMF	Dimethyl fumarate
DNA	Deoxyribonucleic acid
ERs	Estrogen receptors
ERK	Extracellular signal-regulated kinase
GPx	Glutathione peroxidase
HDL	High-density lipoprotein
HIF-1α	Hypoxia-inducible factor-1α
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
ICAM-1	Intercellular adhesion molecule-1
INSIGs	Insulin-induced proteins
LDL	Low-density lipoprotein
LXR	Liver X receptor
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
MIP-1β	Monocyte inflammatory protein-1β
MMF	Monomethyl fumarate
MS	Multiple sclerosis
NCBI	National Centre for Biotechnology Information
NF-κB	(nuclear factor kappa light chain enhancer of activated B cells)
NMDA	*N*-methyl-D-aspartate
ORP5	Oxysterol-binding protein-related protein-5
OSBP	Oxysterol-binding protein
PD	Parkinson's disease
PDAC	Pancreatic ductal adenocarcinoma
PNETs	Pancreatic neuroendocrine tumors
PSA	Prostate-specific antigen
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RRMS	Remittent recurrent multiple sclerosis
SARS-CoV 2	Severe acute respiratory syndrome coronavirus 2
SERMs	Selective estrogen receptor modulators
SOD	Superoxide dismutase
SREBP	Sterol regulatory element-binding protein
SULT2B1b	Sulfotransferase
TCA	Tricarboxylic acid
TLR	Toll-like receptors
TNFα	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion protein-1
VEGF	Vascular endothelial growth factor

9.1 Introduction

Cholesterol is a ubiquitous membrane sterol, present in a broad variety of organisms and with diverse roles that impact the structure, cellular biology, and physiology of all organisms that synthesize or consume it (Barenholz 2002). It is the obligate precursor for numerous biologically critical molecules such as steroid hormones and bile acids, which are synthesized from cholesterol via a series of oxidation and reduction reactions which tailor the structure of the parent sterol to create a particular biological activity (Chiang and Ferrell 2019; Schiffer et al. 2019). Oxysterol or cholesterol oxidation product is a general term

used to refer to sterol molecules which have one of more additional oxygen functions and includes ketosterols, hydroxysterols, and sterol hydroperoxides (Poli et al. 2022; Samadi et al. 2020).[1] They are created when cholesterol is oxidized, either enzymatically or nonenzymatically, adding oxygen atoms to the structure. The addition of oxygen atoms to the cholesterol moiety leads to modifications in its ketone, hydroxy or epoxy groups, resulting in distinct chemical structures and unique properties, different from the cholesterol molecule. Several well-known oxysterols include, 7-α Hydroxycholesterol, 7-β Hydroxycholesterol, 7-Ketocholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 5α, 6α-epoxycholesterol, 5β, 6β-epoxycholesterol, cholesterol-3-β, 5-α, 6-α-triol, and cholesterol-3-β, 5-α, 6-β-triol among others.

Oxysterols are a fascinating class of compounds that have recently attracted a lot of attention, due to their diverse biological activities and potential implications in human health. They serve as important signaling molecules involved in a wide range of physiological processes and play crucial roles in regulating lipid metabolism, cholesterol homeostasis, and bile acid synthesis (Olkkonen and Lehto 2004; Brown and Jessup 2009). Moreover, oxysterols have been implicated in various cellular functions, including inflammation, immune response, neuronal signaling, cell proliferation, and apoptosis. In recent years, research has shed light on the potential involvement of oxysterols in the pathogenesis of several diseases. Dysregulation of oxysterol metabolism has been linked to the development of cardiovascular diseases, age-related macular degeneration, cataract, neurodegenerative disorders such as Alzheimer's and Parkinson's disease, and several cancers (Brown and Jessup 2009; Olkkonen et al. 2012). Furthermore, oxysterols have been recognized as key players in the modulation of cellular cholesterol transport and the formation of atherosclerotic plaques (Zmysłowski and Szterk 2017). Additionally, the development of analytical techniques for accurate quantification of oxysterols in biological samples has paved the way for studying their roles as potential biomarkers for disease diagnosis and monitoring (Griffiths and Wang 2011). In this chapter, we will review a selection of physiologically relevant oxysterols, with a focus on their formation, properties, and roles in health and disease, while also delving into potential of natural and synthetic molecules for mitigating oxysterol-mediated cell damage (Fig. 9.1).

9.2 Biogenesis and Associated Signaling Pathways of Oxysterols

As discussed above, oxysterols are synthesized by transformation of the sterol ring in cholesterol by the addition of oxygen. These modifications may arise due to the action of specific enzymes/enzyme pathways, or in conjunction with various "spontaneous" low-temperature processes commonly referred to as cholesterol auto-oxidation (Schroepfer 2000). While some oxysterols may arise from either pathway, in a steady-state cellular and physiological context, the most abundant oxysterols are typically due to enzyme-mediated formation (ibid). As many of the enzymes that modify oxysterols are also active on auto-oxidation products, the metabolic pathways associated with oxysterols are complex and, at present, incompletely defined, despite being studied for more than a century.

9.2.1 Formation of Oxysterols by Auto-oxidation

Environmental stressors (e.g., temperature, radiation) may trigger the formation of oxysterols, via a well-recognized phenomenon generally termed auto-oxidation that does not require specific enzyme catalysts (Schroepfer 2000). In a cellular context, sterol auto-oxidation may be considered a response to direct or indirect oxidative stress and may be driven by various radical species, lipid peroxides, or metals. While the formation of a broad range of oxysterols via autoxidation has been reported under various conditions, in a

[1] For the purposes of this chapter, we do not include oxyphytosterols in the working definition of oxysterols.

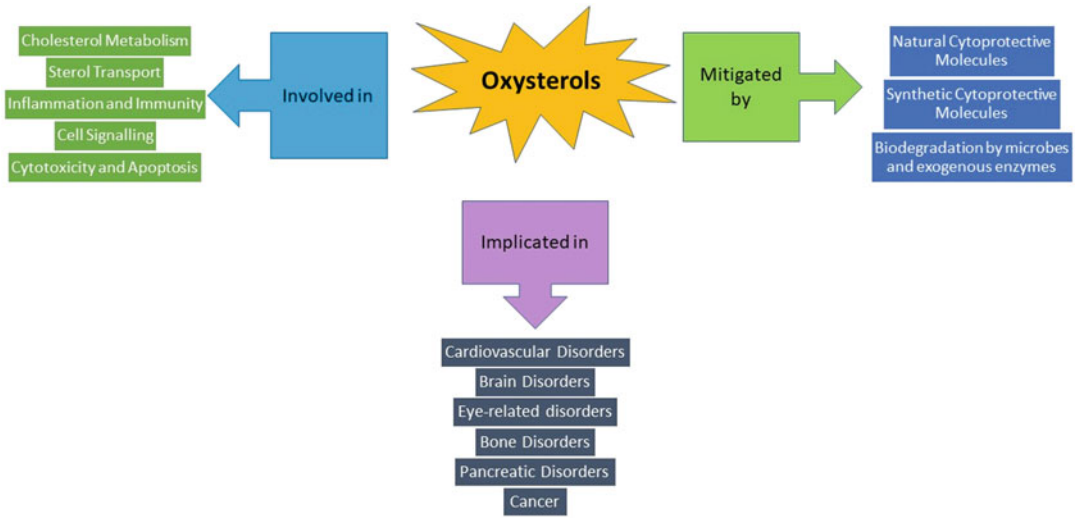

Fig. 9.1 Key roles played by oxysterols and the ambit of the chapter. In the current chapter, we focus on the various roles played by oxysterols as cellular effectors, in age-related disorders, and plausible mitigation strategies

biological context the main oxysterols observed are 7-ketocholesterol (7KC), 7-β-hydroxycholesterol (7β-OHC), the 5,6-epoxycholesterols, and cholestane-3β,5α,6-β-triol. Epoxides and/or hydroperoxides are the primary products of an oxidative attack on cholesterol, although the hydroperoxides typically form under rapid thermal decomposition. For example, at the C7 position, the epimeric cholesterol 7-hydroperoxides decompose to yield a mixture of 7KC, 7α-OHC, and 7β-OHC, although the latter oxysterol is often considered to be a preferential product of this process (Smith and Johnson 1989). In similarity, 5α,6β-EC is regarded as the preferential auto-oxidation product (de Medina et al. 2021).

9.2.2 Formation of Oxysterols by Enzyme Action

Oxysterols are well-recognized intermediates in the formation of bile acids and steroid hormones where they are typically present as an early step in the modification of cholesterol (Olsen et al. 2012). The majority of reactions feeding into bile acid biosynthesis are catalyzed by the action of cytochrome P450 enzymes which introduce a stereoselective hydroxyl (OH) group onto the sterol molecule (Lorbek et al. 2012). In the liver, cholesterol 7α-hydroxylase (CYP7A1) mediates the formation of 7α-hydroxycholesterol (7α-OHC) from cholesterol, the first and flux governing step in the classical (i.e., neutral) pathway of bile acid synthesis (Chiang and Ferrell 2020a). While some authors have provided evidence that there may be some extrahepatic expression of CYP7A1, the consensus is that almost all CYP7A1 is present within the liver (ibid). In contrast, sterol 27-hydroxylase (CYP27A1) is expressed more broadly and indeed has a more broad substrate specificity, acting on many different sterols (including some auto-oxidation products, see below) to introduce an hydroxyl group at the (25R)26 position, a modification often referred to in the literature as 27-hydroxylation. The action of CYP27A1 represents the initial step in the alternative (i.e., acidic) pathway of bile acid synthesis, so called because the same enzyme can further metabolize the primary hydroxysterol product to an acidic metabolic, a C-(25R)26 carboxylic acid (Griffiths and Wang 2020). In the human circulation, (25R) 26-hydroxycholesterol and its acidic metabolites constitute a major portion of the overall circulating oxysterols, which is perhaps not unexpected given the broad expression of CYP27A1 (Uhlén et al. 2015). In contrast to CYP27A1, and

in similarity with the restricted expression of CYP7A1, the enzyme mediating the formation of a third major oxysterol, cholesterol 24-hydroxylase (CYP46A1), is also restricted in expression. CYP46A1 catalyzes the formation of 24(S)-hydroxycholesterol within the brain, which serves as a pathway to eliminate cholesterol from the brain (Moutinho et al. 2016; Pikuleva and Cartier 2021). CYP46A1 has also been shown to mediate the formation of 24S,25-epoxycholesterol (24,25-EC) and (25R)26-hydroxycholesterol from desmosterol (an intermediate in the cholesterol synthesis pathway) (Goyal et al. 2014).

Other oxysterols may be formed enzymatically, for example 25-hydroxycholesterol (25-OHC) despite this oxysterol being historically regarded as an auto-oxidation product. The gene encoding cholesterol 25-hydroxylase (CH25H) was identified 25 years ago and, in contrast to other side-chain oxysterol synthetic enzymes, is a diiron/dioxygenase enzyme (Lund et al. 1998). However, the promiscuous CYP3A4 has also been shown to possess the ability to form 25-OHC from cholesterol(Honda et al. 2011). A significant resurgence of interest in CH25H was triggered by the recognition that it was immunologically active and a direct precursor of the EBI2 ligand 7α,25-dihydroxycholesterol (Bauman et al. 2009; Cyster et al. 2014; Hannedouche et al. 2011).

Although considered an auto-oxidation product, 7KC may also have enzymatic origins. More than 25 years ago, Song and collaborators showed that 7KC could be produced enzymatically from 7α-OHC in hamster liver (Song et al. 1996). Subsequent studies by various investigators have identified a role for 11β-hydroxysteroid dehydrogenases in the interconversion of 7β-OHC and 7KC, although significant species differences are noted (Beck et al. 2019; Odermatt and Klusonova 2015). It has also been shown that the formation of cholestane-3β,5α,6β-triol from 5,6-EC is catalyzed by the concerted action of two cholesterol biosynthetic genes (3-β-hydroxysterol-Δ(8)-Δ(7)-isomerase, (D8D7I) and 3β-hydroxysterol-Δ(7)-reductase (DHCR7)) which together display cholesterol epoxide hydrolase (ChEH) activity (Poirot and Silvente-Poirot 2013).

In most biological contexts, these oxysterols are the quantitatively dominating molecules. However, it is important to note that within the body, there is some evidence that there are discrete anatomical or cellular sites where local concentrations of oxysterols may be significantly elevated (e.g., in lymph nodes, the RPE, foam cells/macrophages) and any evaluation of the physiological effects of oxysterols should consider the anticipated concentrations under in vivo conditions (Dias et al. 2019). Figure 9.2 demonstrates the major enzymatic and non-enzymatic routes of formation of oxysterols.

9.3 Major Classification of Oxysterols and Their Derivatives Implicated in Diseases

Several oxysterols have been reported to occur in food products including 7-α hydroxycholesterol, 7-β hydroxycholesterol, 25-hydroxycholesterol, 7-Ketocholesterol, 27-hydroxycholesterol, 5α, 6-α-epoxycholesterol, 5β, 6β-epoxycholesterol, cholesterol-3-β, 5-α, 6-α-triol, and cholesterol-3-β, 5-α, 6-β-triol. They are often investigated to contribute to the metabolism of lipids and cholesterol, and thus implicated in the pathogenesis of a number of diseases, some of which are described below.

9.3.1 7-α Hydroxycholesterol

7-α hydroxycholesterol is a major precursor molecule in the bile acid synthesis pathway. It is majorly formed by the action of cholesterol 7-α-hydroxylase (CYP7A1) and the synthesis of this compound is the rate-determining step of the bile acid pathway (Chiang and Ferrell 2020a, b). It is steroid by nature and has been an intermediate in many enteric disorders. Along with HMG CoA (β-Hydroxy β-methylglutaryl-CoA), 7-α hydroxycholesterol acts as a regulatory molecule in the bile acid synthesis and function. This oxysterol facilitates the formation of 7-α,25-

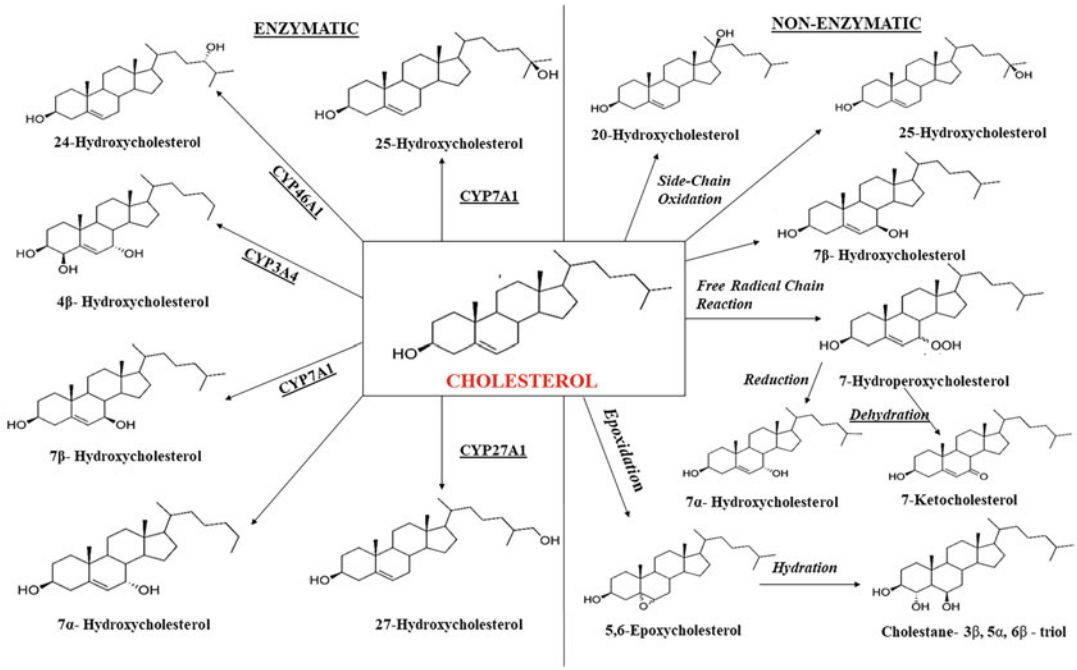

Fig. 9.2 Major pathways of formation of various oxysterols. For the transformation of cholesterol, both enzymatic and non-enzymatic routes are followed

dihydroxycholesterol (7-α,25-OHC) from 25-hydroxycholesterol in cholesterol synthesis pathway (NCBI 2023). In recent studies, it has been found to act as a biomarker in diseases related to liver and gastric function. It is also one of the common oxysterols, that is an indicator of lipid oxidation in various enteric disorders. In a study by Rouhanizadeh et al., it has been seen that oxidation of LDL molecules leads to the loss of proteins and lipids, which may lead to the accumulation of oxysterols and derivatives like cholesterol-β-epoxide, 7-α-hydroxycholesterol, and others (Rouhanizadeh et al. 2008).

9.3.2 7-β Hydroxycholesterol

7-β Hydroxycholesterol is one of the main forms of oxysterols generated as a result of auto-oxidation or enzymatic degradation of cholesterol. It is one of the primary oxysterols found in the LDL profiles. It is steroid in nature and is also found in natural sources of silkworm, *Cryptosula* sp., and other organisms (NCBI 2023). In molecular level, this oxysterol induces oxidative stresses that corresponds to the dysfunction and abnormalities in various organelles which can cause cell death and contribute to the pathophysiology of some cardiovascular diseases, neurodegenerative disorders, and specific eye conditions (Vejux et al. 2020). There have been several attempts made for the evaluation of toxicity of these molecules. In one of the cardiovascular-related studies conducted in Lithuania, a drastic increase in the plasma concentration of 7-β Hydroxycholesterol was noticed in a man suffering from coronary heart disease (Ziedén et al. 1999). This was studied later to be a probable marker of increased in vivo lipid peroxidation as one of the distinguished cellular dysfunctions.

9.3.3 7-Ketocholesterol

7-ketocholesterol, also known as 7-oxo-cholesterol is reported to cause a specific form of cytotoxic activity called oxiapoptophagy, which can be translated as autophagy induced by oxidative stresses. This oxysterol is predominantly found in the food products of animal

origin. It is oxidized in the liver and in the dearth of proper regulation, this molecule may undergo auto-oxidation. However, 7-ketocholesterol is present in large quantities in industrial foods having raw materials containing good amount of cholesterol such as eggs, meat, milk, chocolates, etc., (Clariana and García-Regueiro 2011). This prominence of accumulation of this molecule has been seen in age-related disorders like Alzheimer's, cataract, and age-related macular degeneration (Nury et al. 2021a, b). It can be metabolized in the liver by various processes and one of the major enzymes, 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Anderson et al. 2019). The side effects of this molecule have also been reported in Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV 2). It also acts as a neuroprotective agent and marker in diseases like atherosclerosis and necrotic processes of various disease. In vitro, 7-ketocholesterol has been reported to induce cytokinic and non-cytokinic inflammation that further leads to autophagy.

9.3.4 25-Hydroxycholesterol

25-hydroxycholesterol, of 25 hydroxy steroid acts as an active metabolite in the pathway of cholesterol synthesis. It has been reported to show active functionality in immune cells as a response to infection. Sterol regulatory element-binding protein (SREBP) and liver X receptor (LXR) are the two major transcriptional regulators for the maintenance of cellular cholesterol homeostasis. 25-hydroxycholesterol helps in the negative regulation of these transcriptional regulators and thus contributes to the maintenance of homeostasis in cholesterol metabolism (Liu et al. 2013a). The molecule has been seen to actively participate as an immune mediator in atherosclerosis and play major roles in antiviral complex formation (Gold et al. 2014). Further studies have shown that the deletion of Ch25h gene helps in encoding for 25-hydroxycholesterol production. This has further shown protection against inflammatory-induced pathology of various viral diseases like influenza. It further acts in the activation of macrophages and dendritic cells via toll-like receptors (TLR) (Zhao et al. 2020). The levels of this oxysterol were reported to be lower than the normal in patients suffering from Behcet Disease, which is an inflammatory vasculitis dysfunction. Hence, 25-hydroxycholesterol, along with 7-ketocholesterol can act as potential combined markers for this disease (Messedi et al. 2022).

9.3.5 27-Hydroxycholesterol

Cholesterol is a vital component present in the cell membrane, helping in giving the membrane its fluidity and performing other vital functions like cell signaling which are crucial for the proper functioning of the cell. However, cholesterol plays a vital role in the pathophysiology and development of cancer (Revilla et al. 2019). One of the major metabolites of cholesterol, namely, oxysterol 27-hydroxycholesterol (27HC), is produced by a mitochondrial enzyme called Cytochrome P450 27A1 (CYP27A1). 27-hydroxycholesterol (27HC) is an endogenous metabolite of cholesterol which is produced when the mitochondrial sterol 26-hydroxylase enzyme hydroxylates the carbon atom at the 27th position (CYP27A1). At values ranging from 67 ng/mL (0.17 μM) to 199 ng/mL (0.5 μM), 27-hydroxycholesterol is the most prevalent oxysterol circulating in humans (Duane and Javitt 1999). In terms of physiology, 27-hydroxycholesterol (27HC) is important for regulating the homeostasis of cholesterol which ultimately contributes to cholesterol efflux via the liver receptor (LXR, the orphan receptors) along with the insulin-induced proteins (INSIGs) to prevent the production of cholesterol. 27-hydroxycholesterol (27HC) is also a selective estrogen receptor modulators (SERMs) and its contribution to causing Breast Cancer is significantly noticed (McDonnell et al. 2014).

The 27-hydroxycholesterol axis fosters the development of the tumors. Estrogen receptors (ERs) and LXRs serve as ligands for the 27HC. 27HC encourages proliferation in a way that is specific to cancer cells by working with the ERs

expressed in some malignancies (ERs are one of the nuclear hormone receptor superfamily's members). ERs are expressed in the breast as well as in the prostate. In addition to increasing the chemotherapy efflux pump Pgp and promoting chemoresistance in cancer cells, activation of the LXR has also been found to increase cholesterol efflux and inhibit cell growth. In a cancer cell-extrinsic approach, 27HC inhibits T cell function by acting on LXRs in antigen (Ag)-presenting macrophages and other myeloid immune cells like neutrophils, which eventually inhibits immune surveillance and promotes cancer growth (Carbo et al. 2020). DuSell et al. originally discovered that 27HC could have similar roles to E2 in breast cancer cell lines in the year 2008, shortly after 27HC was identified as an endogenous SERM. These responsibilities include promoting ER activation and consequent cellular proliferation of ER+ cell lines. This shows that processes unique to cancer cells may help 27HC signaling through ER promoting the spread of malignancy (DuSell et al. 2008). To support this theory, Wu et al. discovered that 27HC stimulated ER+ breast cancer cell proliferation in vitro, and methyl piperidine-pyrazole, a selective ER antagonist, eliminated this effect, showing that ER is necessary for 27HC-driven breast cancer cell proliferation. It has been in our knowledge for a long time that 27HC plays a vital role in maintaining homeostasis and regulating cholesterol metabolism, but its contribution and involvement in causing Breast and Prostate cancer has also been discovered over the year and is yet to be deciphered by the researchers (Wu et al. 2013).

9.3.6 5α, 6α-Epoxycholesterol

5α, 6α-epoxycholesterol is a B-ring oxysterol which is formed by the photo-oxidation and autoxidation of cholesterol (Smith and Johnson 1989). 5 α, 6α-epoxycholesterol is found to be an alkylating substance and hints at being mutagenic and carcinogenic.

There are two distinct diastereoisomers of 5,6-epoxycholesterol: the 5α, 6α--epoxycholesterol and the 5β, 6β-epoxycholesterol. They result from cholesterol's mono-oxygenation on its 5, 6 double bonds. With the help of the process called free radical lipid peroxidation, they can be created as a mixture in living systems (Yin et al. 2011). They can be found in animals' solid tissues and bodily fluids (Silvente-Poirot and Poirot 2014). In recent studies, it has been demonstrated that 5α, 6-α-epoxycholesterol is involved in a metabolic branch that is certainly involved in the development of breast cancer and has discovered novel 5α, 6α-epoxycholesterol metabolites with opposing characteristics with regard to Breast Cancer oncogenesis. Metabolites which have antiproliferative as well as cancer cell redifferentiation properties can be produced by 5α, 6α-epoxycholesterol. The epoxycholesterol can get sulfated in Breast Cancer cells by the enzyme called sulfotransferase (SULT2B1b), which results in 5,6-epoxy-cholesterol-3-sulfate formation (De Médina et al. 2013). It has also been found that 5α, 6α-epoxycholesterol builds up in MCF-7 breast cancer cells in a reactive oxygen species-dependent manner and triggers the biosynthesis of triacylglycerol by binding to the liver X receptor (LXRs, the orphan receptors) (Poirot and Silvente-Poirot 2018). DDA (Dendrogenin A) was found through research on the 5α, 6α-epoxycholesterol metabolism in healthy cells. Unexpectedly, it was discovered that DDA (Dendrogenin A) exhibited tumor-suppressor properties in Breast Cancer cells through the activation of cell differentiation, death programs, and the inhibition of oncosterone biosynthesis, pointing to the metabolic balance between the tumor promoter oncosterone and the Dendrogenin A which is a tumor suppressor. This gives a new path to explore the opportunity to create drugs that target oncosterone in developing Breast Cancer drugs (Silvente-Poirot et al. 2018).

9.3.7 Cholesterol 3β, 5α, 6β Triol

Cholestane-3β, 5α, 6β-triol or Cholesterol 3β, 5α, 6β triol is also abbreviated as triol and is the most profuse form of oxysterol. Triol is a derivative of cholesterol formed due to the oxidation of the

compound (Lin et al. 2013). However, in this case, the cholesterol does not directly get converted into Cholestane-3β, 5α, 6β-triol, in fact 5α, 6α-epoxycholesterol and 5β, 6-β-epoxycholesterol are the two important intermediate products in the conversion process, which themselves are oxysterols and play an important role in promotion as well as suppression of tumors.

The triol is an important oxysterol as studies have shown that when glutamate-induced neurotoxicity was done by negatively modulating the NMDA (*N*-methyl-D-aspartate) receptor, Cholestane-3β, 5α, 6β-triol showed a neuroprotective effect, in vitro (Hu et al. 2014). The NMDA receptor is found to function abnormally diminished inside of the brain and is related to memory impairments and psychosis. The research was conducted with rabbits as the model organism where it was found that Cholestane-3β, 5α, 6β-triol aided in decreasing the neuronal injury significantly when the rabbits suffering from ischemia were subjected to the compound (Hu et al. 2014).

The most peculiar feature of Cholestane-3β, 5α, 6β-triol is that it induces cell death with the help of apoptosis (Programmed Cell Death) in Cancer cells like human prostate cancer cell line (PC-3), human prostate cancer cell line (DU-145) and in human LNCaP CDXR-3 (Lin et al. 2013). Cholestane-3β, 5α, 6β-triol also causes short-term apoptosis in human lung fibroblast cell lineages and human mammary gland/breast cells. (Levy et al. 2018).

Cholesterol 3β, 5α, 6β triol has been noticed as a vital suppressing factor of cancer cell lineages making it a potential oxysterol for cancer treatment.

9.3.8 5β, 6β-Epoxycholesterol

5β, 6β-epoxycholesterol (5β, 6β-EC) is an oxysterol which is closely associated with the pharmacology of tamoxifen, a potent anticancer treatment (Kloudova-Spalenkova et al. 2021). The bimolecular interaction between an unoxidized sterol molecule and a hydroperoxyl radical yields 5β, 6β-epoxycholesterol, which can then experience an oxirane ring opening in the presence of water in an acidic medium to transform into sterol triols (Garcia-Llatas et al. 2021). When it comes to cholesterol epoxides, such as 5β, 6β-epoxycholesterol, which is a stable molecule, these can be created either via type I autoxidation or type II autoxidation (with O3), and they can produce cholestane-triol, which can also be formed biochemically from cholesterol epoxide hydrolase (ChEH) (Ghzaiel et al. 2022). One of the most powerful oxysterols that significantly activate autophagy and is cytotoxic is 5β, 6-β-epoxycholesterol. It promotes metabolic stress, mitochondrial as well as lysosomal abnormal functions, along with enhanced plasma membrane absorptivity (Sassi et al. 2021). Oxysterol action as well as hormonal therapy is related in more ways than just through the regulation of Endoplasmic Reticulum properties. Tamoxifen helps some oxysterols to be synthesized. 5β, 6-β-epoxycholesterol then functions as intermediate for Tamoxifen activity and aids in its anticancer activity (Kloudova-Spalenkova et al. 2021). Blocking cells during particular stages in the cell cycle is a property of 5β, 6β-epoxycholesterol's cytotoxic action. The findings suggest that new anticancer treatments may be developed from compounds obtained by cholesterol, hybrid bile acids, and oxysterols. These compounds might even be utilized in glioblastoma antitumor metabolic therapy due to their probable role in oxysterol biosynthesis, which is downregulated in glioblastoma (Sassi et al. 2021). In Alzheimer's disease, modifications in the quantities of oxysterols produced nonenzymatically, including 5β, 6β-epoxycholesterol, were detected in the brain tissue (Varma et al. 2021). By altering the synthesis of oxysterols, that are generated under oxidative stress, the antioxidant effect of zinc might result in the reduced concentration of total oxysterols in a group that received dietary doses. Specific oxysterols, such as 5β, 6-β-epoxycholesterol, are well-known to have strong pro-inflammatory qualities. Many disease entities are assisted in development by

inflammation. As reported in literature, a greater level of COPs is linked to a greater risk of cancer, notably breast cancer (Stawarska et al. 2021).

9.3.9 Cholesterol 3β, 5α, 6α Triol

Cholesterol is either oxidized by enzymes or via the non-enzymatic routes method. Cholesterol undergoes autoxidation and forms 7α or 7β hydroperoxide, or 7α or 7β hydro cholesterol it can also form 7-ketocholesterol, 5α, 6β epoxycholesterol, as the intermediate products and finally forms cholesterol 3β, 5α, 6β triol or 3β, 5α, 6α triol based on what pH the environment it is in Watson et al. (1994).

In the year 1996, an experiment was conducted in which human low-density lipoprotein (LDL) was isolated and treated with cupric ions or soybean lipoxygenase and linoleic acid to oxidize the low-density lipoprotein (LDL). Following the oxidation, isotope dilution gas chromatography-mass spectrometry was performed. The expectation was to receive an oxysterol due to the oxidation, the most striking observation which was made was of the formation of a new compound which was unknown earlier, called Cholesterol 3β, 5α, 6α triol. Along with Cholesterol 3β, 5α, 6α triol, two epimers were also discovered in the ratio of 1:1 in the atherosclerotic vessels and it was concluded that due to cholesterol autoxidation, these epimers cholest-5-ene-313,4-diols were formed in atherosclerotic plaques due to autoxidation of cholesterol (Breuer et al. 1996).

9.4 Potential Role of Oxysterols in Age-Related Disorders

Oxysterols have been linked to a number of age-related disorders such as atherosclerosis, age-related macular degeneration, cataract, cancer, Alzheimer's disease, and chronic inflammatory diseases and they can negatively impact a number of physiological functions if they are accumulated or dysregulated. There exists a complex interplay between cellular processes involving cholesterol and oxysterols which play an important role in health and disease causation. Moreover, it is pertinent to investigate underlying oxysterol-mediated signaling pathways and their impact on cellular function to understand the implications in disease models, some of which are discussed in the following section.

9.4.1 Cardiovascular Disorders

Oxysterols have been described as being involved in cardiovascular diseases. The most common pathology involving 7KC is atherosclerosis, which generally occurs with age and in people with a high-risk lifestyle (sedentary lifestyle, smoking, high cholesterol, high blood pressure). This pathology is characterized by the progressive appearance of atheromatous plaques in the arterial wall. These plaques appear when low-density lipoproteins (LDLs) accumulate locally in arteries. These LDLs, when oxidized (formation of 7KC), become pro-inflammatory, leading to the recruitment of macrophages. The macrophages gorge on these oxidized LDLs to become foam cells, leading to their death by apoptosis. The apoptotic bodies then stagnate at the site and accumulate to contribute to atherosclerotic plaque formation. The smooth muscle cells in the vessel wall will then form collagen fibers that calcify, making the plaque increasingly brittle and fragile, potentially leading to artery rupture (Liguori et al. 2018). In hypercholesterolemic patients, 7KC is the majority oxysterol, accounting for 57% of plasma oxysterols: 7KC (15 ng/mg of free cholesterol, i.e. 57.0%) > 7α/β-OHC (20.7%) > 5,6-β-epoxycholesterol (11.7%) > cholestane-3β,5α,6β-triol (10.5%) > 5,6α-epoxycholesterol (less than 5 ng/mL). At plaque level, 7KC accounts for 55% of the oxysterols: 7KC (607 ng/mg free cholesterol, i.e. 54.8%) > Triol (13.5%) > 7-α/β-OHC (11.6%) > 5,6-β-EC (10.1%) > 5,6-α-EC (10.0%) (Helmschrodt et al. 2013). Faced with these data, we will focus this paragraph on 7KC, triol, and 7α/β-OHC. 7KC induces an increase in the expression of cell adhesion molecules (inter-

cellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein-1 (VCAM-1), and E-selectin) due to the elevation of ROS at the level of vascular endothelial cells, which favors the recruitment of macrophages to the plaques in the early stages of the pathology (Anderson et al. 2019; Lemaire et al. 1998). 7KC has also been shown to disrupt macrophage polarization, inducing the M1 inflammatory phenotype at the expense of the M2 anti-inflammatory phenotype (Saha et al. 2020). 7KC also induces an increase in the secretion of pro-inflammatory interleukins (ILs) such as IL-6, IL-1β, tumor necrosis factor-α (TNFα) or monocyte chemotactic protein-1 (MCP-1) and monocyte inflammatory protein-1β (MIP-1β) by macrophages (Prunet et al. 2006). 7α-OHC induces IL-8, MCP-1/CCL2 expression in monocytes/macrophages (Cho et al. 2017; Kim et al. 2015). 7β-OHC also induces IL-8 secretion (Lemaire-Ewing et al. 2009). The pro-inflammatory response induced by 7KC in monocytes/macrophages is also seen in a mouse model of myocardial ischemia-reperfusion (IR) injury where an involvement of reticulum stress and oxidative stress is observed (Uchikawa et al. 2022). The pro-inflammatory activities of 7KC and 7β-OHC are most commonly associated with effects observed in oxidative stress and cell death. In different cell models: endothelial cells, smooth muscle cells, monocytic cells, 7KC and 7β-OHC have been described to induce cell death and oxidative stress (Ryan et al. 2004). In endothelial cells, 7KC induces apoptosis, oxidative stress as well as inflammation by stimulating, e.g., ATM/Chk2, ATR/Chk1, p53, and PI3K/Akt signaling pathways (Chang et al. 2016; Luchetti et al. 2015; Lizard et al. 1996, 1997a). In promonocytic cells, it was also shown that 7KC and 7β-OHC could induce apoptosis and interleukin-1β secretion, which could be partially inhibited by Bcl-2 overexpression (Lizard et al. 1997b). 7KC induces apoptosis, oxidative stress but also the formation of multilamellar structures, called myelin figures because of their resemblance to the myelin structure (Miguet-Alfonsi et al. 2002). These structures are stained by monodansylcadaverine, so they have an acidic character, they are also stained by Nile Red, and therefore rich in lipids, but their formation is not dependent on caspases (Vejux et al. 2005, 2007a, b, 2020). In these same cells, 7KC induces caspase-dependent polar lipid accumulation (Vejux et al. 2020; Vejux et al. 2007a). The PI3-K/Akt signaling pathway appears to be a common 7KC-induced signaling pathway, as it is found induced following 7KC treatment in promonocytic cells (Vejux et al. 2009) but also in smooth muscle cells (Royer et al. 2009). This signaling pathway is not only involved in cell death but also in the migration and proliferation processes potentially induced by 7KC and cholesterol-5α, 6α-epoxide, processes involved in the formation of atherosclerotic lesions (Liao et al. 2010). The pro-apoptotic effects of 7KC on vascular smooth muscle cells increase the risk of plaque rupture and thrombosis (Liguori et al. 2018). In the same cell type, 7KC induces apoptosis and reticulum stress mediated by NAD(P)H oxidase Nox-4 (Pedruzzi et al. 2004). In both monocytic and smooth muscle cells, 7KC induces apoptosis through mechanisms involving calcium (Berthier et al. 2004, 2005; Sasaki et al. 2007), as may also be the case for 7β-OHC (Lordan et al. 2009; Ryan et al. 2006; Ares et al. 2000). The onset of apoptotic features occurs after the onset of mitochondrial dysfunctions and actin fiber alterations (Zahm et al. 2003). 7-Ketocholesterol not only induces apoptosis but also autophagy via Nox4 and Atg4B (He et al. 2013). 7KC also induces smooth muscle cell calcification, in a caspase-independent manner but in relation to oxidative stress dependent lysosomal dysfunction (Sudo et al. 2015; Saito et al. 2008) as well as the formation of sterol crystals such as those found in advanced atherosclerotic lesions (Phillips et al. 2001). 7KC, in addition to modulating inflammation and affecting cell death, may also have effects on lipid metabolism. Indeed, 7KC, in cardiac cells, promotes cholesteryl ester accumulation and reprograms lipid metabolism by modifying the transcription of several genes such as those encoding sterol O-acyltransferase and phospholipase A2 (Cheng et al. 2021). 7KC, in contrast to 7β-OHC, 25-OHC, and (22R)-OHC, is able to promote THP-1 cell differentiation and foam cell

formation through pathways that have not yet been characterized (Hayden et al. 2002). For triol, there is less data. Triol induces cell death involving the ERK (Extracellular signal-regulated kinase) and NF-KB (nuclear factor kappa light chain enhancer of activated B cells) signaling pathways and oxidative stress (Liu et al. 2011). Like 7KC, triol promotes calcification of vascular smooth muscle cells (Liu et al. 2004).

9.4.2 Brain Disorders

Lipids, which are major components of the brain, have numerous biological and physiological functions. Among lipids, cholesterol and oxysterols formed by cholesterol autoxidation and/or enzymatically (Mutemberezi et al. 2016) could be involved in major brain disorders such as Alzheimer's disease (AD) (Zarrouk et al. 2018) and Parkinson's disease (PD) (Paul et al. 2015). It is now well established that cholesterol is one of the most abundant lipid species present in the central nervous system (CNS). Thus, cholesterol represents around 25% of the total amount of cholesterol present in humans; the brain is also rich in phosphatidylcholine, sphingomyelin, ceramides, glucosyl ceramides, and sulfatides (Björkhem and Meaney 2004; Cermenati et al. 2015). These lipids identified in different parts of the brain are present in all brain cells: glial cells (astrocytes and oligodendrocytes), microglial cells, and neurons. They are also present in myelin which is a lipoprotein structure produced by the oligodendrocytes and which wraps the axons of neurons to favor an efficient conduction of the nerve influx; there are several evidences that some oxysterols which can modify cell membrane properties (permeability, fluidity, raft microdomains) (Guardiola et al. 1996; Filomenko et al. 2015) can impact nerve influx (Bezine et al. 2018). Among the brain lipids, there are lot of evidence that cholesterol and its precursors as well as some of its oxidized derivatives (oxysterols) can play critical roles in neurodegeneration associated with AD and PD (Pfrieger 2021; Pingale and Gupta 2021; Staurenghi et al. 2021).

At the moment, in AD as well as in PD, the role played by cholesterol or by its precursors is still unclear (Zarrouk et al. 2014; Doria et al. 2016). Cholesterol could be synthesized via the desmosterol pathway in young mice, while in aged mice, lathosterol could be the major precursor (Lütjohann et al. 2002). In PD, a study revealed an increase in cholesterol in the visual cortex but not in the anterior cingulate cortex, where the levels of two cholesterol precursors, lathosterol and 7-dehydrocholesterol, were lesser than that in the controls (Cheng et al. 2011). In AD, there are several evidences supporting the hypothesis that amyloid-β (Aβ) can perturb cholesterol homeostasis (Wood et al. 2014). Cholesterol transport and metabolism probably play key roles in AD: the strongest genetic risk factor for AD is APOE-ε4 (Corder et al. 1993). Apolipoprotein E (apo E) is a basic component of very low-density lipoprotein and high-density lipoprotein, which plays an important role in the clearance of cholesterol from the circulation and which is mainly synthetized by astrocytes in the brain.

These glial cells also produce cholesterol which is transported to neurons via apoE (Björkhem and Meaney 2004). There are three isoforms of apoE (apoE2, apoE3, and apoE4), which are encoded by three alleles, APOE-ε2, APOE-ε3, and APOE-ε4, with an allele frequency of 7, 78, and 15%, respectively (Strittmatter and Roses 1996). The ε4 allele increases the risk of developing AD compared with the more common ε3 allele, whereas the ε2 allele decreases the risk (Liu et al. 2013b).

Since oxysterols are thought to reflect cerebral cholesterol turnover, they have a great interest in the diagnostic and prognostic of AD, and a better knowledge of their impact on microglial, glial, and nerve cells can contribute to better understanding of the pathophysiology of neurodegenerative diseases. Oxysterols are able to modulate neuroinflammation, Aβ accumulation, and cell death (Gamba et al. 2015). 24(S)-hydroxycholesterol (24S-OHC), also named cerebrosterol, constitutes a potential biomarker of AD (Jeitner et al. 2011). This compound was described for the first time in 1953 in the human brain which contain around 80% of the total

24-hydroxycholesterol (24-OHC) in the body (Di Frisco et al. 1953).

24S-OHC is an enzymatically oxidized product of cholesterol synthesized by neurons in the brain via CYP46A1 (Lund et al. 1999). This enzyme CYP46A1 is present in hippocampal and cortical neurons, which are important for learning and memory (Russell et al. 2009).

Abnormal levels of 24S-OHC in the cerebrospinal fluid (CSF) and/or in the plasma of AD patients could be a putative biochemical marker of altered cholesterol homeostasis in the brain and/or of neuronal degradation. Decreased 24S-OHC concentrations in the plasma of AD patients were attributed to brain atrophy, which could be a consequence of the decrease in metabolically active neurons (Leoni and Caccia 2011). However, higher plasma and CSF levels of 24S-OHC have also been reported in AD patients and in patients with vascular dementia (Lütjohann et al. 2000; Papassotiropoulos et al. 2002). The selective expression of CYP46A1 around neuritic plaques and the potent inhibition of amyloid precursor protein (APP) processing in neurons by 24S-OHC support the hypothesis that CYP46A1 affects the pathophysiology of AD (Brown et al. 2004). On the other hand, a significant increase in 24S-OHC correlating with an increase in the tau protein level has been described in the CSF of patients with mild cognitive impairment and AD (Shafaati et al. 2007). As PD is associated with an aggregation of α-synuclein in dopaminergic neurons, the effects of 24S-OHC and 27-hydroxycholesterol (27-OHC) which is formed from cholesterol at the mitochondrial level by the enzyme CYP-27A1 (Russell 2000) were studied on SH-SY5Y cells (Marwarha et al. 2011). Indeed, 27-OHC which is present at high level in the plasma can be found to increase in the CSF of some PD patients (Björkhem et al. 2009, 2013) and cross the blood brain barrier when this barrier is altered either in AD and PD (Björkhem 2006). On SH-SY5Y cells, exposure of cells to 27-OHC led to an increase in both monomeric and trimeric α-synuclein and apoptosis whereas no effects were detected with 24S-OHC which however enhances the level of tyrosine hydroxylase and phospho-tyrosine hydroxylase involved in dopamine synthesis (Marwarha et al. 2011). Whereas 27-OHC is less studied than 24S-OHC, adverse effects of 27-OHC have been shown in AD on memory, learning, and Aβ aggregation (Zhang et al. 2015, 2019). In PD, the CSF level of 24S-OHC correlates with the duration of the diseases (Björkhem et al. 2013).

As elevated concentrations of 24S-OHC induce a mode of cell death by oxiapoptophagy (OXIdative stress + APOPTOsis + AutoPHAGY) on nerve cells (Nury et al. 2015), it has been suggested that 24S-OHC might also favor neurodegeneration and brain atrophy (Zarrouk et al. 2015). The presence of 24S-OHC in the vicinity of amyloid plaques has also been shown to enhance the adhesion of large amounts of Aβ to the plasma membrane of neurons and to amplify reactive oxygen species (ROS) production (Gamba et al. 2011). Interestingly, the increase of Aβ peptides and of neuronal death following inhibition of Cyp46a1 expression in the APP23 mouse model of AD suggests that increased amounts of neuronal cholesterol within the brain may contribute to inducing and/or aggravating AD (Djelti et al. 2015). It is, however, important to underline that 24S-OHC is probably not a specific biomarker of AD since abnormal levels of this compound have also been reported in other major neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, and Huntington's disease (Leoni and Caccia 2011; Boussicault et al. 2016).

In AD, increased levels of 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-OHC) have also been found in the cortex of patients in advanced stages of the diseases (Testa et al. 2016) and these later could contribute to the pathophysiology of the disease (Zarrouk et al. 2020; Mahalakshmi et al. 2021). Since these two oxysterols trigger oxidative stress, organelle dysfunction at the mitochondrial and/or peroxisomal level, inflammation and cell death (Vejux et al. 2020), which are hallmarks of several neurodegenerative diseases, they could also contribute to the pathophysiology of AD. Cerebrospinal fluid 7KC level is also associated with Aβ42 and white matter microstructure in cognitively healthy adults (Iriondo et al. 2020). It is also important

to underline that 7KC and 7β-OHC are inducers of oxiapoptophagy as it is the case with 24S-OHC (Nury et al. 2021b). Therefore, identifying molecules capable of inhibiting oxiapoptophagy (Nury et al. 2021a) could open new therapeutic avenues for highly debilitating neurodegenerative diseases such as AD and PD for which no effective treatments are available.

9.4.3 Eye-Related Disorders

Age-related eye diseases for which oxysterol involvement is highly likely in pathophysiology include cataract and age-related macular degeneration (AMD). In cataracts that result in an opacity of the crystallin and for which an involvement of oxysterols by autoxidation is suspected, especially that of 7-ketocholesterol (7KC) (Vejux et al. 2011) (Reyes et al. 2023), effective surgical treatment is available. Due to the rather high price of this latter, it is however not necessarily accessible to all patients. Identifying a pharmacological treatment, which is inexpensive, is therefore an important challenge (Lian and Afshari 2020) (Lu et al. 2022). In the context of cataract, the identification of natural and/or synthetic molecules as well as mixture of molecules allowing to easily treat this disease constitutes an important alternative to the surgical treatment. Lanosterol, and 5-cholesten-3b,25-diol (VP1-001) and other oxysterol compounds, have been reported to combat the aggregation of crystallin in vivo and ex vivo (Wang et al. 2022; Lee and Afshari 2023). Identifying molecules which counteract oxysterols-induced cytotoxicity in this context is an important issue.

AMD, which affects the retina and results in a loss of central vision, exists in two forms: the dry form, which is the most common, and the exudative form, which is rare and results from the evolution of the first to an advanced stage (https://www.nei.nih.gov/learn-about-eye-health/eye-conditions-and-diseases/age-related-macular-degeneration, consulted on February 2, 2023). AMD is characterized by an accumulation of lipids, called drusen between the Bruch's membrane and the basement membrane of retinal pigment epithelial cells (Malvitte et al. 2006). The accumulation of lipids leads to a thickening of the Bruch's membrane disrupting exchanges with the choriocapillary and impaired vision. These lipid deposits are very rich in oxysterols formed by autoxidation mainly 7KC but also 7-β-hydroxycholestrol (7β-OHC) (Rodriguez et al. 2014). Due to the pro-oxidant, pro-inflammatory, and pro-angiogenic activities of 7KC (Vejux and Lizard 2009; Amaral et al. 2013) that can contribute to the destruction of the retina and the formation of retinal neovessels, the cytotoxic effects of 7KC have been extensively studied to clarify its role in the pathophysiology of AMD (Pariente et al. 2019). In AMD, the receptors and signaling pathways on which 7KC acts were mainly studied on ARPE19 cells but also on primary cultures of bovine retinal epithelial cells (Joffre et al. 2007; Dugas et al. 2010). In retinal epithelial cells, cell death has been characterized and cell death by apoptosis has often been described (Neekhra et al. 2007), although a type of death involving the lysosomal pathway, which can be considered lethal autophagy, has also been described with 7β-OHC (Malvitte et al. 2008). In the induction of cell death induced by 7KC on retinal epithelial cells, P2X7 plasma membrane transporter appears to be activated and contributes to ROS overproduction, mitochondrial depolarization, and caspases activation (Olivier et al. 2016). In terms of inflammation on retinal cells, 7KC promotes inflammatory cytokine production via TLR4 and non-cytokinic inflammation via PLA2 activation leading to the release of arachidonic acid which is a precursor of leukotrienes and prostaglandins (Pariente et al. 2019). These results support that countering the deleterious effects of 7KC in the context of AMD requires inhibiting or reducing oxidative stress, inflammation, and cell death. It is also reported that 7KC can induce the expression of VEGF and ABCA1 by an LXR-dependent mechanism that may involve ORP2 (Moreira et al. 2009; Escajadillo et al. 2016). However, it is known that 7KC is not an LXR agonist (Janowski et al. 1996) and that LXR agonists or antagonists do not protect retinal cells from oxysterol-induced toxicity (Dasari et al. 2013). It is important to emphasize that at

the level of the retina other oxysterols, formed enzymatically via the enzyme CYP-46A1 to give 24(S)-hydroxycholesterol (24S-OHC) and via the enzyme CYP-27A1 to give 27-hydroxycholesterol (27-OHC), are also detected not only in cone and rod cells of the retina, but also in nerve cells such as Müller cells (Léger-Charnay et al. 2019; Zhang et al. 2021). These oxysterols depending on their concentrations can also influence the activity of the cells in the different layers of the retina. So, in the pathophysiology of AMD, decrease and/or increase expression and/or activity of CYP46-A1 and CYP27-A1 could also be involved. In agreement with this hypothesis, mice deficient in CYP46-A1 and CYP27-A1 enzymes in the retina have been shown to have disturbed electroretinograms indicating a decrease of the vision (Petrov et al. 2019). The involvement of oxysterols in AMD is still a broad field of investigation that will provide new information to better understand and treat this frequent eye disease in the elderly.

9.4.4 Bone Disorders

The effects of oxysterols on osteogenesis have been reported as early as 2004 using murine pluripotent cells M2-10B4 (Kha et al. 2004) and are well referenced (Vejux and Lizard 2009; Zarrouk et al. 2014). On these cells, pro-osteogenic effects have been reported with some oxysterols, (22(R)-hydroxycholesterol (22R-OHC), 20(S)-hydroxycholesterol (20S-OHC), and 22(S)-hydroxycholesterol (22S-OHC), while others have been shown to be anti-osteogenic, in particular 7-ketocholesterol (7KC) and cholestane-3beta-5alpha-6beta-triol (Kha et al. 2004; Liu et al. 2005). While 22(R)-OHC is an LXR agonist (Janowski et al. 1996; Lala et al. 1997) and 22(S)-OHC is an LXR-antagonist (Janowski et al. 1996), these two oxysterols have opposing effects on the production of ROS which contributes to inhibit osteogenesis (Shouhed et al. 2005). On murine pluripotent cells, M2-10B4 and C3H10T1/2, osteogenesis induced by 20(S)-OHC and 22(S)-OHC is associated with PKA and PKC-dependent signaling pathways (Richardson et al. 2007). The Hedgehog pathway is also activated (Dwyer et al. 2007; Kim et al. 2007). It is noteworthy that 20(S)-OHC inhibits PPAR-gamma expression and adipogenesis (Kim et al. 2007). It has also been shown that 27-hydroxycholesterol (27-OHC), which can be found at high plasma levels in post-menopausal women and can promote breast cancer by interacting with the estrogen receptor (Nelson et al. 2014) (Biasi et al. 2022), may also promote osteoporosis (Chang et al. 2019). 25-hydroxycholesterol (25-OHC) formed from cholesterol by the enzyme cholesterol 25-hydroxylase (CH25H) is another oxysterol that can inhibit differentiation into osteoblasts (Moseti et al. 2020). The cytotoxic activities of 25-OHC in human osteoblast-like MG-63 cells and of 7KC in murine preosteoblasts MC3T3-E1 lead to cell death by oxiapoptophagy characterized by OXIdative stress, APOPTOsis and autoPHAGY previously described on various murine and human cell types (Nury et al. 2021b; Ouyang et al. 2022; Seo et al. 2023). This particular type of cell death, induced by 25-OHC and described in human MG-63 osteoblasts, is also suspected in vivo on female mouse femurs of ovariectomized mice; a mouse model of osteoporosis, in which overexpression of CH25H, beclin-1 and caspase-3, which are biomarkers of autophagy and apoptosis, respectively, is observed (Seo et al. 2023). Consequently, there are several proofs in favor of an involvement of oxysterols formed either by autoxidation and enzymatically in osteoporosis. It is important to note that molecules inhibiting oxiapoptophagy (Nury et al. 2021a) may have a beneficial role in preventing and treating osteoporosis.

9.4.5 Pancreatic Disorders

Oxysterol levels can be used as precise diagnostic indicators for a wide range of disorders or to estimate the occurrence of diseases such as diabetes mellitus (Samadi et al. 2021). The identification of cellular mechanisms that protect pancreatic cells from the endoplasmic reticulum

tension and apoptotic pancreatic death of cells emphasizes the function that high-density lipoprotein serves in oxysterol equilibrium and the stimulation of the hedgehog signaling receptor (Yalcinkaya et al. 2020). All of these major discoveries highlight the fact that the cytoprotective action of high-density lipoprotein (HDL) on model pancreatic β-cells requires the migration of oxysterols for enhancing the upregulation of the hedgehog signaling receptor (Chapman 2022). A variety of lipid peroxide end products, including oxysterols, can be used to measure the oxidative stress caused by peroxidation. As the body's lipid peroxidation end products rise, oxidative stress is developed, which in turn stimulates the development of several complex diseases, such as cancer and neurogenic disorders. In healthy humans, the most frequent final agents of lipid peroxidation accumulate in the pancreas and, over time, induce organ-related disease. These DNA adducts are hazardous and are created when the more similar end constituents of lipid peroxidation engage with DNA (Ali et al. 2022). Oxysterols may play a role in the attraction of neutrophils to hypoxic cancerous cells in pancreatic neuroendocrine tumors (PNETs). So, by lowering tumor-invading neutrophils and endothelial cells in oncogenic islets, unique interaction in pancreatic islets of oxysterol sulfurization delays PNET development (Vasseur and Guillaumond 2022).

Oxysterol-binding protein 2 (OSBP2) is essential for fostering the growth and development of malignancies, yet it is still unexplained how OSBP2 influences pancreatic ductal adenocarcinoma (PDAC). It has been observed that OSBP2 is a potent tumor-associated protein that causes PDAC to have severely lethal traits. It was found that PDAC patients had poorer survival times when their original tumors expressed more OSBP2 than general. As a result, researchers examined the amounts of OSBP2 expression via immunohistochemistry (IHC) in PDAC tissues and neighboring paracancerous tissues (Huang et al. 2022).

Besides, oxysterols reduce immunological response by activating liver X receptors (LXR). Dendritic cell migration caused by LXR-activation by oxysterols may also encourage tumor development. Individuals with adenocarcinoma of the pancreatic duct or other pancreatic disorders have levels that are uneven in either direction, which may be a sign by the exocrine pancreas either hypo or hyperprocessing (Patel and Kashfi 2022). The position of oxysterol production downstream of HIF-1α (hypoxia-inducible factor-1α) signaling in tumors is a process that results in the buildup of oxysterols in tumors. These results demonstrate a complex network underlying tumor angiogenesis in pancreatic neuroendocrine tumors: on the one hand, HIF-1 upregulation results in vascular endothelial growth factor (VEGF) transcription as well as the direct formation of neoangiogenesis, while on the other side, 24S-HC retention and neutrophil procurement further enhance neovessel formation (Soncini et al. 2016).

The oxysterol 25-OHC referred to as an ERα agonist (ERα (NR3A1) is a nuclear regulator activated by estrogens), whereas 27-OHC is referred to as a SERM (selective estrogen receptor modulator) since its ERα-mediated actions are tissue-dependent. The pancreas is one of the tissues that serve a role in maintaining the balance of lipids and glucose. It has been demonstrated that ERα is necessary for the viability of β cells and insulin secretion in the pancreas using human pancreatic islets and mice lacking ERα (Guillemot-Legris et al. 2016).

9.4.6 Cancer

Oxysterols are oxygenated cholesterol derivatives that induce carcinogenesis by getting generated in the body or by external sources like food. Oxysterols also exert pro-apoptotic and cytotoxic effects on tumor cells (Decker et al. 2023). Oxysterols have mechanisms of cancer progression in different parts of the body. Initially, they might contribute to the development of the tumors by increasing the generation of Reactive Nitrogen and Oxygen Species (RNS/ROS). Then, the elevated regulation of proteins like cyclooxygenase-2 (COX-2) results in the modification of phenotypes of the cells, leading the

oxysterols to boost tumor propagation. Finally, the growth of cancer is aided by oxysterols by stimulating migration and causing impact by attaching to particular proteins and initiating signaling cascades (Aggarwal et al. 2019). Breast, prostate, lung, and gastrointestinal cancers are a few types caused by oxysterols.

Prostate cancers are hormone-dependent malignancies influenced by androgens whose effect is mediated by an androgen receptor protein. Furthermore, it impacts the amounts of oxysterols in prostate tissue and aids in regulating the levels of cholesterol efflux. Prostate cell proliferation, prostate-specific antigen (PSA) production, and AR transcriptional activity may also be modulated by 27-HC (27-Hydoxycholesterol), which may also have an impact on prostate cancer (Kloudova et al. 2017).

A trial assessing the levels of 7β-HC (hydroxycholesterol), 7α-HC, 5α, 6α-epoxy-3 β-o, cholestane-5β,6 β-epoxy-3β-ol, and 7-Ketocholesterol has been identified as a positive correlation between 7β-HC levels and lung cancer risk, indicating its potential predictive usefulness. Lately, the oxysterols also serve a role in the development of lung cancer facilitating the behavior of OSBPs (oxysterol-binding proteins), as indicated by the higher expression of ORP5 (oxysterol-binding protein-related protein-5) in cases of lung cancer with metastatic disease roughly equivalent to individuals who have locally advanced cancerous cells, which raises the possibility that OPR5 may have clinical utility in lung cancer patients (Kloudova et al. 2017; Kloudova-Spalenkova et al. 2021).

Breast cancer cells MCF7 and MDA-MB-231 spread more effectively when oxysterols produced by osteoblast-like MG63CM cells, are present. This demonstrates that oxysterols play a significant role in promoting the growth of bone metastases in patients with breast cancer. Moreover, breast cancer MCF7 cells exposed to 27-HC (27-Hydoxycholesterol) had decreased the amount of E-cadherin and b-catenin expression, showing that 27-HC plays a part in the epithelial to mesenchymal transformation. In the MCF7 cytotoxic effect, 27-HC also boosts neurogenesis and progression. In addition, 12 oxysterols (including 25-hydroxycholesterol (HC), 24S-hydroxycholesterol (HC), 7α--hydroxycholesterol (HC), and 5α, 6-α-epoxycholesterol) and 5 sterols were tested. Observations indicate a significant trend between the concentrations of the majority of free and total oxysterols/sterols (Decker et al. 2023; Kloudova-Spalenkova et al. 2021).

Oxysterols move through the gastrointestinal tract and are digested by the intestine; it is not unusual that these substances are considered to have an impact on gastrointestinal cancers. Also, some oxysterols manufactured as byproducts of the synthesis of bile salts can influence the emergence of bile duct and gallbladder cancer. Colorectal cancer is similar to other malignancies in that oxysterols target LXRs stimulation of LXR receptor, restricting the multiplication of both colon and colorectal cancer cell lines. Moreover, 25-HC (Hydroxycholesterol) can promote anoikis in the DLD-1 colon tumor cellular line, a sort of apoptotic cellular loss in consequence to loss of attachment. But oxysterols also have pro-inflammatory actions that might promote the growth of cancer in specific tissues, such as the colon (Passarelli et al. 2023; Liu et al. 2023; Kovač et al. 2019).

9.5 Strategies for Mitigation of Oxysterol-Mediated Cytotoxicity

As evident above, oxysterols have been found to be implicated in a number of lifestyle and age-related disorders. Mitigation of oxysterol-mediated cytotoxicity refers to the reduction or prevention of the harmful effects caused by oxysterols on cells. This can be achieved through dietary components, natural products, exogenous enzymes, and other drugs which can either prevent the formation, degrade post-production, or target oxysterol-associated cytotoxic pathways, some of which are discussed here.

9.5.1 Natural Cytoprotective Molecules and Oils Against Oxysterols

Oxysterols, such as 7β-hydroxycholesterol (7β-OHC) and 7-ketocholesterol (7KC), have been shown to induce mitochondrial dysfunction and oxidative stress in different types of cells. Mitochondria are known to play a key role in regulating energy metabolism and cell survival, and mitochondrial dysfunction has been linked to various diseases, including neurodegenerative disorders, cardiovascular diseases, and cancer. Natural molecules and oils can protect against the cytotoxic effects of these oxysterols (Kessas et al. 2022). For example, *Pistacia lentiscus* L. seed oil has been reported to have a protective effect against 7β-OHC-induced cell death, with potential mechanisms involving the upregulation of antioxidant enzymes and the downregulation of lipid peroxidation and protein carbonylation (Ghzaiel et al. 2021). Milk thistle seed oil, olive oil, and argan oil have also been reported to have protective effects against 7KC-induced oxidative stress and cell death, potentially via the modulation of oxidative stress, inflammatory and apoptotic pathways (Badreddine et al. 2020; Zarrouk et al. 2019; Debbabi et al. 2017).

Resveratrol and tocopherol, two natural compounds with potent antioxidant properties, have also been reported to protect against the cytotoxic effects of oxysterols. Resveratrol has been shown to reduce 7KC-induced oxiapoptophagy by reducing oxidative stress and modulating apoptosis (fragmentation and/or condensation of the nuclei; caspase-3 cleavage and PARP fragmentation) and autophagy (activation of LC3-I to LC3-II) (Yammine et al. 2020), while α-tocopherol has also been reported to prevent 7KC-induced oxiapoptophagy (Nury et al. 2018).

9.5.2 Synthetic Cytoprotective Molecules Against Oxysterols

In addition to natural compounds, synthetic molecules have also shown important protective activities against oxysterol-induced cytotoxicity. For instance, dimethyl fumarate (DMF) and monomethyl fumarate (MMF) are two synthetic molecules that have been reported to protect nerve cells against 7KC-induced oxidative stress and mitochondrial dysfunction, potentially via the activation of Nrf2-mediated antioxidant pathways. Currently, fumaric acid esters are best known in the treatment of psoriasis and multiple sclerosis. Patients with psoriasis and multiple sclerosis (MS) who were treated with DMF experienced improvements in their MS. In 2013, DMF (also known as BG-12 or Tecfidera, Biogen) was approved for the treatment of remittent recurrent MS (RRMS). DMF undergoes rapid first-pass metabolism to form monomethyl fumarate (MMF), which is also pharmacologically active. DMF, with the molecular formula C6H8O4, is a methyl ester of fumaric acid with a molecular weight of 144 g/mol. DMF (1, 25, and 50 μM) was tested on 158N mouse oligodendrocyte cells, which are myelin-synthesizing cells, treated with 7-ketocholesterol (7KC; 25, 50 μM) and 7-β-hydroxycholesterol (7β-OHC, 50 μM). MMF was tested on 7β-hydroxycholesterol-treated 158N cells. DMF attenuates 7KC-induced ROS overproduction, apoptosis, and autophagy in 158N cells (Zarrouk et al. 2017). On 158N cells, DMF (25 μM) and MMF (25 μM) are able to reduce 7β-OHC-induced toxicity, which is characterized by inhibition of cell growth; decreased cell viability; mitochondrial dysfunction (decrease in succinate dehydrogenase activity, loss of $\Delta\Psi m$, increase in mitochondrial superoxide anion ($O_2^{\bullet -}$) production, alteration of the tricarboxylic acid (TCA) cycle and cardiolipin content); induction of oxidative stress (overproduction of ROS, alteration of glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activities, increased levels of lipid peroxidation products (malondialdehyde (MDA), carbonylated dienes (CDs)) and carbonylated proteins (CPs); changes in fatty acid and cholesterol metabolism; and induction of cell death associated with apoptotic and autophagic criteria (caspase-3 cleavage, activation of LC3-I into LC3-II) (Sghaier et al. 2019a). DMF and MMF can also prevent

ultrastructural changes of mitochondria and peroxisomes (Sghaier et al. 2019a).

Biotin (B7) was also used to counteract oxysterol toxicity when tested in patients with primary and secondary progressive MS. MedDay Pharmaceuticals company had developed the experimental neurometabolic modulator Qizenday® (MD 1003 - biotin), which was designed to target both neurodegenerative and demyelinating processes when used at high doses through its non-immunological mechanism. This drug was given to patients from 2016 to 2019, but its provisional approval in MS has been withdrawn due to inconclusive clinical trials. Biotin used at 10 and 100 nM in vitro was able to attenuate 7β-OHC (50 μM)-induced cytotoxicity in 158N mouse oligodendrocyte cells (Sghaier et al. 2019b). A reduction in the loss of cell adhesion, normalization of antioxidant activities, and a decrease in the overproduction of ROS, as well as of protein and lipid oxidation products were also observed (Sghaier et al. 2019b). In addition, biotin partially restores mitochondrial functions: attenuation of $\Delta\Psi m$ loss; reduction of mitochondrial $O_2^{\bullet-}$ overproduction levels; normalization of cardiolipin and organic acid levels. There is also biotin-induced normalization of cholesterol and fatty acid synthesis, and prevention of apoptosis and autophagy (oxiapoptophagy) (Sghaier et al. 2019b).

At the moment DMF, MMF, and MD1003 are the only drugs used in humans capable of attenuating 7KC- and 7β-OHC-induced cytotoxicity in vitro.

In conclusion, natural and synthetic molecules and oils, especially oils associated with the Mediterranean diet, offer promising strategies against oxysterol-induced cytotoxicity. These findings provide new insights to develop new treatments which could be easy to use for diseases associated with increased levels of 7KC and 7β-OHC.

9.5.3 Biodegradation of Oxysterols

Biodegradation of oxysterols proposes the remarkable ability of enzymes to break down and metabolize oxysterols, preferably to lesser toxic metabolites in order to reduce the cytotoxicity. In the body, biodegradation of oxysterols involves enzymatic reactions catalyzed by cytochrome P450 enzymes. These enzymes, particularly members of the CYP27A1 and CYP7B1 families, are responsible for converting oxysterols into bile acids or other metabolites that can be further processed and eliminated from the body. Often with age, due to the enzymatic insufficiency of the body to degrade these molecules, accumulation and further cytotoxicity are caused by the oxysterols. An alternative to endogenous enzymes is the application of exogenous enzymes produced from microbial sources to counteract this catabolic inadequacy to convert them to less toxic products or completely mineralize them. An early idea of "medical bioremediation" was proposed as part of the SENS project, where microbes capable of degrading toxic substrates could be isolated, and their relevant enzymes screened and engineered for a therapeutic effect and further used as an enzyme-replacement therapy (De Grey et al. 2005). Further, several target molecules such as 7-Ketocholesterol were identified to be microbially degraded and enzyme-delivery models proposed (Mathieu et al. 2009). The same group later identified several strains of bacteria of genus *Nocardia, Phyllobacterium*, *Gordonia*, and *Rhodococcus* isolated from the environment and capable of metabolizing 7-ketocholesterol; enzymes such as cholesterol oxidases, oxygenases, and peroxidases were implicated (Rittmann and Schloendorn 2007; Schloendorn et al. 2009). In another study, the authors identified *Rhodococcus* sp. RHA1, *Sphingomonas* sp. JEM-1, *Proteobacterium* Y-134, *Nocardia nova*, and *Pseudomonas aeruginosa*, to be utilizing 7KC as a sole carbon and energy source, leading to its complete mineralization (Mathieu et al. 2008). High levels of 7KC degradation was reported by *Rhodococcus jostii* RHA1. This pathway upregulated genes located mainly in three of four large clusters of putative steroid catabolism genes, cholesterol oxidase was identified as the initial enzyme involved, and several intermediate products identified (Mathieu et al. 2010). Further studies by a different group identified

Pseudomonas aeruginosa PseA and *Rhodococcus erythropolis* MTCC 3951 as potential degrader strains of 7KC with 4-cholesten-3,7-dione (via cholesterol oxidase route), androsta-4-ene-3,7,17-trione, and Chol-5-en-3,7-dione found to be major intermediates of the process with side-chain degradation preceding the ring cleavage (Ghosh and Khare 2016, 2017). They further immobilized cholesterol oxidase enzyme from these two strains and used them to biotransform cholesterol and 7KC into their enone derivative in vitro (Ghosh et al. 2018a, b, 2019). Some recent studies also identified *Thermobifida fusca* IP1, *Alcanivorax jadensis* IP4, *Streptomyces auratus* IP2, and *Serratia marcescens* IP3 as degrader strains of 7KC (Perveen et al. 2016, 2018). The above studies thus point at a promising area of investigation of oxysterol toxicity mitigation via exogenous microbial enzymes. The primary caveat to this technology is that the biological activities of these degradative enzymes are often high in vitro, but their pharmacological effect in humans is often limited or remains to be demonstrated.

9.6 Future Prospects and Conclusion

In conclusion, oxysterols represent an attractive area of research at the intersection of biochemistry, cell biology, and medicine. These oxidized derivatives of cholesterol exhibit diverse biological activities and are involved in various physiological and pathological processes. The implications of oxysterols in age-related cardiovascular diseases, bone disorders, pancreatic disorders, neurodegenerative disorders are reported. Thus, in order to mitigate these toxic effects, several natural and synthetic molecules and enzymes have been investigated. However, actual applications in terms of in vivo and clinical trials remain to be demonstrated. Oxysterols have also garnered attention as potential biomarkers for various diseases. Their levels can be measured in biological samples, such as blood or cerebrospinal fluid, and abnormalities in oxysterol profiles have been observed in several conditions. Therefore, monitoring oxysterol levels may provide valuable diagnostic and prognostic information in clinical settings. Understanding the functions of oxysterols and interactions within the body can provide insights into the development of new therapeutic approaches and diagnostic tools for a wide range of diseases.

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Enzymatically Formed Oxysterols and Cell Death

10

Yasuomi Urano and Noriko Noguchi

Abstract

The side-chain hydroxylation of cholesterol by specific enzymes produces 24(*S*)-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and other products. These enzymatically formed side-chain oxysterols act as intermediates in the biosynthesis of bile acids and serve as signaling molecules that regulate cholesterol homeostasis. Besides these intracellular functions, an imbalance in oxysterol homeostasis is implicated in pathophysiology. Furthermore, growing evidence reveals that oxysterols affect cell proliferation and cause cell death. This chapter provides an overview of the pathophysiological role of side-chain oxysterols in developing human diseases. We also summarize our understanding of the molecular mechanisms underlying the induction of various forms of cell death by side-chain oxysterols.

Keywords

Side-chain oxysterols · 24(*S*)-hydroxycholesterol · 25-hydroxycholesterol · 27-hydroxycholesterol · Endoplasmic reticulum · Cell death · Sterol regulatory element-binding protein · Liver X receptor · Acyl-CoA:cholesterol acyltransferase · Integrated stress response

Y. Urano (✉) · N. Noguchi
Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Kyoto, Japan
e-mail: yurano@mail.doshisha.ac.jp

10.1 Enzymatically Formed Oxysterols

Oxysterols are sterols derived from the oxidation of cholesterol or its precursors and can be generated by autoxidation, enzymatically, or by both processes (Mutemberezi et al. 2016). Cholesterol oxidation can occur on the hydrocarbon rings (A or B) with an additional hydroxyl, epoxide, or ketone group, or on the side chain with a hydroxyl group. Several major oxysterols act as intermediates in the synthesis pathways of bile acids and steroid hormones (Wang et al. 2021). The hydroxylation of the side chain of cholesterol by specific enzymes generates oxysterols, particularly 24(*S*)-hydroxycholesterol (24S-OHC, also known as cerebrosterol), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC, also known as [25*R*]-26-hydroxycholesterol), and other products (Fig. 10.1). This reaction is mediated by mitochondrial or microsomal cytochrome P450 enzymes, except for 25-OHC, which is produced by cholesterol 25-hydroxylase (CH25H). Side-chain oxysterols serve as physiological key regulators of cholesterol homeostasis and primarily function as signaling molecules for excess cholesterol levels (Griffiths and Wang 2022; Poli et al. 2022). This

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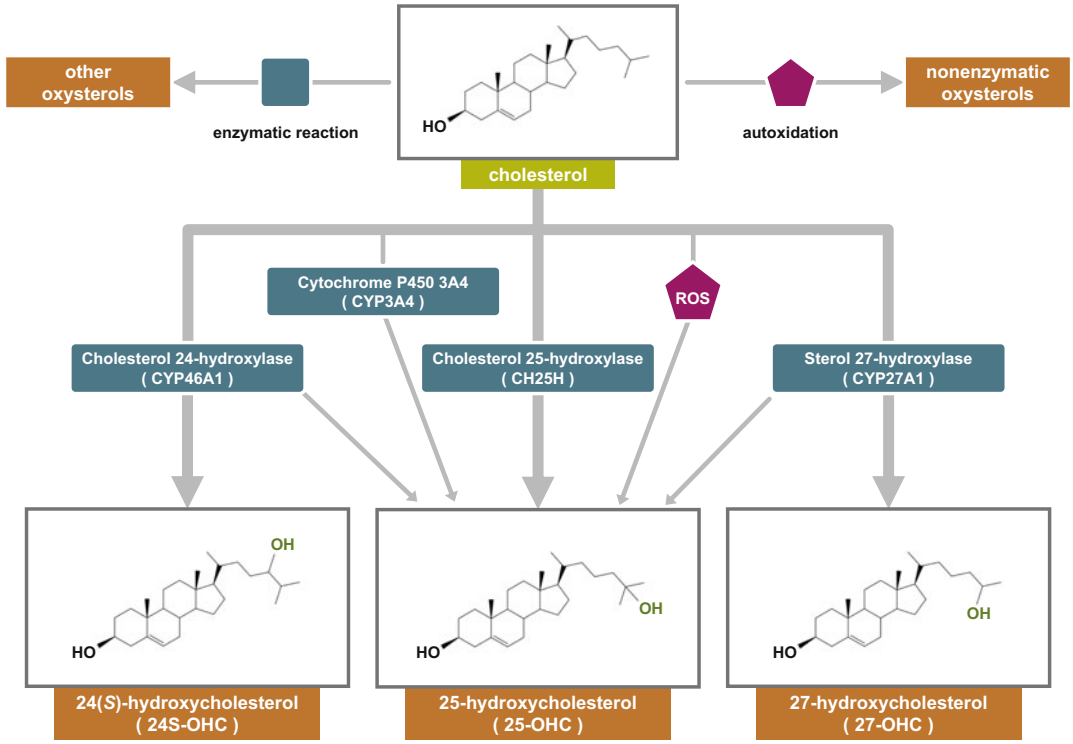

Fig. 10.1 Synthesis and structures of enzymatically formed side-chain oxysterols. Oxysterols are formed by enzymatic activity or nonenzymatic reactions (autoxidation). Cytochrome P450 enzymes and cholesterol 25-hydroxylase (CH25H) catalyze the enzymatic formation of side-chain oxysterols

is due to the increased hydrophilicity of oxysterols with that of cholesterol, which allows them to move more rapidly between the intracellular membrane organelles and facilitates access to their receptors (Dias et al. 2019). While physiological levels of oxysterols are involved in numerous biological processes, an imbalance in oxysterol homeostasis has been implicated in pathophysiology. This chapter focuses on the importance of enzymatically formed oxysterols, especially side-chain oxysterols (24S-OHC, 25-OHC, and 27-OHC), in understanding the regulation of cholesterol homeostasis. We also provide an overview of the pathophysiological role of oxysterol homeostasis imbalance in the development of human diseases and discuss our understanding of the molecular mechanisms by which side-chain oxysterols induce cell death.

10.2 The Role of Oxysterols in Cholesterol Homeostasis

To maintain cellular cholesterol homeostasis, cholesterol levels are tightly regulated by a balance in uptake, storage, endogenous biosynthesis, and efflux (Luo et al. 2020). When cellular cholesterol levels become excessive, some of the cholesterol is enzymatically converted to side-chain oxysterols. Besides serving as substrates for bile acid biosynthesis in cholesterol excretion pathways, these oxysterols play essential roles in the feedback loop of cholesterol homeostasis. Because oxysterols can be transported more easily than cholesterol, they can function as important signaling molecules.

Oxysterols play critical roles in cholesterol homeostasis at the transcriptional and

posttranslational levels (Fig. 10.2). One of the primary regulators by which oxysterol regulates cellular cholesterol homeostasis at the transcriptional level is the sterol regulatory element-binding protein 2 (SREBP-2), which is encoded by *sterol regulatory element-binding factor 2* (*SREBF2*) (Brown et al. 2018). The SREBP family comprises three protein subtypes, termed SREBP-1a, -1c, and -2. SREBP-1 isoforms mainly regulate the expression of enzymes involved in fatty acid biosynthesis pathway, whereas SREBP-2 regulates the expression of proteins involved in cholesterol biosynthesis and uptake. When the endoplasmic reticulum (ER) cholesterol levels are high, SREBP-2 resides as an inactive precursor protein within the ER membrane through the formation of a complex with the SREBP cleavage-activating protein (Scap) (Hua et al. 1996) and insulin-induced genes (Insigs) (Yang et al. 2002). Cholesterol causes a conformational change in Scap, which triggers the association with Insigs and the retention of the Scap/SREBP-2 complex in the ER. When the ER cholesterol levels become low, Scap dissociates from the Insigs, allowing the transport of the Scap/SREBP-2 complex from the ER to the Golgi via coat protein complex II (COPII)-mediated vesicles. Two Golgi-resident proteases (Site-1 protease and Site-2 protease) sequentially cleave SREBP-2, releasing an NH_2-terminal transcriptionally active mature form, which is transported to the nucleus. The mature form of SREBP-2 binds to SREs in the promoters of target genes and transactivates the genes involved in cholesterol biosynthesis (e.g., 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGCR] and HMGCS) and low-density lipoprotein receptor (LDLR) for cholesterol uptake (Horton et al. 2003). Whereas Scap senses cholesterol, Insigs sense 25-OHC and other side-chain oxysterols, causing the formation of SREBP-2/Scap/Insig complexes in the ER, thereby preventing SREBP-2 cleavage and the production of its active form (Adams et al. 2004; Radhakrishnan et al. 2007).

Another transcription factor that controls cholesterol homeostasis through oxysterols is the liver X receptor (LXR) (Wang and Tontonoz 2018). There are two isoforms of LXR in mammals, termed LXRα (NR1H3) and LXRβ (NR1H2), which belong to a subclass of the nuclear receptor superfamily. Side-chain oxysterols are ligands of LXR (Janowski et al. 1996, 1999). Ligand-activated LXR forms heterodimers with retinoid X receptor (RXR), which is the receptor for 9-*cis* retinoic acid. Activating these transcription factors promotes cholesterol efflux and excretion by upregulating target genes, including ATP-binding cassette transporter A1 (ABCA1), ABCG1, and apolipoprotein E (ApoE).

Side-chain oxysterols also regulate cholesterol homeostasis at the posttranslational level. The mevalonate pathway is pivotal for the biosynthesis of cholesterol and other essential biomolecules, such as coenzyme Q (ubiquinone), dolichols, and isoprenoids (Goldstein and Brown 1990). The ER-resident protein HMGCR is a rate-limiting enzyme in the mevalonate pathway and a target of statins, which are cholesterol-lowering drugs (Schumacher and DeBose-Boyd 2021). HMGCR catalyzes the conversion of HMG-CoA to mevalonic acid. The increase in oxysterol level causes the binding of HMGCR to the Insigs, which initiates HMGCR ubiquitination and proteasomal degradation, thereby slowing cholesterol biosynthesis (Sever et al. 2003). HMGCR is a target gene of SREBP-2, suggesting that side-chain oxysterols regulate HMGCR both at the levels of transcription and enzymatic activity. Because cholesterol does not affect this Insig-mediated degradation of HMGCR, cholesterol synthesis can be inhibited by oxysterols even more potently and more rapidly than cholesterol.

Another posttranslational regulator of cholesterol homeostasis is acyl-CoA:cholesterol acyltransferase (ACAT), which is also known as sterol *O*-acyltransferase (SOAT). ACAT is an ER transmembrane protein that utilizes long-chain fatty acyl-CoA and cholesterol as substrates to form cholesteryl esters (Chang et al. 2009). Two ACAT isoenzymes, ACAT1 and ACAT2, have been identified in mammals. ACAT1 is expressed in many tissues, whereas ACAT2 is expressed predominantly in the liver and small intestine. Cholesteryl esters are stored in cytoplasmic lipid

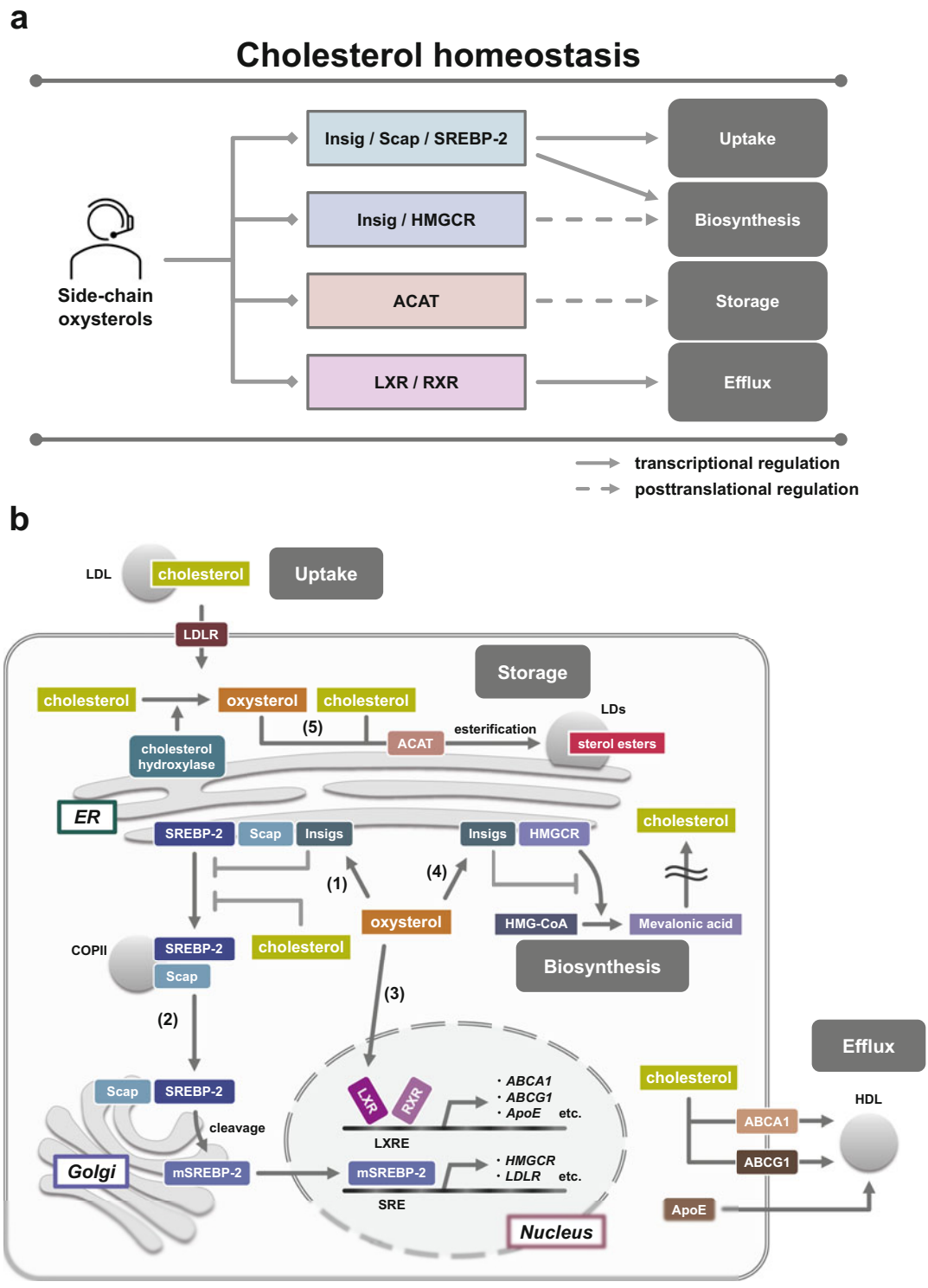

Fig. 10.2 (**a**) The role of side-chain oxysterols in cholesterol homeostasis. Oxysterols are important regulators of cholesterol homeostasis. The transcriptional regulation by side-chain oxysterols is mediated by Insig/Scap/SREBP-2 for cholesterol uptake and biosynthesis or by LXR/RXR for cholesterol efflux. At the posttranslational regulation levels, side-chain oxysterols promote Insig-mediated degradation of HMGCR, which is a key enzyme in cholesterol biosynthesis, or allosteric activation of ACAT for cholesterol storage. (**b**) Mechanisms of side-chain oxysterol

droplets (LDs) to prevent cytotoxic accumulation of free cholesterol or to serve as the precursors for steroid hormone production. In addition to cholesterol, other sterols that possess the 3β-hydroxy group, including oxysterols (such as 24S-OHC and 27-OHC), plant sterols, and pregnenolone, could be ACAT substrates (Rogers et al. 2015; Takabe et al. 2016). As a posttranslational regulation, both ACAT1 and ACAT2 function as allosteric enzymes activated not only by cholesterol but also by oxysterols (Zhang et al. 2003; Liu et al. 2005), which suggests that an increase in oxysterol levels promotes the conversion of excess cholesterol to cholesteryl esters. Because ACAT1 and ACAT2 do not have SRE in their promoter regions, oxysterol-mediated regulation of ACAT activity modulates cholesterol homeostasis independent of SREBP/Scap/Insigs-mediated regulation.

As noted, cellular cholesterol homeostasis is controlled by the precise regulation of transcriptional and posttranslational mechanisms that are sensitive to oxysterol levels. By controlling the production of nascent proteins, transcriptional regulation can respond efficiently to changes in cellular cholesterol concentrations but on relatively slow time scales of hours. In contrast, posttranslational regulation directly alters protein activities, allowing faster cellular responses to changes in cholesterol levels (Olsen et al. 2012). Notably, the physiological importance of side-chain oxysterols as regulators of cholesterol homeostasis in vivo remains uncertain because several studies have reported that mice with transgenic or knockout genes of side-chain oxysterol-producing enzymes showed modest changes in cholesterol homeostasis (Meir et al. 2002; Rosen et al. 1998; Lund et al. 2003; Björkhem 2009).

10.3 24S-OHC

10.3.1 24S-OHC and Brain Disorder

The brain is the most cholesterol-rich organ and contains approximately 25% of the total amount of cholesterol in the body (Björkhem and Meaney 2004). Because the blood–brain barrier prevents cholesterol translocation between the brain and systemic circulation, brain cholesterol levels are not affected by dietary cholesterol levels (Björkhem et al. 1997). In the adult brain, the major source of cholesterol is in situ biosynthesis by astrocytes; this cholesterol is delivered to neurons via ApoE-dependent transportation (Li et al. 2022). Therefore, to maintain a steady-state level of cholesterol in the brain, excess cholesterol is transported outside the brain mainly after its conversion to 24S-OHC by the neuronal cytochrome P450 enzyme cholesterol 24-hydroxylase (CYP46A1) (Russell et al. 2009). 24S-OHC can readily cross the blood–brain barrier by diffusion or using an organic anion transporting polypeptide 2 (Björkhem et al. 2019; Ohtsuki et al. 2007), after which 24S-OHC is transported to the liver, where it is metabolized to bile acids (Björkhem et al. 1997). In addition to their pivotal roles in maintaining brain cholesterol homeostasis, CYP46A1 and 24S-OHC affect higher-order brain functions, such as memory and learning, in a physiological setting (Li et al. 2022).

Fig. 10.2 (continued) action in cholesterol homeostasis. (1) When cholesterol concentrations in the ER membrane are high, cholesterol binds to Scap, causing the retention of Scap/SREBP-2 in the ER, whereas oxysterols bind to Insigs, triggering their complex formation with Scap/SREBP-2 in the ER. (2) When the sterol levels are low, Scap dissociates from the Insigs, eliciting the transport of SREBP-2 to the Golgi, where the active form of SREBP-2 (mSREBP-2) is generated. mSREBP-2 subsequently induces the transcription of SRE-containing genes in the nucleus, which regulates cholesterol biosynthesis and uptake. (3) Oxysterols also act as ligands for LXR signaling, which regulates associated with cholesterol efflux. (4) Moreover, oxysterols cause the association of Insigs with HMGCR, promoting its proteasomal degradation. (5) Cholesterol and oxysterols induce allosteric ACAT activation, producing sterol esters that are incorporated into LDs for storage

Growing evidence suggests that dysregulation of cholesterol homeostasis in the brain is associated with several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Dai et al. 2021; Chang et al. 2017). Moreover, increasing evidence suggests that CYP46A1 and 24S-OHC could be possible biomarkers of neurodegenerative diseases and may have a role in the pathogenesis or progression (Leoni and Caccia 2013; Noguchi et al. 2015; Sodero 2021). For example, polymorphisms in *CYP46A1* are associated with AD (Kölsch et al. 2002). Selective expression of CYP46A1 around neuritic plaques has also been reported (Brown 3rd et al. 2004). Production of amyloid-β (Aβ) peptides is inhibited (Brown 3rd et al. 2004; Urano et al. 2013) or promoted by 24S-OHC (Gamba et al. 2014). Measurement of 24S-OHC levels in four different brain areas of healthy controls and patients with AD showed that 24S-OHC existed at an approximate concentration of 20 ng/mg tissue in controls, and its concentration was lower in patients with AD (Heverin et al. 2004). Assuming a volume of 1 μL for 1 mg tissue, the level of 24S-OHC in the brain tissue may be approximately 50 μM. Several studies reported that plasma 24S-OHC levels were lower in patients with AD than in controls (Bretillon et al. 2000; Kölsch et al. 2004), which may be caused by decreased CYP46A1 levels due to neuronal cell death. However, several studies also reported elevated 24S-OHC levels in the plasma (Lütjohann et al. 2000; Zuliani et al. 2011) and cerebrospinal fluid (CSF) (Schönknecht et al. 2002) of patients with AD or mild cognitive impairment. This finding may suggest increased brain cholesterol turnover as a result of the early stage of neurodegeneration. Differences in disease progression between the patients may explain the conflicting findings of these increased and decreased 24S-OHC levels.

In the case of patients with PD, unchanged or lower levels of 24S-OHC in the plasma but significantly increased levels of 24S-OHC in the CSF relative to the levels in the controls were reported (Björkhem et al. 2013). Furthermore, a significant correlation was observed between 24S-OHC levels in the CSF and disease duration. In the case of HD, plasma 24S-OHC levels were lower in patients with HD than in controls at all disease stages (Leoni et al. 2013). Change in 24S-OHC levels in the plasma or CSF was further reported in brain disorders, such as multiple sclerosis (MS) (Leoni et al. 2002; Fellows Maxwell et al. 2019), amyotrophic lateral sclerosis (ALS) (Hartmann et al. 2022), and autism spectrum disorders (ASD) (Grayaa et al. 2018). In the case of drug-free patients with schizophrenia (SZ), 24-OHC levels in plasma were not significantly different between SZ patients and healthy controls (Guidara et al. 2022). In the SZ group, plasma 24-OHC levels were positively correlated with the positive and negative syndrome scale indicating the severity of symptoms. Furthermore, a link exists between CYP46A1 and retinal diseases, such as age-related macular degeneration (AMD) and glaucoma. For example, plasma 24-OHC levels were specifically associated with AMD (Lin et al. 2018). An association between *CYP46A1* polymorphism and glaucoma has also been reported (Fourgeux et al. 2009).

Moreover, 24-OHC is a positive allosteric modulator of N-methyl-D-aspartate receptors (NMDARs) (Paul et al. 2013; Sun et al. 2016). Because NMDARs are ionotropic glutamate receptors for excitatory neurotransmission throughout the central nervous system, NMDAR functions are crucial for synaptic plasticity and cognition. In HD neurons and mouse models, CYP46A1 overexpression shows neuroprotective effects (Boussicault et al. 2016, 2018).

Although some conflicting findings remain to be clarified, the quantification of 24S-OHC is important in advancing our understanding of neurological disorders. More neurological status likely needs to be assessed for changes in 24S-OHC levels in the plasma or CSF.

10.3.2 24S-OHC-Induced Neuronal Cell Death

The types of cell death are broadly divided into accidental cell death (ACD) and regulated cell death (RCD). ACD is an uncontrolled process of

cell death. In contrast, RCD is a tightly controlled process regulated by complex signaling pathways to eliminate cells that are no longer needed, damaged, or harmful in a targeted fashion. It plays a significant role in both physiological and pathophysiological processes, such as embryonic development, tissue homeostasis, aging, inflammation, and immunity (Galluzzi et al. 2018). Inappropriate neuronal cell death is involved in many neurodegenerative diseases. Beyond the classically defined apoptosis, growing evidence has revealed various forms of nonapoptotic RCD and new pathways of cell death machinery (Tang et al. 2019).

Because 24S-OHC at higher concentrations has been shown to induce neuronal cell death, it is presumed to be involved in the etiology of neurodegenerative diseases (Noguchi et al. 2014, 2015). Using human neuroblastoma SH-SY5Y cell lines, the cytotoxic mechanism of 24S-OHC has been well investigated. At concentrations higher than 10 μM, 24S-OHC induces cell death in SH-SY5Y cells and primary cortical neuronal cells (Kölsch et al. 1999; Yamanaka et al. 2011). Moreover, 24S-OHC-treated SH-SY5Y cells exhibited neither typical apoptotic features, such as nuclear fragmentation and caspase-3 activation, nor the necrotic feature, ATP depletion. Instead, 24S-OHC-treated cells showed necroptosis-like cell death features. Necroptosis is characterized as a type of RCD that is necrosis-like, caspase-independent, and mediated through a pathway dependent on receptor-interacting serine/threonine kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL) and (at least in some settings) the kinase activity of RIPK1 (Yuan et al. 2019). Caspase-8 is known to be the molecular switch for extrinsic apoptosis and necroptosis (Yuan et al. 2016). SH-SY5Y cells lack caspase-8- expression, which is responsible for the absence of apoptotic features in response to 24S-OHC (Yamanaka et al. 2011). Although 24S-OHC-induced cell death is effectively inhibited using a RIPK1 inhibitor or RIPK1 siRNA-mediated knockdown, neither RIPK3 nor MLKL is expressed, suggesting that 24S-OHC induces a necroptosis-like but unconventional type of RCD in SH-SY5Y cells (Vo et al. 2015). When SH-SY5Y cells were differentiated using all-*trans*-retinoic acid in which caspase-8- expression is induced, the exposure to 24S-OHC induced apoptosis as evidenced by caspase-3 activation (Kölsch et al. 2001; Nakazawa et al. 2017). In caspase-8-expressing human T-lymphoma Jurkat cells, 24S-OHC also induced apoptosis (Yamanaka et al. 2014).

Further studies demonstrated that ACAT1-catalyzed esterification of 24S-OHC at the 3-β-hydroxyl group with unsaturated long-chain fatty acids in the ER is responsible for initial key pro-cell death events during 24S-OHC-induced cell death in SH-SY5Y cells (Yamanaka et al. 2014; Takabe et al. 2016) (Fig. 10.3). Consequently, abnormal accumulation of 24S-OHC esters between the two leaflets of the ER membrane bilayer evoked the formation of an LD-like structure coupled with an enlarged ER structure, resulting in the disruption of ER membrane integrity and release of ER luminal chaperone proteins into the cytosol (Urano et al. 2019). In general, a decrease in protein folding capacity in the ER causes unfolded protein response (UPR) to restore the ER homeostasis and maintain the fidelity of protein folding (Hetz 2012). Although UPR is a prosurvival adaptive response, under unresolvable ER stress conditions, the UPR represses the adaptive response and even triggers cell death (Kim et al. 2008; Tabas and Ron 2011). Indeed, 24S-OHC-induced ER dysfunction is accompanied by the activation of pro-death UPR. The ER dysfunction and integrated stress response (ISR) described below are the key signaling pathways for 24S-OHC-induced cell death (Urano et al. 2019, 2022).

ISR and UPR constitute overlapping signaling pathways mediated by eukaryotic translation initiator factor 2α (eIF2α) (Hetz et al. 2020; Pakos-Zebrucka et al. 2016). UPR is activated by the accumulation of unfolded/misfolded proteins in the ER. In mammalian cells, the UPR employs three main signaling pathways, including activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase R-like ER kinase (PERK) pathways. Of these, the PERK pathway is responsible for a part

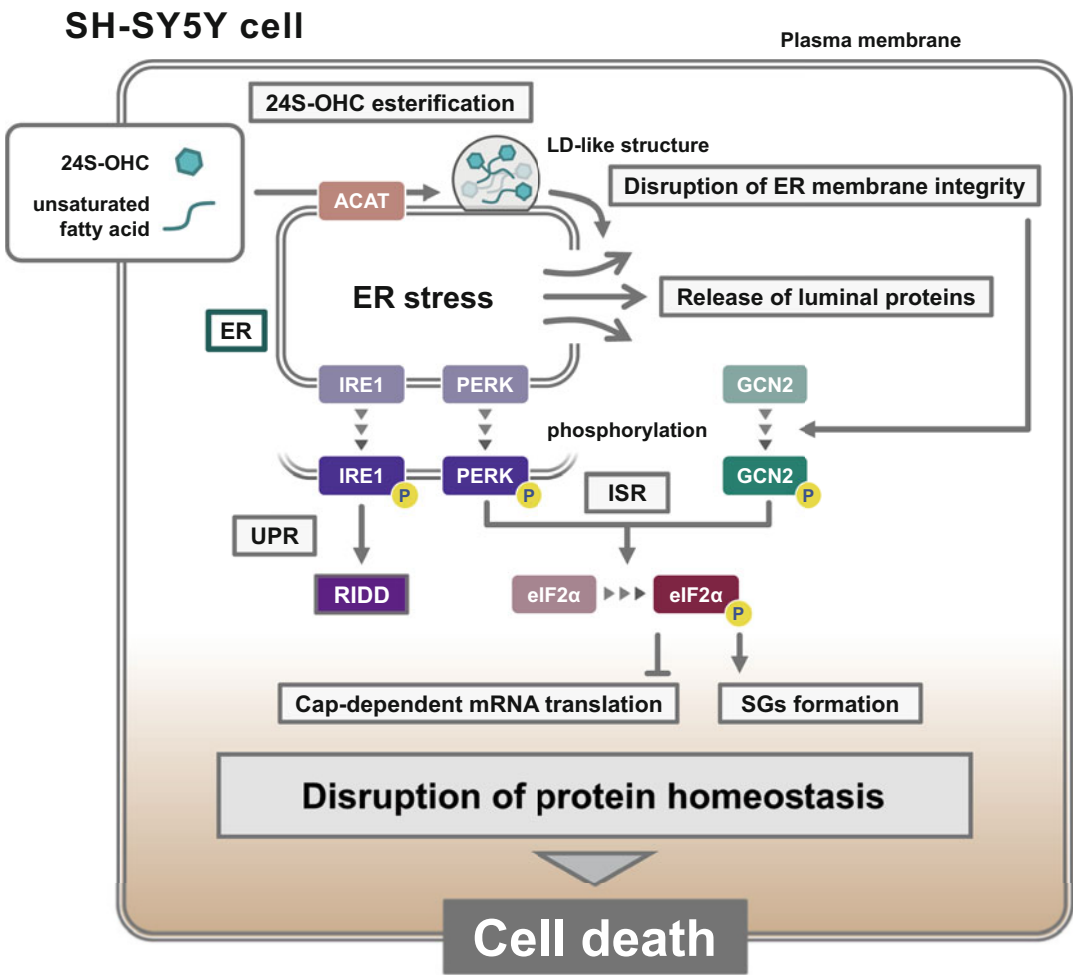

Fig. 10.3 Schematic representation of the mechanism proposed for 24S-OHC-induced cell death in SH-SY5Y cells. Under conditions of excess 24S-OHC levels in SH-SY5Y cells, ACAT1 catalyzes 24S-OHC esterification with unsaturated long-chain fatty acid in the ER. Accumulation of 24S-OHC esters within the ER membrane bilayer leads to the formation of an LD-like structure coupled with an enlarged ER structure, resulting in the disruption of ER membrane integrity, which in turn induces the release of ER luminal proteins into the cytosol. ER stress and/or disruption of the ER membrane integrity activates pro-death UPR signaling, including RIDD. ISR activation through PERK and GCN2 phosphorylation causes the phosphorylation of eIF2α and subsequent inhibition of protein synthesis and the formation of SGs. ISR, UPR, and ER dysfunction together lead to disruption of protein homeostasis and ultimately result in the induction of an unconventional type of cell death by 24S-OHC

of the ISR signaling pathway (Pakos-Zebrucka et al. 2016). ISR is an evolutionarily conserved signaling pathway activated in response to intracellular and extracellular disturbances (Costa-Mattioli and Walter 2020). Disturbance in protein homeostasis is the main cause of ISR signaling activation. Although ISR primarily serves as a prosurvival cell response, severe or prolonged stress leads ISR signaling toward cell death (Liu et al. 2015; Rutkowski et al. 2006). Activated PERK (via autophosphorylation) functions as an eIF2α kinase. In addition to PERK, the ISR sensor relies on three other eIF2α kinases, including general control nonderepressible 2 (GCN2), heme-regulated eIF2α kinase (HRI), and double-stranded RNA-dependent protein kinase R

(PKR). GCN2 is activated by amino acid deprivation and UV light exposure, HRI responds to heme deficiency, and PKR is activated during viral infections. Phosphorylated eIF2α sequesters the eIF2, thereby inhibiting the assembly of a 43S translation initiation complex and causing the general attenuation of 5′ cap-dependent protein synthesis (Liu et al. 2015). Moreover, phosphorylation of eIF2α enhances the translation of selective mRNAs, including not only those that encode prosurvival proteins but also those that encode proapoptotic proteins. The global inhibition of protein translation caused by ISR induces the formation of cytoplasmic membraneless ribonucleoprotein-based compartments known as stress granules (SGs) by liquid–liquid phase separation. While SGs sequester mRNAs upon stress and maintain a translation arrest until recovery from stress, persistent or aberrant SG formation is implicated in disease pathology and cell death (Reineke and Neilson 2019).

Intriguingly, 24S-OHC triggers eIF2α phosphorylation and subsequent SG formation (Urano et al. 2022). Inhibition of ISR signaling suppresses 24S-OHC-induced SG formation and cell death, suggesting that ISR and SG formation are involved in 24S-OHC-induced cell death. Moreover, 24S-OHC-induced ISR is activated through PERK and GCN2 activation, which downregulates global protein de novo synthesis. Inhibition of ACAT1 suppresses the activation of both PERK and GCN2, as well as downstream eIF2α activation, suggesting that ACAT1-catalyzed esterification of 24S-OHC is responsible for the activation of ISR. Furthermore, 24S-OHC-induced ER dysfunction has been proposed to drive the activation of PERK signaling, although it is not clear how 24S-OHC causes the activation of GCN2 signaling. Because 25-OHC has also been reported to activate GCN2 in an LXR- and SREBP-independent manner in bone-marrow-derived macrophages (Shibata et al. 2013), oxysterols may have some common machinery for GCN2 activation. In addition to the PERK pathway, the IRE1 pathway via regulated IRE1-dependent decay (RIDD) is also implicated in 24S-OHC-induced pro-death UPR signaling (Urano et al. 2019). RIDD is the mechanism by which the RNase activity of activated IRE1 upon UPR induces posttranscriptional degradation of a subset of ER-localized mRNAs (Hollien and Weissman 2006; Maurel et al. 2014).

From food-derived products, vitamin E homologs α- and γ-tocopherol have been found to significantly suppress 24S-OHC-induced cell death in SH-SY5Y cells (Nakazawa et al. 2017; Kimura et al. 2018). Vitamin E is a family of eight isoforms, namely α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols. All vitamin E homologs comprise a chromanol ring and an aliphatic side chain. Tocopherols have a saturated side chain, whereas tocotrienols have an unsaturated side chain containing three unsaturated double bonds. Both tocopherols and tocotrienols are potent lipid-soluble antioxidants, but only tocopherols exert inhibitory effects on 24S-OHC-induced cell death in SH-SY5Y cells. Cotreatment with α-tocopherol but not α-tocotrienol effectively suppressed 24S-OHC-induced UPR and ER membrane disruption (Chiba et al. 2023). Neither reactive oxygen species (ROS) generation nor lipid peroxidation was observed in 24S-OHC-treated cells. Therefore, α-tocopherol with a saturated side chain may protect the ER membrane from 24S-OHC-induced disruption through its nonantioxidant activity.

10.3.3 24S-OHC-Induced Cell Death in Other Cell Types

The cytotoxicity of 24S-OHC has also been examined in other cell types. In human hepatic cells (HepG2 cells), 24S-OHC caused caspase- and ACAT-mediated esterification-independent cell death that was suppressed by both α-tocopherol and α-tocotrienol, suggesting the involvement of free radical-mediated lipid peroxidation in 24S-OHC-induced cell death in hepatic cells (Suzuki et al. 2021). In human keratinocytes (HaCaT cells), 24S-OHC caused a caspase-dependent but ACAT-mediated esterification-independent cell death that was inhibited by α-tocopherol but not α-tocotrienol (Suzuki et al.

2021). In murine oligodendrocytes 158N cells, microglial BV-2, and neuroblastoma N2a cells, 24S-OHC induced a type of cell death termed oxiapoptophagy, which was characterized by oxidative stress and several features of apoptosis and autophagy (Nury et al. 2015, 2021). Furthermore, α-tocopherol prevented 24S-OHC-induced oxiapoptophagy. However, it is unclear whether ACAT-mediated esterification is involved in 24S-OHC-induced oxiapoptophagy.

Interestingly, *CYP46A1* was identified as one of the most dramatically dysregulated cholesterol metabolism genes in glioblastoma (Han et al. 2020). A reduction in CYP46A1 expression was associated with an increase in tumor grade and a worse prognosis in patients with glioblastoma. Ectopic expression of CYP46A1 suppressed cell proliferation of glioblastoma cells in vitro and in xenografts by increasing the 24S-OHC levels. Treatment of glioblastoma cells with 24S-OHC increased apoptosis in a dose-dependent manner. Furthermore, restoration of CYP46A1 activity using its activator, efavirenz, inhibits glioblastoma growth by regulating LXR and SREBP-1 activities. These data demonstrated that the CYP46A1/24S-OHC axis is a potential target for glioblastoma therapy.

Together, these findings suggest that 24S-OHC induces different types of cell death through various mechanisms in a cell-type-dependent manner. Considering the involvement of 24S-OHC in neurodegenerative diseases, further studies in a nonproliferative neuronal culture, including iPS cell-derived neuronal culture, stem cell-derived neuronal culture, and the neuron-glia coculture system, are warranted to understand the molecular actions of 24S-OHC.

10.3.4 Protective or Damage-Promoting Effects of 24S-OHC Against Other Cytotoxic Stimulations

The cytoprotective effects of 24S-OHC have also been demonstrated. 7-Ketocholesterol (7-KC) is an oxysterol generated by the autoxidation of cholesterol. Because 7-KC has been shown to have a highly cytotoxic potential in neuronal cells, it may contribute to the pathogenesis of neurodegenerative diseases. Treatment with 24S-OHC at sublethal concentrations showed a significant reduction in cell death induced by subsequent treatment with 7-KC in both undifferentiated and retinoic acid-differentiated SH-SY5Y cells (Okabe et al. 2013). Cotreatment of 24S-OHC with the RXR ligand promoted the cytoprotective effects of 24S-OHC against 7-KC-induced cell death. Knockdown of LXR or ABCG1 using siRNA significantly diminished 24S-OHC-induced cytoprotective effects. This finding suggests that 24S-OHC at sublethal concentrations induces adaptive responses via the activation of the LXR/RXR pathway, thereby protecting cells from the subsequent 7-KC-induced cytotoxicity. It has also been reported that 24S-OHC at low concentrations protects cells from cell death induced using staurosporine, which is a toxic substance that induces apoptosis (Emanuelsson and Norlin 2012). In addition, a low concentration of 24S-OHC stimulated cellular processes critical to maintaining redox homeostasis and showed a protective action against oxidative stress in human glioblastoma U-87 MG cells (Cigliano et al. 2019).

Regarding the damage-promoting effects of 24S-OHC, it was reported that 24S-OHC at low concentrations but not 27-OHC or 7-β-hydroxycholesterol enhanced Aβ binding to human differentiated neuronal cell lines by the upregulation of a multireceptor complex involving CD36 and β1-integrin (Gamba et al. 2011). In addition, 24S-OHC promoted the generation of NADPH oxidase-dependent generation of ROS, resulting in the disruption of the redox equilibrium and potentiation of Aβ42 neurotoxicity.

10.4 25-OHC

10.4.1 25-OHC and Related Diseases

In the plasma, 25-OHC has been reported to be a minor side-chain oxysterol compared with 24S-OHC and 27-OHC (Mutemberezi et al.

2016). ER-resident CH25H is a key enzyme for the production of 25-OHC (Cao et al. 2020). Expression of CH25H is found in macrophages and the liver, and it is regulated in response to immune and inflammatory conditions and in an LXR-dependent manner (Liu et al. 2018; Park and Scott 2010). Besides CH25H, other cytochrome P450 enzymes, such as CYP3A4, CYP46A1, and sterol 27-hydroxylase (CYP27A1), and even ROS-induced reactions can generate 25-OHC (Diczfalusy and Björkhem 2011; Honda et al. 2011; Diczfalusy 2013). However, the importance of these reactions in vivo remains unclear.

Growing evidence indicates that 25-OHC is involved in antiviral process and inflammatory immune response (Cyster et al. 2014). CH25H expression is induced in response to toll-like receptor ligands and the subsequent signaling through the interferon receptor/Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1) pathway in human macrophages (Park and Scott 2010). The subsequently produced 25-OHC shows antiviral activities against a broad range of enveloped viruses (Blanc et al. 2013; Liu et al. 2013). Furthermore, 25-OHC has been shown to block membrane fusion between the virus and host cell, ultimately inhibiting viral replication, but the exact mechanism may vary depending on the virus and cell type. Recently, 25-OHC has been reported to suppress the replication of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which causes novel coronavirus disease-2019 (COVID-19), by blocking membrane fusion (Zang et al. 2020; Wang et al. 2020). Regarding cytokine production, 25-OHC can have both pro-inflammatory and anti-inflammatory roles (Reboldi et al. 2014).

10.4.2 25-OHC-Induced Cell Death

It has been reported that 25-OHC induces cell death by different mechanisms in various types of cells. For example, it induces apoptosis in human aortic smooth muscle cells (Ares et al. 1997). Moreover, it induces Ca^{2+} influx through plasma membrane channels. Using Ca^{2+} entry blocker effectively inhibited apoptosis, suggesting that Ca^{2+} plays a crucial role in 25-OHC-induced cell death. Furthermore, 25-OHC induced apoptosis in vascular smooth muscle cells, resulting in the promotion of vascular calcification (Dong et al. 2020). Activation of pro-death UPR signaling, including increases in ATF4 and CCAAT/enhancer-binding protein homologous protein (CHOP) expression, has been implicated in 25-OHC-induced apoptosis and vascular calcification. Another report showed that soluble adenylyl cyclase (sAC) played a key role in the 25-OHC-induced apoptosis of rat vascular smooth muscle cells by controlling protein kinase A (PKA)-dependent phosphorylation as well as mitochondrial translocation of Bax and mitochondrial ROS formation (Appukuttan et al. 2013).

Oxysterol-binding protein-related protein 4L (ORP4L) has been reported to bind 25-OHC and be involved in cell proliferation and survival (Charman et al. 2014). In macrophages, excessive 25-OHC led to the disassembly of the ORP4L/$G\alpha_{q/11}$/phospholipase C (PLC)-β3 complex, resulting in disruption of Ca^{2+} signaling, a decrease of antiapoptotic Bcl-XL expression, and an increase in apoptosis (Zhong et al. 2016). In thioglycolate-elicited peritoneal macrophages, 25-OHC caused ER stress, including CHOP induction and subsequent apoptosis (Sekiya et al. 2014). A deficiency of neutral cholesterol ester hydrolase 1 (NCEH1) promoted the accumulation of the 25-OHC ester in the ER due to its defective hydrolysis, resulting in the augmentation of 25-OHC-induced apoptosis. Moreover, ACAT1 inhibition suppressed NCEH1-dependent augmentation of 25-OHC-induced ER stress and apoptosis. These findings suggest that the esterification of 25-OHC in the ER plays an important role, and ACAT and NCEH1 can exert proapoptotic and antiapoptotic effects, respectively, in 25-OHC-induced macrophage cell death.

Oxysterol-binding protein-related protein 8 (ORP8) is another member of the ORP family and it binds to 25-OHC (Yan et al. 2008). In hepatic cell lines, HepG2 and Huh7, 25-OHC

induced apoptosis via the pro-death UPR pathway (Li et al. 2016). ORP8 knockdown diminished 25-OHC-induced ER stress and apoptosis, suggesting that ORP8 mediates the cytotoxicity of 25-OHC. Cotreatment with either α-tocopherol or α-tocotrienol effectively suppressed 25-OHC-induced HepG2 cell death (Suzuki et al. 2021). The accumulation of lipid hydroperoxides was also observed in 25-OHC-treated HepG2 cells, suggesting the involvement of free radical-mediated lipid peroxidation in 25-OHC-induced cell death. In a rat liver tumorigenesis model, antitumor effects of 25-OHC were reported to induce the entry of rat AH136B ascites hepatoma cells into the sub-G1 phase (Yokoyama et al. 1999). Anticancer effects of 25-OHC were also observed in head and neck squamous cell carcinoma cells (You et al. 2020).

UV irradiation has been shown to increase 25-OHC levels but not 24S-OHC or 27-OHC levels in human primary keratinocytes (Olivier et al. 2017). In human keratinocyte HaCaT cells, 25-OHC caused caspase-3-dependent apoptosis (Suzuki et al. 2021) and caspase-1-dependent pyroptosis (Olivier et al. 2017). Pyroptosis is characterized as a type of RCD that is caused by the caspase-1/4/5/11-dependent formation of gasdermin family-mediated pores on the plasma membrane, leading to the release of pro-inflammatory cytokines and cell rupture (Galluzzi et al. 2018; Vandenabeele et al. 2022). Because the P2X7 receptor and caspase-1 were activated by 25-OHC, the P2X7/NLRP3 inflammasome signaling pathway was implicated in 25-OHC-induced pyroptosis in HaCaT cells.

In the CSF and serum of patients with untreated ALS, the 25-OHC levels were higher than those in the control and treatment groups, and the serum 25-OHC levels were associated with the disease severity and progression rate (Kim et al. 2017). Increased expression of *CH25H* and *CYP3A4* mRNA was also observed in the early symptomatic stages of ALS model mice. In the motor neuron-like cell line (NSC-34) expressing human G93A mutant of superoxide dismutase 1, 25-OHC was shown to induce apoptosis. Cotreatment of 22(*S*)-hydroxycholesterol as an LXR antagonist significantly attenuated cell death, indicating the involvement of the LXR signaling pathway in 25-OHC-induced motor neuron cell death.

In addition to the observation in thioglycolate-elicited peritoneal macrophages (Sekiya et al. 2014), ACAT inhibition also suppressed 25-OHC-induced cell death in SH-SY5Y cells, but not in HepG2 and HaCaT cells (Suzuki et al. 2021). As noted, because UPR/ER stress causes 25-OHC-induced cell death in many types of cells, it is of interest to verify the cytoprotective effects of ACAT inhibition in those cells.

10.5 27-OHC

10.5.1 27-OHC and Related Diseases

It has been shown that 27-OHC is the most abundant oxysterol in circulation and an important intermediate in cholesterol catabolism to bile acid biosynthesis (Kim et al. 2022). In the mitochondria, CYP27A1 mediates the enzymatic synthesis of 27-OHC from cholesterol. In the liver, 27-OHC is further catabolized toward bile acids by oxysterol 7α-hydroxylase (CYP7B1). Although CYP27A1 is mainly expressed in the liver, it is also expressed in macrophages, the brain, and the lung, indicating that 27-OHC concentration can be locally regulated in nonhepatic tissues (Kim et al. 2022). Bile acids have been reported to suppress the transcription of *CYP27A1* (Chen and Chiang 2003). Mice with CYP27A1 deficiency had normal plasma levels of cholesterol and markedly reduced levels of bile acid synthesis (Rosen et al. 1998) and showed hepatomegaly and hypertriglyceridemia (Repa et al. 2000). Mutations in human *CYP27A1* have been linked to a rare autosomal recessive lipid storage disease called cerebrotendinous xanthomatosis, which is characterized by progressive dementia, xanthomatosis, and accelerated atherosclerosis (Cali et al. 1991). The diverse phenotypes observed in patients with cerebrotendinous xanthomatosis demonstrate the importance of CYP27A1 in bile acid biosynthesis, reverse cholesterol transport, and vitamin D_3 biosynthesis.

Accumulating evidence indicates that 27-OHC is related to neurodegenerative diseases. This oxysterol is synthesized at low levels in neurons and glial cells (Russell 2000). The peripherally derived 27-OHC crosses the blood–brain barrier via free diffusion (Heverin et al. 2005). In the case of patients with AD, 27-OHC levels in the CSF are elevated in AD and mild cognitive impairment subjects compared with those in the controls (Wang et al. 2016b), which could be attributed to dysfunction of the blood–brain barrier and blood–CSF barrier. Moreover, 27-OHC increased Aβ levels by increasing the levels of β-secretase (Marwarha et al. 2013) and decreasing the levels of the insulin-degrading enzyme (IDE), which is known as an Aβ-degrading enzyme (Zhang et al. 2019). Furthermore, 27-OHC also increased phosphorylated tau levels (Marwarha et al. 2010). In the case of PD, 27-OHC reduced the levels of tyrosine hydroxylase, which is the rate-limiting enzyme in dopamine synthesis, and increased the levels of α-synuclein, which is the major component of Lewy bodies in SH-SY5Y cells (Marwarha et al. 2011). In patients with hereditary spastic paraplegia type 5 (SPG5) with mutations in *CYP7B1*, a marked accumulation of 27-OHC was observed (Schüle et al. 2010).

It has been established that 27-OHC functions as an endogenous selective estrogen receptor modulator (SERM). SERM is an estrogen receptor ligand that shows agonist or antagonist activity in a cell- and promoter-dependent manner. In the vasculature, 27-OHC acted as an estrogen receptor α antagonist and promoted atherosclerosis progression via pro-inflammatory processes (Umetani et al. 2014). However, 27-OHC also acted as an agonist for estrogen receptor α and promoted the proliferation of cancer cells and tumorigenesis (Kim et al. 2022). The pro-tumor properties of 27-OHC were found to be associated with various cancers, notably breast cancer (Nelson et al. 2013). Furthermore, 27-OHC increased tumor metastasis due to increased myeloid immune cell function and decreased cytotoxic $CD8^{+}$T lymphocytes (Baek et al. 2017). In addition, it also induced hematopoietic stem cell mobilization and extramedullary hematopoiesis during pregnancy by regulating estrogen receptor α function (Oguro et al. 2017).

10.5.2 27-OHC-Induced Cell Death

It has been shown that 27-OHC induces apoptotic cell death in different cell types. There are two different types of apoptotic pathways: one is initiated when stress occurs within the cell (the intrinsic pathway) and the other is triggered by the binding of ligands to cell surface death receptors (the extrinsic pathway). It has been reported that 27-OHC activates both intrinsic and extrinsic apoptotic pathways. For example, 27-OHC treatment increased apoptosis via increased ROS levels and ER stress in hematopoietic stem and progenitor cells (Woo et al. 2022). Exogenous 27-OHC treatment also promoted ROS production and apoptosis in leukemia cells. In contrast, 27-OHC was shown to evoke the extrinsic pathway by inducing the production of tumor necrosis factor-α in macrophages (Kim et al. 2013; Umetani et al. 2014).

Other types of cell death processes are also observed in 27-OHC-induced cell death. In colon cancer Caco-2 cells, a high concentration of 27-OHC induced nonapoptotic cell death (Warns et al. 2018). These effects are independent of LXR or estrogen receptor activation and are due to decreased activation of Akt, which is associated with cell proliferation. In cocultured SH-SY5Y cells and astrocyte C6 cells, 27-OHC induced apoptosis associated with the reduction of mitochondrial membrane potential (Wang et al. 2016a) and pyroptosis by causing lysosomal membrane permeabilization and subsequent cathepsin B leakage into the cytosol (Chen et al. 2019). However, it remains unclear how 27-OHC damages mitochondria and lysosomes.

Ferroptosis is a type of programmed cell death that is characterized by iron-dependent peroxidation of polyunsaturated phospholipids in cell membranes (Galluzzi et al. 2018; Yang and Stockwell 2016). Recent work suggests that ferroptosis plays a pivotal role in tumor suppression (Li et al. 2020). In estrogen receptor

α-negative cancer cell lines, acute exposure to 27-OHC attenuated cell growth and migration by disrupting lipid metabolism through interfering with SREBPs signaling (Liu et al. 2021). Chronic exposure to 27-OHC led to the selection of 27-OHC-resistant cells, which induced adaptive responses to ferroptosis by upregulating the activity of processes that allowed the cells to withstand lipid oxidative stress. Moreover, 27-OHC-resistant cells were more tumorigenic and metastatic when evaluated in an in vivo model. The enhanced tumorigenic and metastatic activities of 27-OHC-resistant cells were attenuated by inhibition of the phospholipid hydroperoxide-reducing enzyme glutathione peroxidase 4 to increase the sensitivity to ferroptosis. Because circulating 27-OHC levels are elevated in hypercholesterolemia, the possibility that 27-OHC impacts cancer pathogenesis by selecting cells that are resistant to ferroptosis was considered (Liu et al. 2021).

10.6 Concluding Remarks

Growing evidence indicates the importance of side-chain oxysterols in various pathogeneses. Side-chain oxysterols induce various forms of cell death depending on the hydroxylated site of cholesterol and the cell types; they also exert protective effects against cell death caused by other stresses. These functions are thought to be due to the various physiological activities of side-chain oxysterols at the transcriptional and post-translational levels. Suppression of oxysterol-induced cell death is considered effective for disease treatment. Inhibitors of side-chain oxysterol-producing enzymes may be therapeutic agents for diseases that are caused by oxysterol-induced cell death. ACAT inhibitors are also promising drugs for cell death caused by the esterification of oxysterols. Thus, further studies of the roles of side-chain oxysterols in various pathogeneses may pave the way for the development of therapeutic reagents for oxysterol-related diseases.

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Oxysterols in Vascular Cells and Role in Atherosclerosis

11

Celine Luquain-Costaz and Isabelle Delton

Abstract

Atherosclerosis is a major cardiovascular complication of diseases associated with elevated oxidative stress such as type 2 diabetes and metabolic syndrome. In these situations, low-density lipoproteins (LDL) undergo oxidation. Oxidized LDL displays proatherogenic activities through multiple and complex mechanisms which lead to dysfunctions of vascular cells (endothelial cells, smooth muscle cells, and macrophages). Oxidized LDLs are enriched in oxidized products of cholesterol called oxysterols formed either by autoxidation, enzymatically, or by both mechanisms. Several oxysterols have been shown to accumulate in atheroma plaques and to play a key role in atherogenesis. Depending on the type of oxysterols, various biological effects are exerted on vascular cells to regulate the formation of macrophage foam cells, endothelial integrity, adhesion and transmigration of monocytes, plaque progression, and instability. Most of these effects are linked to the ability of oxysterols to induce cellular oxidative stress and cytotoxicity mainly through apoptosis and proinflammatory mediators. Like for excess cholesterol, high-density lipoproteins (HDL) can exert antiatherogenic activity by stimulating the efflux of oxysterols that have accumulated in foamy macrophages.

Keywords

Oxysterol · Oxidized low-density lipoprotein · Atherosclerosis · Macrophages · Endothelial cells · Apoptosis · Inflammation

Abbreviations

4β-OHC	4β-Hydroxycholesterol
5,6α-EC	5α,6α-Epoxycholesterol
5,6β-EC	5β,6β-Epoxycholesterol
7α-OHC	7α-Hydroxycholesterol
7β-OHC	7β-Hydroxycholesterol
7KC	7-Ketocholesterol
20S-OHC	20(S)-Hydroxycholesterol
22R-OHC	22(R)-Hydroxycholesterol
22S-OHC	22(S)-Hydroxycholesterol
24S-OHC	24(S)-Hydroxycholesterol
25-OHC	25-Hydroxycholesterol
27-OHC	27-Hydroxycholesterol
CH25H	Cholesterol 25-hydroxylase
CT	Cholestane-3β-5α-6β-triol
EC	Endothelial cells
ER	Endoplasmic reticulum
HDL	High-density lipoprotein

C. Luquain-Costaz · I. Delton (✉)
CNRS 5007, LAGEPP, Université of Lyon, Université Claude Bernard Lyon 1, Villeurbanne, France

Department of Biosciences, INSA Lyon, Villeurbanne, France
e-mail: isabelle.delton@insa-lyon.fr

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IL	Interleukin
MMP	Matrix metalloproteinase
oxLDL	Oxidized low-density lipoprotein
SMC	Smooth muscle cells
TNFα	Tumor necrosis factor

11.1 Introduction

Atherosclerosis is a chief cause of morbidity and mortality in Western countries. It is characterized by the formation in the intima arteries of atherosclerotic plaques consisting of the accumulation of lipids, complex carbohydrates, blood cells and products, fibrous tissue, and calcium deposits. Since the regions with low fluid shear stress—the sites of arterial branching or curvature—are much more susceptible, the abdominal aorta, coronary arteries, and carotid bifurcations are the predilection vessels for the formation of atherosclerotic plaques. The formation of atherosclerotic plaques also called atherogenesis is a long-term and evolutionary process. Initial lesions (fatty streaks) can be observed very early in life and stay asymptomatic for a long time. Advanced atherosclerotic lesions develop with the accumulation of lipids, cell debris, and fibrous tissue in a long-term processes (20–40 years) The thickening of the plaques causes cardiovascular diseases (CVD) such as coronary heart disease, myocardial infarction, and stroke when disrupting. The risk factors for atherosclerosis are multiple including non-modifiable factors such as age, sex, and genetic background, and modifiable factors such as smoking, lack of exercise, and high-fat diet. It is well acknowledged that atherosclerosis is a major cardiovascular complication of diseases associated with chronic inflammatory status, increased oxidative stress and disorders of lipid metabolism, such as type 2 diabetes, obesity, and metabolic syndrome.

Arteries are constituted of three morphologically distinct layers: the outerlayer adventitia, the media, and the innermost layer intima where the atherosclerotic plaques are formed. Atherogenesis begins with the infiltration and sub-endothelial accumulation of low-density lipoproteins (LDL). In physiopathological situations, LDL becomes oxidized (oxLDL) closely related to oxidative stress prevailing in the arterial wall that causes oxidation of the protein and lipid moieties of LDL through both enzymatic or non-enzymatic (ROS-induced) pathways. LDL oxidation can be catalyzed by metal cations (copper, iron) and several enzyme systems, including 12/15-lipoxygenase strongly expressed strongly in atherosclerotic plaques, myeloperoxidase, NOS (nitric oxide synthase), xanthine oxidase, and NADPH oxidase. The oxidative products are various, including oxidized derivatives of fatty acid (e.g., malondialdehyde (MDA), 13-HPODE, and 13-HODE), and lipids (e.g., lysophosphatidylcholine and oxysterols) as well as protein carbonyls. The early oxidation of LDL can only produce the minimally oxidized LDL because of the presence of antioxidants, such as vitamin E, A, and β carotene, and the efficient antioxidant enzymes (e.g., superoxide dismutase). In the narrow sub-endothelial space, LDL undergoes further oxidation to form the highly oxidized LDL involving the ROS produced by macrophages and endothelial cells (EC), and also several enzymes form these cells. OxLDL displays atherogenic activities through multiple and complex mechanisms. Globally, oxLDL altered homeostasis of vascular cells resulting in loss of EC integrity, migration and proliferation of smooth muscle cells (SMC), and macrophage foam cell formation through different signals linked to proinflammatory and proapoptotic effects (Nègre-Salvayre et al. 2017, 2020).

The LDL receptor (LDL-R) is located on the plasma membrane and internalizes LDL after binding to apoB-100. At a minor stage of oxidation, the apoB is simply no longer recognized by the LDL-R. At a major stage of oxidation, the modified apoB allows the recognition of multiple scavenger receptors (SR) including SR-A1 (scavenger receptor class A1), SR-A2, CD36, SR-B1 (scavenger receptor class B1), and LOX1 (lectin-like oxidized LDL receptor 1) (Zingg et al. 2021). In contrast with LDL-R, even though the uptake of modified LDL induces the elevated cholesterol

content in macrophages, the expression of SR is not regulated by the intracellular cholesterol quantity, which results in high uptake of oxLDL by macrophages and cholesterol (as its ester form) accumulation in macrophages. The cholesterol-engorged macrophages are called foam cells. The accumulation of foam cells in artery walls, which is easily recognized by light microscopy, is a sign of early atherosclerotic lesions.

Among oxidized lipids in oxLDL, cholesterol oxidation products of cholesterol also called oxysterols have recently gained growing attention with respect to their role in atherogenesis. This review is an overview of the oxysterols that accumulate in atheroma plaques and that exert biological activities on vascular cells including EC, SMC, and macrophages, thereby contributing to atherogenesis and ultimate plaque rupture.

11.2 Oxysterols in Atherosclerotic Plaque and in Serum from Patients with CVD

As mentioned above, oxLDLs are enriched in oxysterols, some of which are involved in the ability of oxLDL to induce cellular oxidative stress and cytotoxicity, mainly through apoptosis. Oxysterols are also associated with the regulation of lipid metabolism and inflammation and are considered as factors contributing to clinical complications of atherosclerosis (see Sect. 11.3).

Oxysterols are produced from cholesterol oxidation through enzymatic pathways with oxidative position mainly on the side chain of cholesterol generating hydroxycholesterols (OHC) including 20S-OHC, 22R-OHC, 22S-OHC, 24S-OHC, 27-OHC, 25-OHC, and 7α,25-dihydroxycholesterol. Non-enzymatic pathways cholesterol autoxidation mainly on the cholesterol ring led to 7-ketocholesterol (7-KC), 7β-OHC, 7α-OHC, 4β-OHC, 5α,6-α-epoxycholesterol (5,6α-EC), 5β,6-β-epoxycholesterol (5,6β-EC), and cholestan-3β,5α, 6β-triol (CT). 7α-OHC and 25-OHC can be formed by both pathways.

11.2.1 Levels of Oxysterols in Atheroma Plaque and Plasma of Atherosclerotic Subjects

The first evidence for the presence of oxysterols in atherosclerotic plaques dated from mid-1960s (Brooks et al. 1966; Fumagalli et al. 1971). Increased amounts of 27-OHC and 7-KC were reported in these studies. Since then, many studies have confirmed the association between the presence of oxysterol in atheromatous lesions and atherosclerotic plaque formation, progression, and stability. Overall, 27-OHC is the major oxysterol recovered in advanced atherosclerotic plaques, followed by 7-KC, 7β-OHC, and 7α-OHC. These oxysterols account for 75–85% of the total oxysterols detected in atherosclerotic plaques from various sites, the others being 25-OHC, 24-OHC, and 5,6-EC (Vaya 2013).

Elevated levels of 27-OHC were found in human carotid atherosclerotic plaques compared to non-atherosclerotic human vessels in correlation to high expression of 27-hydroxylase (the enzyme converting cholesterol to 27-OHC) in macrophage-rich core regions of complicated lesions (Crisby et al. 1997). Since 27-OHC can be eliminated from macrophages, it was proposed that 27-OHC formation could be a defense mechanism against deleterious cellular accumulation of cholesterol.

Analysis of oxidized lipids in human aortic advanced atherosclerotic plaques revealed elevated amounts of 27-OHC and 7β-OHC compared to normal aorta (Carpenter et al. 1993). This group also highlighted the presence of these two oxysterols at different stages of atherosclerotic lesions (fatty streaks, intermediate, and advanced lesions) from aorta and common carotid artery, compared to normal human artery. 27-OHC was significantly more abundant in advanced lesions than in intermediate lesions or fatty streaks (Carpenter et al. 1995). 27-OHC and 7β-OHC were also detected in all the samples of human atheromatous lipid core and fibrous cap of individual advanced atherosclerotic plaques (Garcia-Cruset et al. 1999). It is also noteworthy that pharmacological lowering of 27-OHC was

associated with coronary plaque regression (Nakano et al. 2022).

7-Oxygenated sterol (7β-OHC, 7α-OHC, and 7KC) have also been widely detected in atherosclerotic lesions; 7-KC is the second most abundant oxysterol and the major 7-oxygenated sterol found in atheromatous plaques(Brown et al. 1997; Garcia-Cruset et al. 2001; Ravi et al. 2021), although it was not detected in a recent study (Pinto et al. 2022). The presence of 7β-OHC in atherosclerotic lesions together with 27-OHC has been reported in early studies (Carpenter et al. 1993; Garcia-Cruset et al. 1999). Importantly, the in situ formation of ROS-derived 7-oxygenated sterol in human carotid plate was confirmed—rather than auto-oxidation during sample processing—as well as that of ROS-derived EC (Helmschrodt et al. 2013).

Other oxysterols such as 24-OHC, 25-OHC, 5,6β-EC, and 5,6α-EC were found in human fatty streaks and advanced atherosclerotic lesions (Garcia-Cruset et al. 2001; Helmschrodt et al. 2013). Increased levels of 24-OHC, 25-OHC, 27-OHC, 7α-OHC, and 7β-OHC but not 7-KC were measured in symptomatic subjects with carotid atherosclerotic plaques compared to asymptomatic subjects (Pinto et al. 2022). Arterial intima accumulation of 27-OHC and 24S-OHC is associated with severe peripheral artery disease (Virginio et al. 2015). The accumulation of 25-OHC was reported in coronary atherosclerotic lesions (Canfrán-Duque et al. 2023) but not in arterial tissue from subjects with severe peripheral artery disease (Virginio et al. 2015).

In addition to high detection in atheromatous plaques, elevated plasma concentrations of several oxysterols including 25-OHC, 27-OHC, and 7β-OHC were shown to correlate with symptoms of coronary and peripheral artery diseases and atherogenic risk profile (Ziedén et al. 1999; Yasunobu et al. 2001; Rimner et al. 2005; Virginio et al. 2015). Increased levels of 7β-OHC in plasma were proposed to be a biomarker for high risk of developing cardiovascular disease and coronary atherosclerotic plaques (Khatib and Vaya 2014). Elevated plasma 7-KC levels have also been associated with higher risk of cardiovascular disease events in the general population and in patients with coronary artery disease (Hitsumoto et al. 2009; Song et al. 2017; Wang et al. 2017).

Atherosclerosis is a major complication of diseases associated with high oxidative stress such as diabetes, metabolic syndrome, and dyslipidemia. The level of total oxysterols was markedly increased in the serum of diabetic subjects compared to healthy controls, mainly due to high amounts of 7-KC, 7α-OHC, 7β-OHC, and 5,6α-EC (Khatib and Vaya 2014). Elevated plasmatic oxysterol levels especially 7-KC and CT were correlated to hyperglycemia and glycation index as well as a number of coronary risk factors, particularly in type 2 diabetic patients (Samadi et al. 2019; Samadi et al. 2020; Ahmed et al. 2022).

11.2.2 In Vitro and In Vivo Formation of Oxysterols

In vitro oxidation of LDL using different acellular oxidizing conditions (copper, peroxynitrite, 2,2′--azobis(2-amidinopropane)dihydrochloride-AAPH, hypochlorous acid) has confirmed the formation of oxysterols in the particle through auto-oxidation process, including 7-KC, 7α-OHC, 7β-OHC that were reproducibly detected. The formation of 5,6α- and 5,6β-EC, as well as 24-OHC, 25-OHC, or 27-OHC has also been reported but to a lesser extent and not in all studies (Matsunaga et al. 2009; Vaya et al. 2011; Arnal-Levron et al. 2013; Orsó et al. 2015; Chen et al. 2015). The level of oxysterols in LDL varies according to the oxidizing conditions and is the highest in copper highly oxidized LDL (Orsó et al. 2015).

Although less explored, cell-mediated generation of oxysterols was also reported. Macrophages, as well as EC and SMC, were shown to mediate radical-dependent LDL oxidation leading to the formation of lipid hydroperoxides and consequent increase of 7-OHC and 7-KC (Müller et al. 1998). It is commonly assumed that accumulation of oxysterols in atheroma macrophages results from the massive uptake of oxLDL that itself contains oxysterols (Brown et al. 1996, 1997).

Accordingly, the levels of 7-KC, 7α-OHC, 27-OHC, and cholest-4-en-3-one were found to increase in oxLDL-loaded mouse macrophages proportionally to the degree of LDL oxidation (Paul et al. 2019).

The intracellular formation of oxysterols in cultured macrophages is very low and usually under the detection limit but some species (i.e., 7-KC, 25-OHC, 24-OHC, and 24,25-EC) were highly increased upon excessive cholesterol loading or exposure to the endotoxin Kdo2-Lipid A (Fu et al. 2001; Dennis et al. 2010; Maurya et al. 2013). Intracellular formation of oxysterols is promoted in macrophages after uptake of modified LDL that undergo oxidation inside lysosomes leading to the formation of 7-KC and 7β-OHC (Wen and Leake 2007; Yoshida and Kisugi 2010). Our studies confirmed that cellular activity significantly contributes to the accumulation of oxysterols in macrophage cell lines (Arnal-Levron et al. 2013; Chen et al. 2015). Upon exposure to copper-oxidized LDL, both cellular cholesterol and LDL-derived cholesterol were oxidized in murine RAW and human THP1 macrophages resulting in a huge increase of oxysterol production. Major oxysterols originated from non-enzymatic pathway (7-KC and 7α/β-OHC), the enzymatically formed 25-OHC and 27-OHC being recovered in much lower proportions. We also demonstrated that the oxidation of LDL-derived cholesterol occurred mainly in the late endosomal compartment, while oxidation of cellular cholesterol likely occurs at the plasma membrane site (Chen et al. 2015). The intracellular oxysterol production in oxLDL-loaded RAW macrophages was regulated by the specific endosomal phospholipid bis (monoacylglycero)phosphate, also supporting LDL-cholesterol oxidation in this compartment (Arnal-Levron et al. 2013).

11.3 Biological Effects of Oxysterols in Atherogenesis and Plaque Progression

Several oxysterols have been involved in various aspects of atherogenesis, such as formation of macrophage foam cells, endothelial dysfunction, adhesion and transmigration of monocytes, plaque progression, and instability (Poli et al. 2009). Depending on the type of oxysterols, various biological activities are exerted on vascular cells including EC, SMC, and macrophages. Although a subset of oxysterols is likely to exert antiatherogenic effects by regulating cholesterol homeostasis, an overlapping but distinct set of oxysterols display proatherogenic activity by inducing cytotoxicity primarily through apoptosis and stimulating inflammatory pathways.

11.3.1 Oxysterols and Foam Cell Formation

Cholesterol homeostasis in macrophages results from the finely regulated balance between cholesterol acquisition including de novo synthesis of cholesterol dependent on HMGCoA reductase (HMGCoAR) activity and uptake of non-oxLDL by LDL-R, and efflux of excess cholesterol by extracellular acceptors HDL and apoA1. Both mechanisms of cholesterol acquisition are regulated by a complex of three proteins located in the endoplasmic reticulum (ER): SREBP (sterol regulatory element binding protein), SCAP (SREBP cleavage activating protein), and Insig (insulin-induced gene). This complex regulates the expression of LDL-R receptor and HMGCoAR inversely correlated to ER cholesterol content (Sato 2010; Savla et al. 2022). The removal of excess intracellular cholesterol is regulated by the activity of nuclear receptor liver X receptors (LXR) by enhancing the expression of ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 (Matsuo 2022). Atherosclerosis is characterized by excessive accumulation of cholesterol within sub-endothelial macrophages. To sum up, cholesterol accumulates consequently due to the massive unregulated uptake of oxLDL by SR and to the defects in ABC-dependent efflux of cholesterol (Li et al. 2021). In early lesions, excess cholesterol is stored in the form of cholesterol esters produced by acyl-coA:cholesterol ester transferase (ACAT) activity within lipid droplets giving

macrophages their foamy appearance. In advanced lesions, free cholesterol accumulates in ER, leading to ER stress that triggers apoptosis of foamy macrophages.

Side chain oxysterols, in particular 25-OHC, suppress the expression of SREBP2 target genes presumably in an Insig-dependent manner, which negatively regulates cholesterol biosynthesis and uptake (Sato 2010). As natural ligands for LXRα and LXRβ (Janowski et al. 1999), side chains oxysterols including 24,25-EC, 22(R)-OHC, 24 (S)-OHC, 25-OHC, and 27-OHC exert antiatherogenic activity by stimulating the expression of ABCA1 and ABCG1 in macrophages (Töröcsik et al. 2009; Olkkonen 2012; Saito et al. 2023). However, some oxysterols can inversely promote foam cell phenotype. A mixture of 7β-OHC and 7-KC stimulates lipid droplet formation and upregulates SRA that facilitates the ingestion of oxLDL (Yuan et al. 2016; Ward et al. 2017; Saha et al. 2020).

11.3.2 Oxysterol and Apoptosis/ Autophagy/Oxyautophagy

Oxysterols are considered potent regulators of cell death primarily by inducing apoptosis, stress of endoplasmic reticulum, and autophagy (Fig. 11.1).

Apoptosis is a natural, programmed cell death process that occurs in multicellular organisms. Two main pathways trigger apoptosis: the intrinsic pathway (or mitochondrial pathway) and the extrinsic pathway (or death receptor pathway). Both pathways involve a series of complex signaling events that ultimately lead to the activation of caspases and the subsequent destruction of the cell. The intrinsic pathway is activated by intracellular stresses, such as DNA damage, oxidative stress, or nutrient deprivation. It is associated with outer mitochondrial membrane permeabilization and release of cytochrome c that induces the assembly of a caspase-activation complex. The extrinsic pathway is activated by the binding of extracellular ligands, such as Fas ligand or tumor necrosis factor-alpha (TNFα) to death receptors on the cell surface which initiates the caspase cascade. Caspase 3 is considered to be a key mediator of apoptosis; it cleaves a number of key cellular proteins, including cytoskeletal proteins, nuclear proteins, and enzymes, leading to DNA fragmentation, and membrane dismantling (D'Arcy 2019).

The ability of oxysterols to induce apoptosis in vascular cells has been well described. Among the oxysterols found in atheroma plaques, 7-OHC and 7-KC are commonly the most cytotoxic. These oxysterols induce apoptosis in SMC (Hughes et al. 1994; Miyashita et al. 1997; Pedruzzi et al. 2004), EC (Luchetti et al. 2017), and macrophages (Li et al. 2012; Ward et al. 2017; Ravi et al. 2021) contributing to the cause of cell death in core regions of atherosclerotic plaques. Compared to 7-oxysterols, side chain oxysterols exert no or lower apoptotic effect. 27-OHC exerts dual effects in terms of

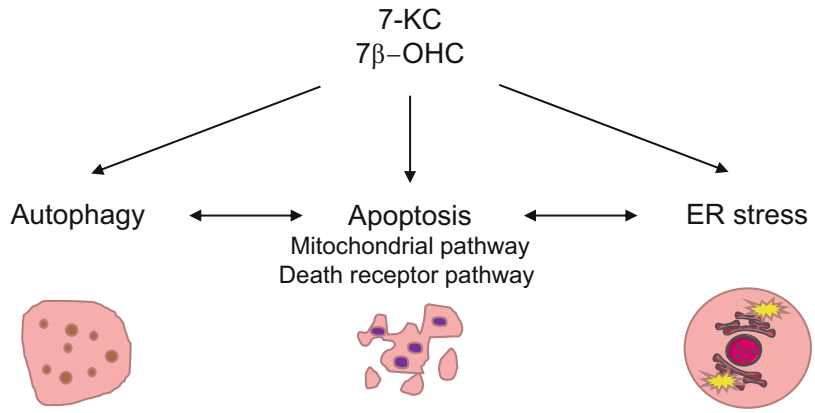

Fig. 11.1 Regulation of cell death by oxysterols.7-KC, 7-ketocholesterol; 7β-OHC, 7β-hydroxycholesterol; ER stress, endoplasmic reticulum stress

cytotoxicity toward macrophages, acting as a protector at a low concentration while triggering apoptosis at high concentrations (Riendeau and Garenc 2009). Low micromolar concentration of 27-OHC evokes survival signals in U937 human promonocytic cell line through the activation of ERK and AKT and inhibition of the proapoptotic protein Bad in response to initial ROS formation (Vurusaner et al. 2016, 2018). Studies using 25-hydroxylase deficient macrophages suggest that 25-OHC increases susceptibility to LPS-induced apoptosis in macrophages through increased caspase 3 activation and reduced efferocytosis capacity (Canfrán-Duque et al. 2023).

7-Oxysterols stimulate both intrinsic mitochondrial and extrinsic death receptor pathways of apoptosis. In macrophages, apoptosis mediated by 7-oxysterols is associated with caspase-3 activation, increased permeability of mitochondrial membrane, release of cytochrome c and endonuclease G (Prunet et al. 2005; Palozza et al. 2010; Li et al. 2012). 7-KC and 7β-OHC can also induce ROS production and decrease cellular antioxidants, therefore inducing mitochondrial oxidative stress in the sub-endothelial space (Tabas et al. 2015; Ravi et al. 2021). Apoptosis mediated by 7-oxysterols also involves the regulation of the expression of Bcl-2 family proteins that exert pro- or anti-apoptotic activity. In macrophages, 7-KC induces proapoptotic pathways associated with the proapoptotic proteins Bax and Bim (Berthier et al. 2005; Palozza et al. 2010; Li et al. 2012) and inhibits the anti-apoptotic protein AKT (Vejux and Lizard 2009; Palozza et al. 2010). However, 7-KC was also reported to trigger a survival response through the activation of PYK2/MEK1/2/ERK pathway allowing BAD phosphorylation (Berthier et al. 2005). 7-KC and 7β-OHC-induced apoptosis is also associated with the induction and nuclear translocation of the tumor suppressor p53 (Li et al. 2012) which has been shown to be highly expressed and to promote apoptosis in human atherosclerotic plaque (Yuan et al. 2010).

Endoplasmic reticulum (ER) stress is a cellular response to an accumulation of misfolded or unfolded proteins within the ER. ER stress triggers a signaling pathway called the unfolded protein response (UPR) that aims to restore ER homeostasis. ER markers include phosphorylated eIF2α and IRE1α and expression of the proteins GRP78 and CHOP. Prolonged activation of UPR leads to cell dysfunction and death, contributing to the development of various diseases, including neurodegenerative disorders, diabetes, cancer, and cardiovascular diseases. The links between oxLDL and ER stress as well as the involvement of ER stress in atherosclerosis initiation and progression have been well documented, but the role of oxysterols is not well established (Sanda et al. 2017; Luchetti et al. 2017). In SMC and EC, 7-KC-induced ER stress is characterized by increased phosphorylation of IRE1α and expression of CHOP and GRP78 (Pedruzzi et al. 2004; Sanson et al. 2009). 7-KC and 7-OHC can induce macrophage apoptosis through moderating ER stress-specific signaling involving CHOP induction (Son et al. 2012; Park et al. 2016).

Autophagy is a process by which cells degrade and recycle damaged or unwanted cellular components to promote cell survival. Autophagy has been recently revealed as a crucial regulator in the formation of early and advanced atherosclerotic plaques (Li et al. 2022). This phenomenon is traditionally regarded as beneficial in atherosclerosis as it prevents EC apoptosis and senescence, regulates the proliferation of SMC cells, and inhibits foam cell formation and lipid-laden macrophage apoptosis. Few studies have examined the role of oxysterols in autophagy of vascular cells. 7-KC was reported to induce autophagy in human SMC promoting survival and stabilizing atherosclerotic plaque (He et al. 2013; Zhang et al. 2020). On the other hand, 7-KC-induced autophagy may exert deleterious effects as it promotes vascular calcification through autophagy-lysosomal pathway (Sudo et al. 2015).

Last decade, cell death induced by oxysterols has been defined as a complex mode of cell death involving oxidative stress, apoptosis, and autophagy, defined as oxiapoptophagy. Oxiapoptophagy is associated with organelle dysfunction and in particular with mitochondrial and peroxisomal alterations involved in the induction of cell death and in the rupture of redox balance.

Oxidative stress can induce both apoptosis and autophagy, and these processes can interact with each other. For example, under certain conditions, autophagy can promote apoptosis by degrading anti-apoptotic proteins, while apoptosis can inhibit autophagy by cleaving essential autophagy proteins. Thus, the interplay between oxidative stress, apoptosis, and autophagy is complex (Nury et al. 2014, 2021). With respect to oxysterols 7-KC, 7β-OHC and 24S-OHC were reported to be strong inducers of oxiapoptophagy in different cell types including U937 monocytic cells (Nury et al. 2021; de Freitas et al. 2021). Other oxysterols have been shown to induce oxiapoptophagy such as 24(S)OHC in oligodendrocytes (Nury et al. 2015), 25-OHC in fibroblast cells (You et al. 2021), and 7α,25-dihydroxycholesterol in osteoblasts (Seo et al. 2023). However, the link between oxysterols and oxiapoptophagy in atherosclerosis is not well documented.

11.3.3 Oxysterols and Inflammation

Oxysterols are considered as potent inducers of inflammation as they can alter endothelial monolayer integrity, recruit the immune cells, stimulate the expression of various inflammatory molecules, regulate macrophage polarization into M1/M2 phenotypes, and promote the rupture of fibrous caps (Testa et al. 2018) (Fig. 11.2).

It is well known that endothelial dysfunction occurs in the early stage of atherosclerosis and some oxysterols have been involved in this process. 25-OHC is able to inhibit EC proliferation, migration, and tube formation and to impair endothelium-dependent vasodilation through inhibition of NO production (Ou et al. 2016). It also reduced the expression of tight junction proteins (Niedzielski et al. 2021). 25-OHC, as well as 7-KC, was also shown to reduce endothelial monolayer impedance and adhesion (Chalubinski et al. 2013).

Integrins are components of cell-matrix adhesions that regulate monocyte adhesion to endothelial cells and their migration to the site of inflammation. The expression of β1-integrin was shown to increase in U937 monocytes exposed to an oxysterol mixture of pathophysiologic relevance through activation of the ERK signaling pathway. Among oxysterols present at concentrations close to those found in vascular lesions, 7α-OHC and CT were the most potent compounds, and 7β-OHC, 5,6α-EC, and 7-KC were the least potent ones (Gargiulo et al. 2012). This oxysterol mixture was also shown to stimulate the expression and synthesis of MCP-1 (monocyte chemotactic protein-1), another monocyte chemoattractant also referred to as CCL2, in U937 macrophages through the activation of ERK pathway and nuclear binding of NF-κB (nuclear factor κB) (Leonarduzzi et al. 2005). It was also reported that 25-OHC is a ligand of α5β1 and αvβ3 integrins to activate integrin-focal

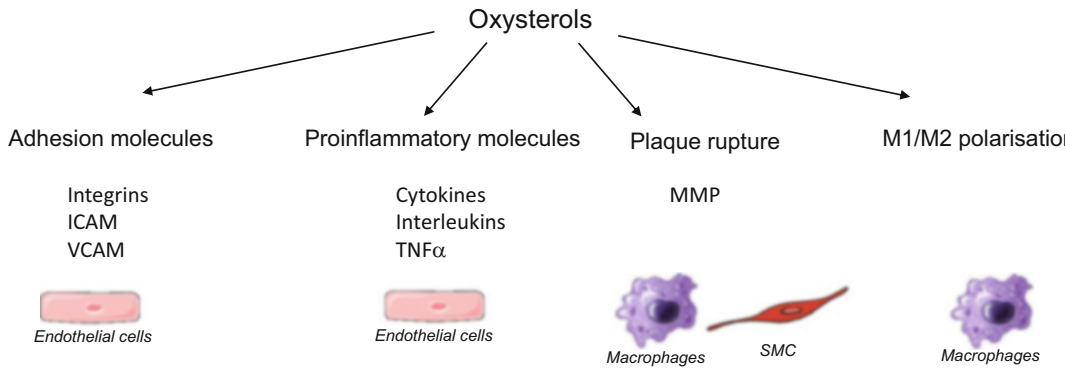

Fig. 11.2 Regulation of inflammation by oxysterols. ICAM, intercellular adhesion molecule-1; VCAM, vascular cell adhesion molecule; TNFα, tumor necrosis factor α; MMPs, matrix metalloproteinases; SMC, smooth muscle cells

adhesion kinase (FAK) signaling (Pokharel et al. 2019).

ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), and E-selectin are adhesion molecules involved in the interaction between leukocyte and EC and subsequent transmigration of monocytes through the endothelial monolayer. Oxysterols, especially those oxidized at C7 (7α/β-OHC and 7-KC), increased the levels of ICAM-1, VCAM-1, and E-selectin expressions in human vascular cells through mechanisms involving p38MAPK pathway or ROS production (Lemaire et al. 1998; Shimozawa et al. 2004; Tani et al. 2018). 25-OHC is also able to induce ICAM-1 synthesis in human EC associated with disruption of endothelial integrity, both effects being counteracted by statin (Niedzielski et al. 2021).

Oxysterols were also reported as modulators of proinflammatory cytokines such as interleukins IL1, IL6, and IL8. In macrophages, 7β-OHC, 7-KC, and 25-OHC regulate IL8 production involving MEK/ERK pathway and AP-1-mediated process (Erridge et al. 2007; Lemaire-Ewing et al. 2009). IL8 induction and secretion in macrophages were also reported after treatment with 7α-OHC through mechanisms dependent of C5a receptor and PI3K and MEK pathways (Cho 2017). 7-KC also stimulates IL12 and IL1α in macrophages (Saha et al. 2020) and 27-OHC stimulates the secretion of IL8 and IL1 in human monocytes through activation of TLR4/NFKB pathway (Gargiulo et al. 2012; Kim et al. 2013). In EC, 7-KC stimulates the expression and secretion of IL-8 associated with ROS production and PI3K/AKT signaling pathways (Chang et al. 2016). 25-OHC also induces proinflammatory cytokines in EC including IL1β, IL-18, IL-23 while anti-inflammatory cytokines IL-10 and IL-37 were repressed (Woźniak et al. 2023). TNFα which is mainly expressed in macrophages is a key regulator of the cytokine cascade and displays proatherogenic activity by promoting the formation of foam cells. The production of TNFα in macrophages is stimulated by 27-OHC and 7α-OHC but not 7-KC in macrophages (Kim et al. 2013; Gargiulo et al. 2015). 25-OHC promotes the production of proinflammatory cytokines including TNF and IL6 through activation of αvβ3 integrin signaling (Pokharel et al. 2019).

Many studies have also highlighted the key role of oxysterols in promoting atherosclerotic plaque instability and rupture (Gargiulo et al. 2018). Matrix metalloproteinases (MMPs) belong to the zinc-metalloproteinases family which are involved in the degradation of extracellular matrix, therefore playing a key role in the rupture of fibrous caps and the formation of advanced atherosclerotic lesions. MMP are primarily produced by activated macrophages but also by vascular SMC and EC. Recently, the role of pro-protein convertase subtilisin/kexin protease (PCSK) 6 in plaque instability and rupture has been suggested related to its ability to stimulate the activity of MMP and its elevated expression in symptomatic carotid plaque. Among oxysterols from a mixture representative of those present in advanced human carotid plaques, 27-OHC, 7α-OHC and to a lesser extent 25-OHC were shown to be the most potent inducers of MMP-9 expression in human monocytes through the activation of TLR4/NFkB pathway (Gargiulo et al. 2012, 2015). It was further demonstrated that the mixture of oxysterols-induced MMP-9 activity through PCSK6 activation in monocytes (Testa et al. 2021). In SMC, 7-KC and 5,6α-epoxide induce the expression of MMP-2 and MMP-9 through the EGFR/PI3K/AKT signaling pathways, which was associated with SMC migration and proliferation (Liao et al. 2010). In atherosclerotic mice, an oxysterol-rich diet containing mainly 7C derivatives and EC induces plaque instability and rupture related to increased MCP1 expression and MMP activity (Sato et al. 2012).

Macrophages exist as two main subsets, the classically activated macrophages—proinflammatory M1 phenotype and the alternatively activated macrophages—anti-inflammatory M2 phenotype. M1 macrophages are primarily found in rupture-prone atherosclerotic plaques, while alternatively activated macrophages accumulate in stable plaque. 7-KC increased the production of the proinflammatory cytokines TNF-α and IL-6 in M1 macrophages (Buttari et al. 2013).

In addition, 7-KC is able to redirect the polarization of M2 macrophages to an M1-like subset by changing the profile of surface markers and cytokines toward an anti-inflammatory phenotype, by decreasing endocyte clearance capacity and by increasing the secretion of MMP9 secretion (Buttari et al. 2013, 2014; Saha et al. 2020). By contrast, 27-OHC was reported to favor plaque stabilization by driving M2 polarization of human macrophages (Marengo et al. 2016). After entering the cells through CD receptors, these oxysterols trigger LXR activation which leads to IL10 secretion and MIF release and contributes to atherosclerotic plaque stabilization.

Other inflammatory mediators such as prostaglandins (PG) whose production is regulated by the enzyme COX2 contribute to plaque development. COX2 expression and synthesis as well as PGE2 production are increased by CT in EC (Liao et al. 2009). 27-OHC promotes upregulation of COX-2 and PGE synthase thereby inducing PGE2 synthesis in human monocytes. This regulation is associated with enhanced production of proinflammatory cytokines including IL8, IL1β, and TNFα and MMP9, which leads to plaque instability (Gargiulo et al. 2018).

11.4 Mechanisms of Oxysterol Efflux

It is well acknowledged that plasma high-density lipoprotein (HDL) levels are inversely related to the risk of atherosclerotic cardiovascular disease, related to multiple anti-atherosclerotic functions exerted by HDL such as antioxidative capacity, anti-inflammatory activity, cytoprotective activity, and protection on endothelium-dependent vasorelaxation. The best-known atheroprotective activity of HDL is its ability to export cholesterol from foamy macrophages and artery wall. Transport proteins ABCA1 and ABCG1 play a central role in cholesterol efflux: ABCA1 regulates the efflux of cholesterol from the macrophage to apoA1/pre-β HDL while ABCG1 mediates efflux to mature HDL.

We and others have shown that HDL can also promote the efflux of oxysterols that accumulate in macrophages after exposure to oxLDL (Terasaka et al. 2007; Xu et al. 2009; Iborra et al. 2011; Chen et al. 2015; Paul et al. 2019). Overall, 7-KC and 7α/β-OHC were the most efficiently released and 7-KC-induced macrophage apoptosis was reduced, which confers protective effect to HDL against pro-atherogenic oxysterols. In contrast to HDL, ABCA1-dependent apoA1 exhibits weak ability to export oxysterols including 7-KC from macrophages (Kritharides et al. 1995; Gelissen et al. 1999; Terasaka et al. 2007; Xu et al. 2009; Chen et al. 2015). Another study reported opposite results showing that apoA1 efficiently export 7-KC, 7α-OHC, 5,6α-EC, cholest4-en-3-one and 27-OHC from oxLDL-loaded macrophages while HDL only exerted a trend toward oxysterol efflux (Paul et al. 2019).

In some pathological situations such as type 2 diabetes and obesity with elevated risk of atherosclerosis, HDL undergoes modifications including oxidation, glycation, and glycoxidation, which impair ability to efflux cholesterol (Denimal 2023). In the study by Chen et al. (2018), we showed that oxidized and glycoxidized HDL as well as HDL isolated from diabetic subjects, have decreased ability to efflux oxysterols from oxidized LDL-laden macrophages compared to HDL from healthy subjects. Efflux of 7-KC was specifically decreased compared to that of cholesterol. This defect of HDL toward oxysterol efflux may potentiate the deleterious effects of oxysterols and especially 7-KC that accumulate in atheroma macrophages. Oxysterols were detected at very low levels in HDL of healthy subjects, mainly represented by 7-KC, and in lower proportions 7α/β-OHC, 25-OHC, and 27-OHC. Similar or even lower amounts were found in HDL from diabetic subjects, which could reflect their strong antioxidant capacities that would protect cholesterol from oxidation, or their weaker capacity to mobilize cellular oxysterols in particular 7-KC. Under high oxidative conditions used to generate oxidized and glycoxidized HDL, a considerable increase of 7C-derived oxysterols, in particular 7-KC, is observed (Chen et al. 2018). It was not evaluated whether the high amounts of oxysterols in these modified HDLs are responsible for their

lower ability to remove oxysterols, as it has been proposed toward cholesterol efflux (Gesquière et al. 1997).

11.5 Conclusions

The involvement of oxysterols in atherosclerotic plaque formation and instability is now well established. Among oxysterols most abundant in atheroma plaque, 7-derivative oxysterols in particular 7-KC, 5,6-EC, and CT exert proatherogenic effects through induction of proapoptotic and proinflammatory mediators. Side chain oxysterols, in particular, 25-OHC and 27-OHC display dual effects as they also exert protective effects by preventing foam cell formation, or plaque instability. It is now well demonstrated that oxidative stress is a determinant for the formation of oxysterols as well as signaling pathways evoked by deleterious oxysterols. The cellular targets of oxysterols including membrane receptors, signaling pathways, and transcription factors are also well documented. Therapeutic strategies to counteract deleterious effects of oxysterols in atherosclerosis and other diseases such as cancer and neurodegenerative diseases have started to be evaluated either based on pharmacological inhibition of oxysterol-activated signaling pathways (Lee et al. 2015; Park et al. 2016; Saha et al. 2020), oxysterol derivatives (de Medina et al. 2021) or antioxidants naturally present in nutrition oils (Nury et al. 2021; Rezig et al. 2022). Pharmacology of oxysterols is a promising route for the development of new drugs against current high-incidence diseases and thus deserves further investigations.

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Conflict of Interest The authors declare no conflict of interest.

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Implication of Oxysterols and Phytosterols in Aging and Human Diseases

12

Solenne Vigne and Caroline Pot

Abstract

Cholesterol is easily oxidized and can be transformed into numerous oxidation products, among which oxysterols. Phytosterols are plant sterols related to cholesterol. Both oxysterols and phytosterols can have an impact on human health and diseases.

Cholesterol is a member of the sterol family that plays essential roles in biological processes, including cell membrane stability and myelin formation. Cholesterol can be metabolized into several molecules including bile acids, hormones, and oxysterols. On the other hand, phytosterols are plant-derived compounds structurally related to cholesterol, which can also have an impact on human health. Here, we review the current knowledge about the role of oxysterols and phytosterols on human health and focus on the impact of their pathways on diseases of the central nervous system (CNS), autoimmune diseases, including inflammatory bowel diseases (IBD), vascular diseases, and cancer in both experimental models and human studies. We will first discuss the implications of oxysterols and then of phytosterols in different human diseases.

Keywords

Oxysterols · Phytosterols · Immune responses · Inflammation · Autoimmune diseases · Cholesterol metabolism · Cancer

Abbreviations

7α,25-OHC	7α,25-Dihydroxycholesterol
7-KC	7-Ketocholesterol
25-OHC	25-Hydroxycholesterol
Aβ	Amyloid beta
ABCG	Adenosine triphosphatase binging cassette G
ACAT2	Acyl CoA, cholesterol acyltransferase
AD	Alzheimer's disease
ALD	Adrenoleukodystrophy
AMN	Adrenomyeloneuropathy
BBB	Blood–brain barrier
CAT	Catalase
CCALD	Childhood cerebral ALD
CCR	C-C motif chemokine receptor
CD	Crohn's disease
Ch25h	Cholesterol 25-hydroxylase
CNS	Central nervous system
ConA	Concanavalin A

S. Vigne · C. Pot (✉)
Laboratories of Neuroimmunology, Service of Neurology and Neuroscience Research Center, Department of Clinical Neurosciences, Lausanne University Hospital and University of Lausanne, Epalinges, Lausanne, Switzerland
e-mail: caroline.pot-kreis@chuv.ch

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COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
CYP7B1	Cytochrome P450 family 7 subfamily B member 1
DSS	Dextran sodium sulfate
EAE	Experimental autoimmune encephalomyelitis
EBI2	Epstein-Barr virus-induced G-protein coupled receptor 2
EBV	Epstein-Barr virus
EC	Endothelial cell
EDSS	Expanded disability status scale
ER	Estrogen receptor
ERs	Estrogen receptors
FDA	Food and Drug Administration
GPR183	G-protein coupled receptor
HD	Huntington's disease
IBD	Inflammatory bowel diseases
ICAM-1	Intracellular adhesion molecule 1
ILC3	Innate lymphoid cells
iNOS	Inducible nitric oxide synthase
JAM-A	Junction adhesion molecule-A
LDL-C	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
LXRs	Liver X receptors
MCP-1	Monocyte chemotactic protein 1
MMP-9	Matrix metalloproteinase 9
MoDC	Monocytes-derived dendritic cells
MS	Multiple sclerosis
NF-κB	Nuclear factor-kappa B
NLRP3	NOD-like receptor family, pyrin domain containing 3
NO	Nitric oxide
NOX	NADPH oxygenase
NPC1L1	Niemann Pick C1 like 1 transporter
Nrf2	Nuclear factor erythroid-related factor-2
PARP-1	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PD	Parkinson's disease
PMS	Progressive multiple sclerosis
RORs	Retinoid acid receptor
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
SCFAs	Short-chain fatty acids
SERMs	Selective estrogen receptor modulator
SNP	Single nucleotide polymorphisms
SOD	Superoxide dismutase and nitric oxide synthase enzymes
SREBP1	Sterol regulatory element-binding protein-1
TH	Tyrosine hydroxylase
TLR4	Toll-like receptor 4
TNBS	Trinitrobenzene sulfonic acid
UC	Ulcerative colitis
VSMC	Vascular smooth muscle cells
ZO-1	Zonulin-1

12.1 Oxysterols: Origin and Formation of Oxysterols

Cholesterol is one of the most essential chemical substances for human life. This abundant lipid molecule has several vital functions that serve normally functioning cells. It contributes to the structural elaboration of the cellular membrane to modulate its integrity, fluidity, and biochemical functions. Furthermore, cholesterol is involved in physiological processes such as signaling, proliferation, and cell apoptosis. Due to its high body distribution and its three-dimensional structure, the cholesterol molecule is prone to oxidation processes leading to its conversion into oxysterols by the addition of a hydroxyl, carbonyl or epoxy, hydroperoxide or carboxyl group to the fused ring structure or side chain. Oxysterols arise from dietary sources, nonenzymatic autoxidation reactions (reactive oxygen species, free radicals), or through enzymatic cholesterol oxidation, mostly by mitochondrial or microsomal cytochrome P450 family enzymes (oxidoreductases, hydroxylases, and reductases). They constitute essential intermediates in the biosynthesis of various fundamental substances such as steroid hormones, vitamin D, or bile acids. Primary oxysterols are synthesized enzymatically directly from cholesterol such as 24S-, 25-, (25R)-26, 27-hydroxycholesterols, 7α-hydroxycholesterol, and 7β-hydroxycholesterol. The secondary oxysterols are derived from primary oxysterols

such as 7α,25-dihydroxycholesterol (7α,25-OHC) generated from 25-hydroxycholesterol (25-OHC). Oxysterol such as 7-ketocholesterol, epoxycholesterol, and cholestane represents the main non-enzymatic oxysterols. Due to the addition of polar groups, oxysterols are more water soluble than cholesterol, facilitating spontaneous diffusion between fat-soluble membranes. Therefore, oxysterols act as signaling lipids that regulate cholesterol biosynthesis, its cellular uptake, and efflux via effects on the major transcription factors responsible for cholesterol homeostasis. In recent years, oxysterols emerged as important players in other biological processes during immunity and inflammation and were shown to display immunomodulatory functions including immune cell trafficking, cytokine secretion promotion from different cell types in several organs as well as having antiviral properties. Oxysterols can interact either on the same receptor (agonist, antagonist effects) or interfere with multiple receptors increasing the complexity of their study (Guillemot-Legris and Muccioli 2022). The most described nuclear receptors include the liver X receptors (LXRs), the estrogen receptor (ERs), the retinoid acid receptor-related orphan receptors (RORs), and the glucocorticoid receptor. Among the membrane receptors, the G-protein coupled receptor (notably GPR 183 also known as Epstein-Barr virus-induced G-protein coupled receptor 2 EBI2) as well as Insig in the endoplasmic reticulum can also be activated by oxysterols.

Particularly, the interactions of oxysterols through LXR and EBI2 receptors were reported to be the most important in the development of immune system responses and signaling. Indeed, some oxysterols like 25-OHC and 7α,25-OHC regulate the function and phenotype of immune cells such as macrophages, lymphocytes, neutrophils, and dendritic cells (Cyster et al. 2014; Poli et al. 2022; Reinmuth et al. 2021). In correlation with its pro-inflammatory function, 25-OHC has been considered to promote the progression of chronic inflammatory and degenerative diseases, including atherosclerosis, Alzheimer's disease, and multiple sclerosis. Under typical conditions, oxysterol concentration is maintained at a very low and precisely regulated level. However, an elevated level of oxysterols is potentially cytotoxic and has been implicated in the process of inflammation by promoting cell death, proliferation, or differentiation. Thus, by their effects on several immune cells, they can contribute to the pathophysiology of various diseases with immune components (Table 12.1).

12.1.1 Neurodegenerative Diseases

In humans, the brain contains high levels of cholesterol representing approximately 20% of the whole-body cholesterol (Björkhem and Meaney 2004). Brain cholesterol is predominantly found (about 70–80%) in myelin (Dietschy and Turley 2004) and is synthesized locally by neuronal cells and astrocytes (Zhang and Liu 2015). Cholesterol metabolism is important in the central nervous system (CNS) not only during early development but also later in life. Brain cholesterol levels are tightly regulated and increasing evidence indicates that abnormal cholesterol metabolism in the brain is linked to neurological disorders. In the CNS, LXR receptors are considered central modulators of inflammation and cholesterol homeostasis (Courtney and Landreth 2016). These receptors mainly act as oxysterol sensors and are activated by the reactions of several oxysterols like 24-OHC. Oxysterols accumulate in the brain certainly playing a crucial role by enhancing oxidative stress and inflammation which represent typical features in various neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), and leukodystrophies. Neurodegenerative disorders can be divided into demyelinating diseases, such as multiple sclerosis or peroxisomal leukodystrophies (X-ALD) or non-demyelinating neurodegenerative disorders including Alzheimer's, Parkinson's, or Huntington's diseases. We will thus continue our discussion based on those two disease categories.

Table 12.1 Implication of oxysterols in aging and human diseases

Diseases	Experimental systems	Enzymes/ oxysterols	Functions	References
Multiple sclerosis	CSF MS patients, EAE	7-KC	Activation and migration of microglial cells induce neuronal damages	Diestel et al. (2003); Leoni et al. (2002, 2004); van de Kraats et al. (2014); Novakova et al. (2015); Karrenbauer et al. (2006); Teunissen et al. (2003); Mukhopadhyay et al. (2017)
	Plasma, CSF MS patients	24-OHC 27-OHC	Increased in early stage of MS, correlation with neurological symptoms and brain atrophy in RRMS, reduced in natalizumab treated patients in RRMS. Decreased in older progressive MS	
	EAE	CH25H 25OHC 7α,25OHC	Promote immune cell trafficking to the CNS, enhance pro-inflammatory response and neuroinflammation, reduce immunosuppressive neutrophils, BBB dysfunction	Chalmin et al. (2015); Wanke et al. (2017); Clottu et al. (2017); Ruiz et al. (2023); Munji et al. (2019)
Leukodystrophies	Plasmas X-ALD patients	7-KC	Increased in plasma, promotes oxidative stress and peroxisomal dysfunction in microglial cells and proliferation	Nury et al. (2020)
	Fibroblasts and oligodendrocytes of AMN and CCALD patients	CH25H 25-OHC	Increase cerebral inflammation via the activation of the NLRP3 inflammasome pathway	Jang et al. (2016)
Alzheimer's disease	Plasma of AD patients	CYP46A1	Associated with neuronal loss and brain atrophy, potential biomarker	Gamba et al. (2021); Staurenghi et al. (2021); Björkhem et al. (1998); Lütjohann et al. (1996)
	Human neuroblastoma	24-OHC	Induce pro-inflammatory responses, synaptotoxic effects. Prevent Tau hyperphosphorylation and Aβ production	
	Mouse primary neurons	27-OHC	Associated with memory deficits, reduces dendritic spine density, and synaptic plasticity	Testa et al. (2016); Gilardi et al. (2009); Dai et al. (2021); Merino-Serrais et al. (2019)
	Human AD and mouse model	CH25H 25-OHC	Upregulated, increases pro-inflammatory mediators during AD	Wong et al. (2020); Guven et al. (2019)
Huntington's disease	HD cell lines, Human plasma, and Brain mouse model	CYP46A1 24-OHC	Decreased in human brain and plasma at early stage of the disease. Improve neuronal atrophy and decrease Huntingtin protein	Kacher et al. (2019, 2022); Kreilaus et al. (2016); Boussicault et al. (2016)

(continued)

Table 12.1 (continued)

Diseases	Experimental systems	Enzymes/ oxysterols	Functions	References
Parkinson's disease	CSF of PD patients Human neuroblastoma	24-OHC	Levels in CSF correlate with duration of the disease and with protein Tau expression. Increases tyrosine hydroxylase expression, reduce α-synuclein accumulation	Björkhem et al. (2018); Doria et al. (2016); Rantham Prabhakara et al. (2008); Raju et al. (2018)
	CSF of PD patients Human neuroblastoma	27-OHC	Levels increased in the CSF. Upregulation of α-synuclein expression via LXR, increases levels of α-synuclein and induces apoptosis, downregulation of tyrosine hydroxylase	
Intestinal diseases	Primary human cells, mouse models	7-KC 27-OH	Decrease the integrity of endothelium and intestinal epithelial barriers, alter intestinal immunity	Deiana et al. (2017); Chalubinski et al. (2013); Wang et al. (2020a)
	Fibrotic tissue of CD patients	7α/β-OHC CH25H	Upregulated and positively correlated with various fibrosis mediators	Raselli et al. (2019)
	Mouse models	25-OHC 7α,25OHC	Negatively regulate humoral response against enteric infection. Positioning of immune cells and regulation of intestinal inflammation	Ceglia et al. (2023); Piper and Mauri (2021); Trindade et al. (2021); Emgård et al. (2018)
Breast cancer	Human tissues, Mouse models, Carcinoma cell line	27-OHC	Elevated concentration in plasma and breast cancer tissues. Correlated with tumor size. Promotes the oncogenic estrogen-dependent signaling, enhances cell proliferation, promotes metastasis, interferes with hormonal therapies	Baek et al. (2017); Torres et al. (2011); Zhen et al. (2021); Nelson (2018); He and Nelson (2017); Dalenc et al. (2017); Kimbung et al. (2017); Wu et al. (2013); Kloudova et al. (2017); Soucek et al. (2018)
Colon cancer	Human plasma and tissues, Carcinoma cell line	7β-OHC 25-OHC, 27-OHC	Induce expression of inflammatory and chemotactic cytokines, associated with poor outcome of colorectal cancer	Mascia et al. (2010); Bai et al. (2005); Swan et al. (2016); Biasi et al. (2012); Rossin et al. (2019); Guo et al. (2018)
Lung cancer	Human plasma, Carcinoma cell line	7β-OHC	Positive association with lung cancer risk, induces apoptosis	Linseisen et al. (2002); Kang et al. (2005); Melloni et al. (2018)
Cardiovascular diseases	Human and mouse endothelial cells, macrophages	25-OHC	Promote the expression of inflammatory genes, increase apoptosis, and reduce efferocytotic capacity	Canfrán-Duque et al. (2023)
		7β-OHC 7α-OHC 7-K 27-OHC	Present in atherosclerotic plaques of hypercholesterolemic patients. Endothelial dysfunction, cell phenotype changes, vascular calcification, oxidative stress, apoptosis, inflammation	Leonarduzzi et al. (2005); Shentu et al. (2012); Ou et al. (2016); Phillips et al. (2001); Liao et al. (2010); Gargiulo et al. (2016)

12.1.1.1 Demyelinating Diseases

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating and neurodegenerative disease of the CNS. The development of MS is under the control of both genetic and environmental factors, among which viral infections, in particular, exposure to Epstein-Barr virus (EBV) and adolescent obesity could be linked to the development of multiple sclerosis (Bjornevik et al. 2022; Bjornevik et al. 2023; Chitnis and Weiner 2022; Kuchroo and Weiner 2022). Most brain cholesterol is contained within myelin, and it was suggested that accumulated levels of circulating or cerebrospinal fluid (CSF) cholesterol metabolites such as oxysterols could reflect dysregulation in brain cholesterol turnover caused by demyelination and BBB integrity. It was further proposed that oxysterols play a key role in the neuroinflammatory processes mediating MS disease progression (Zhornitsky et al. 2016). We previously reviewed the role of oxysterols in various autoimmune diseases (Duc et al. 2019b).

In humans, persons with MS show altered levels of oxysterol in plasma and cerebrospinal fluid compared to healthy controls, and differential patterns of oxysterol expression are described depending on the stage of the disease (Table 12.1). However, the conclusions of differently conducted studies remain controversial in some parts. During MS, myelin sheaths are damaged by immune cells in multiple areas of the brain and spinal cord. In 2003, Diestel et al. proposed that the myelin breakdown product 7-ketocholesterol production was found in the CSF of MS patients and could promote neuronal damage by activation of poly (ADP-ribose)-polymerase (PARP)-1 and regulation of the expression of integrins such as CD11a and intracellular adhesion molecule 1 (ICAM-1), leading to activation and migration of microglial cells in the brain (Diestel et al. 2003). In this line, it was shown that 7-ketocholesterol levels are higher in persons with progressive multiple sclerosis (PMS) compared to the relapsing-remitting form (RRMS) (Fellows Maxwell et al. 2019). 24-OHC is also transitory increased in the plasma and CSF from patients in early stages of the disease whereas, during the latter stages, the levels are reduced because of neuronal cell destruction. Moreover, a similar correlation has been described between the severity of the neurological symptoms and the circulating levels of 24-OHC in a study of 118 patients (among which 65% RRMS) (Leoni et al. 2002). In another study of 88 RRMS patients, both 24- and 27-OHC were found elevated in the CSF of patients with contrast-enhancing lesions compared to healthy controls or patients without contrast-enhancing lesions (Leoni et al. 2004; Guven et al. 2019). In addition, serum level of 24-OHC was positively correlated with brain atrophy during RRMS (van de Kraats et al. 2014). Moreover, in natalizumab-treated persons with RRMS, a reduction of 24- and 27-OHC was observed in the CSF, possibly related to a reduction of neurodegeneration and improvement of BBB permeability (Novakova et al. 2015). However, age and the expanded disability status scale (EDSS) disease scores were negatively correlated to circulating 24-OHC possibly by loss of neuronal cells able to produce 24-OHC in older persons with PMS (Karrenbauer et al. 2006). More recently, translational and longitudinal studies reported that the plasma levels of different oxysterols as 24-OHC, 25-OHC, 27-OHC, 7αHC, and 7KC differed significantly between MS and HC and between RRMS and PMS. They observed a significant decrease of 24-, 27-, and 7a-OHC in RRMS compared to healthy controls and a significant increase of 7KC in PMS compared to RRMS reflecting the important oxidative stress found in PMS (Fellows Maxwell et al. 2019; Mukhopadhyay et al. 2017; Teunissen et al. 2003).

In addition to the potential use of oxysterols as biomarkers, oxysterols have also been shown to impact immune responses, in particular, oxysterols downstream the cholesterol 25-hydroxylase (Ch25h), more specifically 25-OHC and 7α-25-OHC (Table 12.1). Immune cells such as macrophages (Park and Scott 2010), monocytes-derived dendritic cells (MoDC)

(Chalmin et al. 2015), and endothelial cells (Ruiz et al. 2023) are rich sources of Ch25h under inflammatory conditions. 25-OHC and 7α,25-OHC are also broadly implicated in immune responses: 25-OHC suppresses IgA production by B cells and has broad antiviral properties. It can also impair suppressive cell functions, in particular, Type-1 regulatory T cells (Tr1 cells) (Vigne et al. 2017) and myeloid-derived suppressor cells (MDSC) (Ruiz et al. 2023). Furthermore,7α,25-OHC guides B cells, dendritic cells, and macrophages within the germinal follicles of the spleen and lymph nodes during an immune response (Sun and Liu 2015). Indeed, Ch25h-derived oxysterols control the immune response by promoting encephalitogenic T cell trafficking to the CNS using a mouse model of MS, the experimental autoimmune encephalomyelitis (EAE) and thus enhancing a pro-inflammatory response and neuroinflammation. Mice deficient for Ch25h display an attenuated EAE disease course compared with control mice (Chalmin et al. 2015). In line with this study, we and others proposed that elevated expression of 7α,25-OHC and pro-inflammatory cytokines were associated with an increased migration profile of encephalitogenic Thelper 17 (Th17) lymphocytes. Moreover, animal and human Th17 cells express high levels of EBI2 compared to other T cell subsets (Chalmin et al. 2015; Clottu et al. 2017; Wanke et al. 2017). This suggests an important role for oxysterol/EBI2 on T lymphocyte migration not only in murine models but also in human MS disease. Interestingly increased EBI2 expression was observed during the Epstein-Barr virus infection which is itself associated with an increased risk of developing MS (Bjornevik et al. 2022). Genetic analysis of persons with MS revealed a potential association between variants of the Ch25h gene and primary progressive multiple sclerosis (PPMS) (Forwell et al. 2016). More recently we showed that Ch25h expression is increased in CNS endothelial cells during EAE and that 25-OHC is able to further reduce the expansion of immunosuppressive neutrophils (Ruiz et al. 2023). In line with these results, Ch25h gene expression was shown to be upregulated in endothelial cells in various murine models associated with BBB dysfunction (Munji et al. 2019). These promising studies suggest that oxysterols play a detrimental role during autoimmunity. The discovery of oxysterols as mediators of immune cell trafficking during MS may lead to new targets to harness encephalitogenic immune cells in autoimmune diseases.

Leukodystrophies

Adrenoleukodystrophy (ALD), a genetic disorder that follows X-linked inheritance pattern in most cases (X-ALD), is caused by an *ABCD1* mutation. It is a progressive neurodegenerative disorder associated with the accumulation of very long-chain fatty acids (VLCFA). The main manifestation of X-ALD is milder axonopathy of the spinal cord and severe cerebral inflammatory demyelination (Berger et al. 2014). The most severe phenotype of X-ALD is childhood cerebral ALD (CCALD), which is accompanied by acute inflammatory demyelination of the CNS that leads to a vegetative state or death within 3–5 years of onset (Moser et al. 2007). On the other hand, adrenomyeloneuropathy (AMN), the most prevalent ALD phenotype, manifests as a slowly progressive myelopathy in adulthood (Kemp et al. 2016). However, the pathogenesis mechanism of ALD remains obscure, it is believed that oxidation damage and inflammation are the main pathogenesis of neuronopathy found in patients with X-ALD. In the progression of X-ALD, oxidative stress could play, directly and/or indirectly, an important role in the development of the disease. Lipid peroxidation, especially oxysterol, mainly accumulates in the inflammatory demyelinating lesions and adrenal cortex, which are the major affected areas in X-ALD. 7-Ketocholesterol is increased in the plasma of patients with X-ALD. It promotes oxidative stress and peroxisomal dysfunction in microglial cells leading to microglial cell activation and proliferation which could contribute to demyelination and neurodegeneration (Nury et al. 2020). In another study reported in 2016, the oxysterol 25-OHC was identified as a potent mediator in the

pathogenesis of X-ALD by contributing to cerebral inflammation via the activation of the NLRP3 inflammasome pathway. The NLRP3 inflammasome was shown to play a crucial role in demyelination and oligodendrocyte loss in a mouse model which argues in favor of a significant role for NLRP3 inflammasome in cerebral neuroinflammation (Jha et al. 2010). Using a microarray analysis, the authors showed that Ch25h expression was increased in CCALD patients-derived cells, in ex vivo primary fibroblasts and oligodendrocytes of AMN and CCALD patients compared to healthy controls. Mechanistically they have shown that 25-OHC promotes NLRP3 inflammasome assembly and activation by stimulating mitochondrial ROS that leads to the recruitment of microglia, IL-1β release, and oligodendrocytes apoptosis, and consequently severe neuroinflammation and demyelination (Jang et al. 2016). Lipid peroxidation, especially oxysterol, mainly accumulates in the inflammatory demyelinating lesions and adrenal cortex, major affected areas in X-ALD. Thus, cholesterol metabolism is yet considered to be associated with X-ALD pathogenesis.

12.1.1.2 Non-demyelinating Diseases

Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide. It represents 70% of total dementia cases and is clinically expressed by a progressive loss of cognitive abilities and functional independence. AD is associated with the involvement of many factors, including inflammation and impaired cholesterol metabolism. Microglia, astrocytes, and neurons play a role in neuroinflammation during AD and are found to be activated in brain regions surrounding amyloid plaques. It was proposed that alterations in oxysterol synthesis in the brain correlate with AD progression. Cholesterol conversion into oxysterols is the major way to eliminate cholesterol accumulation in the brain. These cholesterol metabolites can cross the BBB from both directions and represent the principal cholesterol transporters between the brain and the peripheral circulation (Gosselet et al. 2014). During AD, increased BBB permeability has been suggested to increase the release of oxysterols into the circulation (Dias et al. 2014). In particular, the major oxysterol studied in the brain is 24-OHC (Björkhem et al. 1998; Lütjohann et al. 1996). 24-OHC is produced by the enzyme CYP46A1 which is responsible for the major brain cholesterol conversion both in neurons and in some astrocytes (Bogdanovic et al. 2001; Brown et al. 2004). The plasma levels of 24-OHC are associated with neuronal loss and brain atrophy. While the role of 24-OHC during AD pathogenesis and its usefulness as a potential biomarker were reported, the literature remains controversial. Between the years 2000 and 2020, numerous human studies investigated the correlation between circulating level of 24-OHC and AD risk (Gamba et al. 2021). Apart from their role as biomarkers, oxysterols contribute to various other pathological mechanisms involved in AD pathogenesis. 24-OHC can elicit a strong pro-inflammatory response in human neuroblastoma by inducing the expression of pro-inflammatory mediators, including the chemokines interleukin 8 (IL-8) and monocyte chemotactic protein 1 (MCP-1), the adhesion molecule β1-integrin, the scavenger receptor CD36 and the matrix metalloproteinase 9 (MMP-9) (Testa et al. 2016). Moreover, oxysterol-treated astrocytes upregulate reactive astrocyte markers, release pro-inflammatory molecules, and have a synaptotoxic effect on mouse primary neurons (Staurenghi et al. 2021). However, while numerous studies highlighted the potential role of 24-OHC in promoting AD development, 24-OHC could also exert beneficial effects against AD progression, such as preventing tau hyperphosphorylation and Aβ production (Gamba et al. 2021). Another enzymatically produced oxysterol, 27-hydroxycholesterol (27-OHC), mainly produced by glial cells (Gilardi et al. 2009) and various oxysterols deriving from cholesterol non-enzymatic oxidation were also related to AD. High levels of 27-OH have been associated with memory deficits both in AD and other neurodegenerative processes

through a receptor LXR-dependent mechanism (Dai et al. 2021). It has been demonstrated that 27-OHC reduces dendritic spine density and synaptic maintenance and plasticity in primary mouse hippocampal neurons (Merino-Serrais et al. 2019). Furthermore, it was suggested that 25-OHC may function as a microglial-secreted inflammatory mediator in the brain. Indeed, Ch25h expression was upregulated in human AD brain tissue and in transgenic mouse brain tissue-bearing amyloid β plaques or tau pathology. Treatment with the toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) markedly upregulates Ch25h and 25-OHC expression leading to the secretion of IL1-β in mouse primary microglia (Wong et al. 2020). Interestingly, Ch25h polymorphism was associated with an increased AD risk in the Turkish population suggesting a role for oxysterols downstream this pathway in the pathogenesis of AD independently from APOE (Guven et al. 2019). More recently, it was demonstrated that oxysterols could also promote astrocyte reactivity in mice, resulting in the release of several mediators including inflammatory cytokines, adhesion, or chemotactic molecules that affect both neuronal health and synapses (Staurenghi et al. 2021). The effects of oxysterols are still controversial but growing evidence suggests that some of them may play a role in AD pathogenesis (Zarrouk et al. 2018) by inducing oxidative stress, inflammation (Testa et al. 2018), Aβ formation and accumulation (Zhang et al. 2019), protein Tau hyperphosphorylation (Marwarha et al. 2010), synaptic dysfunction (Merino-Serrais et al. 2019), and cell death (Jang and Lee 2011).

Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by an elongated polyglutamine repeat in the huntingtin protein and characterized by behavioral abnormalities, cognitive decline, and involuntary movements leading to a progressive decline in function. Studies were mostly performed using HD cell lines and mouse models of HD and showed dysregulation of cholesterol metabolism and reduced levels of oxysterols and more particularly a significant reduction of CYP46A1 and 24-OHC both in the brain and in the plasma of multiple rodent models (Kacher et al. 2022). Interestingly, CYP46A1 expression and 24-OHC concentrations are also decreased in post-mortem brain tissues and in patient plasma at early stages of the disease. 24-OHC concentration correlates with the severity of disease and the degree of brain atrophy (Kreilaus et al. 2016). In mice, CYP46A1 deletion in the striatum reproduces the HC phenotype, with spontaneous striatal neuron degeneration and motor deficits (Boussicault et al. 2016). More recently, gene therapy delivery of CYP46A1 by stereotaxis in the striatum of mice allowed neuroprotection by different cellular and molecular events (Boussicault et al. 2016; Kacher et al. 2019). These studies provide evidence that gene therapy approaches targeting 24-OHC and CYP46A1 are encouraging and could represent a promising therapeutic strategy for the treatment of HD.

Parkinson's Disease

Parkinson's disease (PD) is a progressive, neurodegenerative disorder of aging that affects both motor and cognitive function characterized by the loss of dopaminergic neurons in the substantia nigra and the accumulation of α-synuclein, a protein that aggregates in Lewy bodies. Cholesterol and its associated derivatives are implicated in the pathophysiology of PD consisting of mitochondrial dysfunction, interaction with α-syn and lipid metabolism pathways. Oxysterols particularly 24-OHC and 27-OHC were reported to be associated with PD. Oxysterols could play a relevant role in the aging process of neurodegenerative disease; however, their roles remain controversial. The level of 24-OHC was increased in the CSF, whereas markedly reduced in the circulation of PD patients probably due to a release from dying neuronal cells. Interestingly, a significant correlation was found between 24-OHC and microtubule-associated protein Tau in CSF from the PD patients (Björkhem et al. 2018). 24-OHC was described to activate tyrosine hydroxylase (TH) the rate-limiting dopamine

synthesis enzyme leading to an increase in dopamine synthesis. Using human neuroblastoma, 24-OHC reduces α-synuclein accumulation thereby preventing the formation of Lewy bodies. On the other hand, 27-OHC reduces the expression of tyrosine hydroxylase (TH) and increases α-synuclein levels by increasing oxidative stress and ER stress. This process could lead to the alteration of lipid rafts through sterol regulatory element-binding protein-1 (SREBP1) inducing apoptosis through the estrogen receptors and LXR receptors leading to the inhibition of post-synaptic signaling and thus to neurodegeneration (Doria et al. 2016; Rantham Prabhakara et al. 2008). Furthermore, increased cholesterol level in the neuroblastoma cell line causes depolarization of mitochondrial membrane potential and reduces dopaminergic neurons (Raju et al. 2018). In PD, the role of cholesterol and its metabolites is debatable, but the above evidence suggests that oxysterols could play an important role as a new target for PD. In addition to neurological diseases, oxysterols have also been implicated in the pathogenesis of other diseases, including intestinal diseases that we will now here discuss.

12.1.2 Intestinal Diseases

Several examples of evidence showing a relation between oxysterols and inflammatory bowel disorders (IBD) have appeared throughout the last years (Table 12.1). We and others largely reviewed the implication of oxysterols in two IBDs, ulcerative colitis (UC) and Crohn's disease (CD) (Duc et al. 2019b; Misselwitz et al. 2021; Willinger 2019). The gut, where the oxysterols originated from diet, mainly from cholesterol-rich food, represents the primary site of exposure to oxysterols. These metabolites can interact with the human digestive tract homeostasis, by causing intestinal mucosal inflammation. In 2017, Rossin et al. reviewed the implication of oxidized lipids derived from a rich cholesterol diet in the development of intestinal inflammation and colon cancer progression (Rossin et al. 2017). A cholesterol-rich diet can stimulate the production of type I interferon by enterocytes, which results in increased Ch25h expression and consequently 25-OHC production (Mukherjee et al. 2017). Cholesterol oxidation products also affect human colonic epithelial cells and thus have a potential implication in the inflammatory bowel disease progression (Biasi et al. 2009). Dietary auto-oxidation oxysterols induce the loss of intestinal epithelial layer integrity in CaCo-2 cells, with subsequent hyperactivation of pro-inflammatory cytokines matrix metalloproteinases (MMP)-2 and -9 and decreased levels of tight junction proteins including ZO-1, occludin, and junction adhesion molecule-A (JAM-A) (Deiana et al. 2017). Other oxysterols such as 7-ketocholesterol and 25-hydroxycholesterol were reported to decrease the integrity of endothelium and intestinal epithelial barriers in primary human cell culture and to alter intestinal immunity using animal models (Chalubinski et al. 2013). In addition, in vivo evidence showed that 27-OHC could also induce downregulation of tight junction proteins and anti-inflammatory cytokine IL-10; upregulation of pro-inflammatory IL-1β; as well as modifications in the intestinal tight junction ultrastructure (Wang et al. 2020a, b). The oxysterols 7α-OHC and 7β-OHC were also described to negatively affect intestinal barrier function and induce pro-inflammatory cytokine production (Rossin et al. 2017).

In line with these observations, ch25h gene expression was upregulated in human intestinal fibrotic tissue of CD patients compared with healthy controls and the expression of this oxysterol synthesizing enzyme was positively correlated with various fibrosis mediators (Raselli et al. 2019). In contrast, it was suggested that LXR ligation by oxysterols may protect from colitis by influencing the Treg/Th17 balance during intestinal inflammation. Interestingly, LXR-deficient mice are more susceptible to chemically induced colitis (Jakobsson et al. 2014). This study also reported lower LXR expression in the inflamed colon of IBD patients.

Oxysterol also impacts IgA production in the intestine. Indeed, 25-OHC suppresses IgA

production by inhibiting class switching in vitro. Accordingly, Ch25h-deficient mice have lower amounts of IgA. In contrast, IgA is moderately increased in mice lacking CYP7B1, suggesting that 7α,25-OHC-mediated B cell positioning may stimulate IgA generation (Bauman et al. 2009). Interestingly, enlarged isolated lymphoid structures consisting of B cells form were observed in the small intestine of Ch25h-deficient mice. In addition, experimental studies reported that oxysterols such as 7α,25-OHC and 25-OHC produced from dietary cholesterol in intestinal epithelial cells are able to negatively regulate humoral response against enteric infection (Ceglia et al. 2023; Piper and Mauri 2021; Trindade et al. 2021). In 2012, following the identification of the oxysterol receptor EBI2 (GPR183) as an IBD risk gene (Jostins et al. 2012), experimental observations indicated an important role of EBI2/oxysterol axis in promoting inflammatory cell recruitment during the pathogenesis of colitis (Emgård et al. 2018). EBI2 receptor and its ligand 7α,25-OHC were shown to regulate the positioning of immune cells including innate lymphoid cells which are critical for the formation of lymphoid tissue in the colon and regulation of intestinal inflammation (Misselwitz et al. 2021). It was shown that EBI2 is highly expressed by innate lymphoid cells (ILC3s) and that ILC3s migrate toward 7α,25-OHC (Emgård et al. 2018). Furthermore, EBI2 and its ligand 7α,25-OHC promote ILC3 migration to cryptopatches and isolated lymphoid follicles in the colon and the small intestine. Authors also showed that 7α,25-OHC is produced by fibroblastic stromal cells in the gut creating an oxysterol gradient that attracts ILCs expressing EBI2 to the sites where lymphoid tissues are formed in the colon. Experiments in EBI2-deficient mice demonstrate that these animals were less susceptible to colitis in an innate model of intestinal inflammation. Moreover, it was observed that there was an increased production of 7α,25-OHC, the ligand of EBI2 during colonic inflammation and a significant correlation between oxysterol-producing enzyme expression and colonic inflammation in biopsies of patients with ulcerative colitis (Wyss et al. 2019; Guillemot-Legris and Muccioli 2022). Interestingly, in pediatric patients with ulcerative colitis, *CH25H* and *CYP7B1* expression correlates with the degree of local inflammation, suggesting a role for oxysterols and their receptor GPR183 in human IBD. For instance, genetic, translational, and experimental studies implicate the oxysterol 7α,25-OHC and its receptor EBI2 pathway as an important step in IBD pathogenesis. Notably, higher GPR183 expression is observed on CCR6 and CCR9 expressing lymphocytes as well as on Th17 cells compared to total CD4 memory T lymphocytes and GPR183 surface staining is higher on pro-inflammatory Th17 cells, associated with IBD (Ruiz et al. 2021). In addition, more than 240 single nucleotide polymorphisms (SNP) have been associated with susceptibility to IBD in genome-wide association studies (Jostins et al. 2012; Liu et al. 2015). One of the associated SNPs, rs9557195, is located within the gene for GPR183 (Jostins et al. 2012) and patients carrying this SNP display an increased incidence of the extra-intestinal manifestation of IBD (Ruiz et al. 2021). Interestingly, GPR183 is also expressed on IL-17-producing γδ T (Tγδ17) cells that express CCR6 and GPR183. Severity of skin inflammatory responses in humans is linked to a diet with high-fat content and the IL-17-producing γδ T cells—contributes to psoriasis induction. Mice fed high-cholesterol food (HCF) experience more severe psoriasis, in an GPR183 expressing Tγδ17 cell-dependent manner. Hence, GRP183-oxysterol pathway emerges as the candidate mechanism underpinning high-fat diet-induced aberrant tissue inflammation in humans (Frascoli et al. 2023). Thus, influencing oxysterol synthesis and activity in the intestine and the skin might represent a promising therapeutic target for IBD and their extra-intestinal manifestations.

12.1.3 Oxysterols in Cancer

The hypothesis that certain oxysterols might favor tumor growth and progression was put forward just a few years ago. Many cancers display altered cholesterol metabolism, and recent studies

demonstrate that manipulating systemic cholesterol metabolism may be useful in improving immunotherapy responses. Oxysterols are implicated in various cellular processes and display their metabolic and transcriptional activities mainly through their nuclear LXR receptors. LXRs modulate the cell cycle in a large range of cancer cell lines and are involved in the proliferation/apoptosis balance regulation in various types of cancers (Wang et al. 2021). Accordingly, an increase in their concentration has been often associated with several cancers such as breast, colon, or lung discussed below. Moreover, LXRs also inhibit inflammatory signaling, but on the other hand, tumor-derived oxysterols are potent in recruitment of pro-tumor immune cells and inhibition of anti-tumor immune response and thus exert other pro-cancerous actions, both by LXR-dependent and independent mechanisms. Thus, in literature, the role of oxysterols in carcinogenesis remains controversial (Kloudova et al. 2017; Kloudova-Spalenkova et al. 2021). Among studies, 27-OHC is the most studied oxysterol in cancer (Biasi et al. 2022; González-Ortiz et al. 2021; Nelson 2018). Some studies described its adverse effect by its ability to bind and modulate estrogen receptors, promoting the oncogenic estrogen-dependent signaling, and contributing to breast carcinoma cell line proliferation (Nazih and Bard 2020). Oxysterols 25- and 27-OHC enhance cell proliferation in breast cancer cell lines via the modulation of ERα, suggesting a potential role in resistance to hormonal therapy (He and Nelson 2017; Lappano et al. 2011; Ma et al. 2022). In addition, 27-OHC was designed as a selective estrogen receptor modulator (SERM), in macrophages that infiltrate the tumor environment in breast cancer patients. In vitro and in vivo experimentation suggests that 27-OHC treatment promotes metastasis phenomena due to LXR agonist function, inducing epithelial–mesenchymal transition leading detrimental role in cancer progression (Baek et al. 2017; Torres et al. 2011; Zhen et al. 2021). Elevated 27-OHC concentrations have been reported in plasma and breast cancer tissues on in vivo models (Dalenc et al. 2017; Kimbung et al. 2017; Nelson 2018; Wu et al. 2013). However, not all oxysterols act in the same manner as 27-OHC. Indeed, 22(R)-OHC and 24-OHC suppressed proliferation and induced apoptosis in a breast cancer model cell line (ER+) (Nazih and Bard 2020). In a recent report, serum oxysterols were determined in 58 patients with primary breast carcinoma in different tumor stages before treatment, reporting that the level of oxysterol was correlated with tumor size (Kloudova-Spalenkova et al. 2020). However, the level of 27-OHC was inversely associated with breast cancer risk in a cohort of postmenopausal women possibly explained by the dual effect of oxysterol in combination with estradiol (Lu et al. 2019). It was suggested that oxysterol may also interfere with hormonal therapy and modulate the efficacy of other kinds of therapies like chemotherapy, immunotherapy but also radiotherapy in breast cancer (Kloudova et al. 2017; Kloudova-Spalenkova et al. 2020). In a prospective study, evaluation of oxysterols profile in 29 patients before and after treatment with tamoxifen and aromatase inhibitors showed increased concentrations of 27-OHC after treatment by aromatase inhibitors (Dalenc et al. 2017). In another analysis, samples from 24 patients diagnosed with primary breast carcinoma before and after surgical tumor removal and initiation of therapy were characterized, 27-OHC serum levels were lower after surgery (Kloudova et al. 2017; Soucek et al. 2018).

As described above, dietary oxysterols are initially absorbed in the intestine and could therefore influence carcinogenesis in the gastrointestinal tract. In vitro and in vivo studies show that oxysterols have both antiproliferative and pro-apoptotic effects in human colonic cancer cell lines and colorectal tumors in mouse models (Kloudova et al. 2017). Oxysterols could delay colon cancer growth by blocking cell cycle progression and inducing apoptosis via caspase activation (Rossin et al. 2017). In contrast, studies reported that oxysterol creates an immunosuppressive tissue microenvironment favoring tumor growth and immune escape process. Different human and murine tumor cells can release oxysterols that inhibit chemokine receptor expression on immune cells and thus their

migration into lymphoid organs allowing tumors to escape from immune surveillance (Raccosta et al. 2016). Moreover, oxysterols accumulate in the microenvironment of different tumor grafts in mice where they play a key role in the recruitment of tumorigenic cells (Raccosta et al. 2013). They also affect the expression of inflammatory molecules in colon cancer cells. 7β-OHC induced expression of key inflammatory and chemotactic cytokines in human enterocyte like cells (Mascia et al. 2010). 25-OHC pre-treatment enhanced IL1-β-induced IL-8 production in human colon carcinoma cell lines (Bai et al. 2005). A mixture of oxysterols was shown to increase oxidative stress in cells and the production of cytokines IL-6 and IL-8 (Serra et al. 2018). Interestingly in patients with colorectal cancer, serum level of IL-8 was increasing with the progression of cancer (Biasi et al. 2012). Increased 27OHC was associated with poor outcomes in patients with colorectal carcinoma (Swan et al. 2016). Analysis of tumors from 26 colorectal cancer patients revealed increased levels of 27-OHC in carcinomas at stage III compared to other stages with no differences in healthy tissues (Rossin et al. 2019). The same observation was reported in tissue samples of gastric carcinoma (Guo et al. 2018).

In lung cancer, a human study conducted in 2002 found a positive association between 7β-OHC level and lung cancer risk (Linseisen et al. 2002). In vitro, 7β-OHC was described to induce apoptosis via activation of the intrinsic apoptotic pathway in lung cancer cell line (Kang et al. 2005). Interestingly, in a survival analysis of 5 years, it was reported that the expression of LXR-alpha receptor was a significant prognostic factor of better survival in completely resected Stages II and III lung cancer (Melloni et al. 2018). For instance, other types of cancers was identified to have a potential link to oxysterol as pancreas (Di Gangi et al. 2016; Wang et al. 2017), bladder (Wang et al. 2020b), or glioblastoma (Liu et al. 2019), however, further clinical investigations are necessary to elucidate their precise role in the initiation and progression of carcinogenesis and their potential clinical utility in diagnosis, prevention or therapy.

12.1.4 Oxysterols in Cardiovascular Diseases

12.1.4.1 Atherosclerosis and Vascular Aging

Hypercholesterolemia is a lipid disorder in which total cholesterol and LDL-C are present in elevated concentrations in the blood and represent an important risk factor for atherosclerosis, cardiovascular disease, including cerebrovascular disease, coronary heart disease, and peripheral arterial disease. Increased plasma LDL-C levels were positively correlated with the incidence of atherosclerosis and cardiovascular disease (CVD) (Guijarro and Cosín-Sales 2021; Guillemot-Legris and Muccioli 2022). It is currently well described that preventive measures could reduce the risk of CVD, such as the use of statin treatment but also diet and lifestyle factors. It was suggested that during endothelial dysfunctions, oxysterols were formed by LDL oxidation in the subendothelial space, and significantly contribute to various steps of vascular mechanisms leading to atherosclerosis (Gargiulo et al. 2016; Zmysłowski and Szterk 2017). Interestingly, a large amount of oxysterols has been detected in atherosclerotic plaques of hypercholesterolemic patients (Gargiulo et al. 2016). Due to their evident pro-oxidant, pro-inflammatory, and pro-apoptotic properties, oxysterols represent relevant components in the pathogenesis of vascular aging. They appear able to trigger and sustain an inflammatory process within the vasculature. In the vascular wall, oxysterols contribute to inflammatory process by upregulating adhesion molecules on the surface of endothelial cells and increasing the recruitment of monocytes from the bloodstream and the formation of foam cells which are the first players of atherogenesis (Leonarduzzi et al. 2005). Oxysterols participate in vascular oxidative stress by upregulating the multi-subunit NADPH oxygenase (NOXs) enzymes leading to the alteration of reactive oxygen species (ROS) production (Ou et al. 2016). By inserting into endothelial cell (EC) membranes, it was reported that oxysterols could alter endothelial permeability and modulate

their fluidity and structure (Shentu et al. 2012). Furthermore, oxysterols were described to promote vascular smooth muscle cells (VSMCs) proliferation and migration, thus contributing to collagen deposition and vascular calcification, which leads to arterial stiffness (Phillips et al. 2001; Liao et al. 2010). In a recent study, 25-OHC has been shown to accumulate in human coronary atherosclerotic lesions. Interestingly, Ch25h was highly expressed in both human and mouse inflammatory plaque macrophages. Macrophage-derived 25-OHC was reported to promote the expression of inflammatory genes, increase apoptosis, and reduce efferocytotic capacity (Canfrán-Duque et al. 2023). Due to its contribution during the progression of atherosclerosis, future studies further exploring its role as a biomarker of inflammation and plaque stability in human atherosclerosis will represent a promising issue.

12.1.4.2 Stroke

Atherosclerosis is the main cause of stroke, a devastating disease and the second leading cause of death worldwide associated with a high rate of morbidity, mortality, and disability. Blood–Brain Barrier (BBB) disruption is an important parameter of acute ischemic stroke, leading to brain edema and hemorrhagic transformation. After BBB leakage, blood-borne immune cells (neutrophils, monocytes, and T lymphocytes) infiltrate the central nervous system (CNS) and further increase BBB permeability. In contrast, immune cells can also enhance BBB repair and angiogenesis mostly in the latter phase of ischemic stroke. Transcriptomic analysis of murine CNS endothelial cells identified a subset of upregulated genes in multiple models associated with BBB dysfunctions including experimental models of stroke (Munji et al. 2019). The role of oxysterols in stroke and stroke animal models needs to be further investigated.

12.2 Phytosterols, Their Origin and Functions

Phytosterols or plant sterols are the naturally occurring non-nutritive bioactive compounds in plants. Their structure resembles the one of cholesterol except for the presence of a modification of their carbon side chains. Phytosterols are not synthesized in the human body and must be obtained from the diet. Their dietary source is fat-rich vegetables including vegetable oils, fruits, nuts, legumes, and cereals. The most abundant phytosterols in Western diets are sitosterol, stigmasterol, and campesterol. The common consumption of plant sterols is around 200–400 mg/day which is almost similar to cholesterol, however, phytosterols are poorly absorbed and are excreted faster from the liver than cholesterol and thus are less abundant in human tissues. The primary potential function of phytosterols is to reduce intestinal cholesterol absorption by competition for incorporation and solubilization into the bile salts micelles resulting in a reduction of circulating total and LDL-cholesterol levels (Cedó et al. 2019). In 2010, the FDA approved the beneficial health potential of phytosterol in terms of chronic disease risk reduction, however, due to their poor intestinal absorption, high consumption of vegetables is needed to impact cholesterol levels.

The exact mechanism by which phytosterols decrease serum cholesterol levels is not completely understood. However, phytosterol could act by different mechanisms to decrease cholesterol absorption such as reducing the transport and efflux of cholesterol into the enterocytes through Niemann Pick C1 Like 1 transporter (NPC1L1), reducing cholesterol esterification by acyl CoA, cholesterol acyltransferase (ACAT2) thus less soluble and available for intestinal absorption and recirculation, or increasing trans intestinal cholesterol excretion through the adenosine triphosphatase binging cassette G (ABCG5 and ABCG8) (Kaur and Myrie 2020). The therapeutic use of phytosterols to lower LDL-Cholesterol (LDL-C) concentration has been widely studied as early as 1951. In more recent years, they were introduced as additives in commercially prepared foods such as margarine. Phytosterol supplements are also widely available in many health foods stores, grocery stores, and retail pharmacies (Catapano et al. 2016; Gylling et al. 2014; Jellinger et al. 2017; Piepoli et al. 2016).

In addition to their cholesterol-lowering efficacy, experimental and clinical studies have suggested that plant sterols play an important role in diminishing inflammation (Nani et al. 2021). Incorporation of phytosterols into cell membranes can lead to immunomodulation and influence the production of eicosanoids, leukotrienes, and prostaglandins, which are essential modulators of immune responses. The anti-inflammatory effect of plant sterols has been addressed in different diseases, where inflammation plays a major role. However, the anti-inflammatory effect of phytosterol appears independent of their hypocholesterolemic effect. Phytosterols block the NF-κB pathway both in vitro and in murine inflammation models (Cheon et al. 2006; Shishodia and Aggarwal 2004; Preetha et al. 2006; Badshah et al. 2016; Saleem et al. 2004; Prabhu et al. 2016). β-Sitosterol, the main sterol of several plant species, exerts anti-inflammatory, anticancer, antispasmodic, antioxidant, and antidiabetic activities. Furthermore, the consumption of β-sitosterol was reported to reduce the expression of Il-6 (Bouic and Lamprecht 1999; Valerio and Awad 2011). Stigmasterol showed potent immunomodulatory activity in vitro by suppressing Concanavalin A (ConA)-induced T cell proliferation (Le et al. 2017). Moreover, it has been shown to reduce the release of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) and nitric oxide (NO), as well as the inhibition of cyclooxygenase-2 (COX-2) (la Torre Fabiola et al. 2016; Sharif et al. 2022). The anti-inflammatory properties of phytosterols were reported both in vitro and in vivo using LPS-stimulated macrophages and LPS-induced acute lung injury in mice. Phytosterols provide anti-inflammatory benefits by activating the LXRs/ABCA1 signaling pathway and altering the activation of the TLR4/NF-κB pathway (Yuan et al. 2019; Li et al. 2015). In addition, phytosterols suppress oxidative stress leading to antitumoral and neuroprotective activities (Pattarachotanant et al. 2021; Pratiwi et al. 2021). Recently, Haque et al. found that stigmasterol attenuates excitotoxicity, DNA damage, and mitochondrial dysfunction as well as decreasing ROS production (Haque et al. 2021). Stigmasterol also enhanced the activities of antioxidant enzymes (catalase (CAT), superoxide dismutase (SOD)), and nitric oxide synthase enzymes (iNOS and nNOS), providing neuroprotective effects (Adebiyi et al. 2018). Finally, numerous works have shown the potential antimicrobial activities of phytosterols, they appear to display both bacteriostatic and bactericidal activities against a broad range of Gram-positive and Gram-negative bacteria (Anwar et al. 2022; Dumandan et al. 2022; Pierre Luhata and Usuki 2021).

Therefore, the enrichment of phytosterols in food and dietary supplements might be considered as a new pharmacological approach in the management of various diseases. In this line, different meta-analyses studies have examined the effects of phytosterols in human inflammatory diseases such as neurodegenerative diseases, intestinal disorders, hypercholesterolemia, cardiovascular disease, and also their potential role in several types of cancers.

12.2.1 Neurodegenerative Diseases

As discussed in the first part of this chapter, cholesterol is synthesized in the CNS in situ and its metabolism is tightly regulated. During neuroinflammation, changes in brain cholesterol metabolism have been associated with poor neurological disease outcomes, such as Alzheimer's disease and Multiple sclerosis. It has been also proposed that cholesterol modulates the immune system, and that hypercholesterolemia drives pro-inflammatory responses. However, cholesterol is also indispensable in the CNS for various processes, including brain development, myelination, and neuronal signaling. In contrast to circulating cholesterol, phytosterols, can cross the blood–brain barrier and accumulate in the membranes of CNS cells. Due to their ability to reach the brain, researchers have started to investigate the physiological role of phytosterols in the CNS. In addition to their cholesterol-lowering ability, phytosterols also possess anti-inflammatory and anti-oxidative effects, which are crucial factors contributing to the pathology

of CNS disorders. As of today, only a few studies evaluate the neuroprotective and neurodegenerative properties of phytosterols during CNS disorders such as AD and MS (Catani et al. 2018; Dash et al. 2021). The role of phytosterols in Huntington's and Parkinson's diseases remains to be investigated. We will thus now only focus on their role during one demyelinating disease, MS, and on non-demyelinating disease, AD.

Multiple Sclerosis

It has been proposed that hypercholesterolemia contributes to pro-inflammatory processes and poor outcomes of MS (Stampanoni Bassi et al. 2020; Tettey et al. 2014; Ďurfinová et al. 2018). However, data reporting the role of lowering cholesterol levels during MS, for example, using statin treatments, remain controversial and largely debated. It has been further suggested that the beneficial effect of the lipid-lowering drugs statins is independent of their cholesterol-lowering effects and related to immunomodulatory activities (Aktas et al. 2003; Paintlia et al. 2005). In line with these observations, we recently showed that the sole modulation of circulating cholesterol levels is not sufficient to impact the severity of the MS mouse model, the experimental autoimmune encephalomyelitis (EAE) (Vigne et al. 2022).

Due to their immunomodulatory properties, ability to cross the BBB and capacity to lower blood cholesterol levels, the impact of phytosterols was evaluated in MS patients. Overall, studies suggest rather immunomodulatory and chemotactic properties of phytosterols than a direct effect on lowering cholesterol. Studies involving animal models, as well as some smaller studies with persons with MS, are underway to explore the connection between diet and MS and to understand how different foods could affect inflammatory processes in the body. In the year 2000, studies with animal models as well as clinical trials showed promising results regarding the effects of medicinal plants and their compounds on MS (Rabiei 2019). The effect of phytosterols was first evaluated and compared to statins on the proliferation and release of key pro-inflammatory cytokines from peripheral blood mononuclear cells (PBMC) of MS patients. Phytosterol reduced serum TNF-α levels by 24%, IL-5 by 47%, and IL-11 by 27% in MS patients and increased serum IL-10 levels by 47% in healthy individuals. Moreover, sitosterol has been reported to be effective in regulating the secretion of pro/anti-inflammatory cytokines at physiologically relevant concentrations without the side effects associated with statin therapy (Desai et al. 2009; Claflin et al. 2018). In 2011, Valerio et al. examined the effect of phytosterol administration in the development of EAE and showed their beneficial effects in the reduction of infiltration and inflammatory activity of immune cells into the CNS, thereby decreasing demyelination associated with the disease. Supplementation with a phytosterol cocktail consisting of sitosterol (60%), campesterol (25%), and stigmasterol (15%) suppressed the expressions of pro-inflammatory factors, including TNF-α, CCL2, IFNγ, and interleukin-6, and also upregulated the expression of IL-10 in EAE (Valerio et al. 2011). Walnut oil significantly reduced disease severity, inhibited plaque formation, and altered cytokine production in animal models of MS (Ganji et al. 2019; Conde et al. 2020). More recently, the use of withametelin, a potent phytosterol isolated from the leaves of *Datura innoxa*, showed significant neuroprotective potential in EAE via modulation of the nuclear factor-erythroid-related factor-2 (Nrf2) mediated-oxidative stress and NF-kB mediated inflammation (Khan et al. 2021). Phytosterols have been reported to accumulate in the CNS and CSF, especially in glial cells and to a lesser extent in neurons (Sharma et al. 2021; Vanmierlo et al. 2015). Phytosterols were reported to reduce EAE intensity, reduce lymphocyte and macrophage infiltration in CNS, and suppress inflammatory activities of these immune cells (Valerio et al. 2011). Thus, phytosterols could directly affect the functioning of these cells from oxidative stress and thus impact MS progression. Moreover, considering that phytosterols could activate both ERs and LXR signaling, they could affect CNS repair processes and neuroinflammation. These results provide

evidence to support phytosterols as a preventative agent that could help to protect against the development of inflammation-driven diseases, such as MS. However, those results are only based on limited studies and long-term prospective translational studies are mandatory to elucidate the applicability of phytosterols in MS.

Alzheimer's Disease

Due to the importance of oxysterol regulation as 24-OHC in CNS homeostasis, several studies have looked at the role of phytosterols or oxyphytosterols in the development of CNS disorders. It was proposed that in the early stage of AD, BBB and choroid plexus functions are impaired, resulting in reduced concentration of phytosterols in the CNS that might be relevant biomarkers for AD (Vanmierlo et al. 2011). Interestingly, in a mouse model of AD dietary supplementation of the oxyphytosterol improved memory performance correlated with a reduction of Aβ plaque load (Bogie et al. 2019). Both sitosterol and stigmasterol could have the potential to ameliorate amyloid β deposition in vitro and in vivo in AD models (Shi et al. 2011, 2013; Ye et al. 2020; Burg et al. 2013). More recently, an in vivo study of mice treated with 24(*S*)-saringosterol for 10 weeks revealed a slowing of cognitive decline based on enhanced spatial and object memory assessments. It also reduced the in vivo expression of ionized calcium-binding adapter molecule 1 (Iba1), a marker for microglial activation and inflammation. However, no effect was observed on the number of Aβ plaques (Martens et al. 2021). Overall, in in vitro and in vivo models, phytosterols are able to reduce Aβ plaque and secretase activity suggesting that a diet enriched in plant sterol might be beneficial for neurodegenerative diseases. However, phytosterols have limited intestinal absorption thus higher doses may be required to cross the BBB. Currently, researches are evaluating strategies to improve the bioavailability and delivery of phytosterols (Tolve et al. 2020). In conclusion, further studies should be carried out to explore the therapeutic and AD-specific mechanisms of phytosterols for their role in neuroprotection.

12.2.2 Intestinal Inflammation

Inflammatory bowel disease (IBD), which mainly refers to Crohn's disease and Ulcerative Colitis, is a disordered interplay of genetic, microbial, and environmental factors leading to the activation of the mucosal immune and non-immune response, resulting in active inflammation and tissue destruction. Few studies are available about the effects of phytosterols in experimental models of colitis such as trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS) that has usually been used to generate an animal model which closely mimics Crohn's disease or Ulcerative Colitis, respectively. During TNBS, it was reported that β-sitosterol inhibited colon shortening and led to reduced macroscopic scores and myeloperoxidase activity. Moreover, β-sitosterol reduced the expression of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and the cyclooxygenase COX-2, in the colons of TNBS-induced colitis, as well as the activation of NF-kB signaling (Lee et al. 2012). Other studies showed that in both preventive and therapeutic murine models phytosterols attenuated intestinal inflammation by suppressing NF-kB signaling by targeting IkappaB kinase and chemokine expression in intestinal epithelial cells (Cheon et al. 2006; Islam et al. 2008). Similarly, Aldini et al. evaluated the effects of a combination of phytosterols on the prevention and remission of clinical symptoms and mucosal healing during colitis in mice. Based on histological analyses, they showed that phytosterols pre-treatment significantly decreases clinical severity in the acute phase of the disease, reduces tissue inflammation, and improves significantly mucosal healing during remission. Moreover, phytosterol might have a beneficial effect on the regulation of intestinal microflora (Aldini et al. 2014). Based on this latter study, a metabolomic approach was used to provide further insights into phytosterol's role in colitis. It was reported that metabolic pathways such as glycolysis, Krebs cycle, amino acids, and nucleotide metabolism seem to be re-established by phytosterol treatment (Iaccarino et al. 2019). Recently, Wen et al. reported that stigmasterol

might reduce the Th17 cell response and promote Treg-cell development in mice with DSS-induced colitis. Interestingly authors observed that transplantation of fecal microbiota of stigmasterol-treated mice significantly alleviated inflammation. Additionally, stigmasterol administration resulted in a higher generation of gut microbiota-derived short-chain fatty acids (SCFAs), specifically butyrate in the feces of DSS-induced colitis mice (Wen et al. 2021). From the above study, phytosterols could play an important role in restoring the initial structural and biochemical environment in the gastrointestinal tract of colitis mice. These experimental results suggest that phytosterols may be taken into consideration as potential nutraceutical tools in the management of IBD and other intestinal inflammatory diseases.

12.2.3 Cancers

The mechanisms by which phytosterol consumption enables anticancer responses are varied and not fully understood (Ramprasath and Awad 2015; Woyengo et al. 2009). It was suggested that phytosterols provide potential anticancer properties via various mechanisms including the inhibition of cell cycle progression, cell invasion, migration, and adhesion, the promotion of cell apoptosis, and the stimulation of the immune function. Several in vitro studies have been conducted to provide biological plausibility for the hypothesis. The inhibitory effect of β-sitosterol and stigmasterol on tumor growth has been shown in various human tumor cell lines, including colon cancer (Choi et al. 2003), lung cancer (Dong et al. 2021), hepatic cancer (Kim et al. 2014), human prostate cancer (Awad et al. 2001), endometrial, cervical or ovarian cancer (Bae et al. 2020a, b), and breast cancer (Mallipeddi et al. 2021; Awad et al. 2008). Phytosterol might activate pro-apoptotic proteins such as p53 and BAX, while decreasing antiapoptotic signals (Bcl-2) and promoting the mitochondrial apoptosis signaling pathway, including the upregulation of caspase signaling. They were described to impact kinase signaling pathways such as PI3K/Akt, Akt/mTOR, and JAK/STAT, VEGFR-2 involved in numerous types of cancers and suppressed chemoresistance or enhanced inhibitory activity of some antiproliferative drugs (Alvarez-Sala et al. 2019). Initial observations show that dietary phytosterols play a role in immunomodulatory compounds, in which mixtures of sterols and sterolins enhance the cell responsiveness of T lymphocytes both in vitro and in vivo (Bouic 2001). In addition, phytosterols, which are lipid components of membranes, are thought to influence membrane fluidity, levels of sex hormones, and NF-KB activation, all of which may play vital roles in cancer risk. Limited data from animal studies suggest that very high intakes of phytosterols may inhibit the growth of various types of tumors. In a mouse model of skin carcinoma, stigmasterol reduced the lipid peroxide levels and DNA damage (Ali et al. 2015). Ramalingam et al. observed that the oral administration of β-sitosterol (20 mg/kg, three times per week for 24 weeks) was associated with the inhibition of proliferation and metastasis and the induction of apoptosis in renal cancer cells in rats (Sharmila and Sindhu 2017). Only a few observational studies examined the associations between dietary phytosterol intakes and cancer risk in humans (Woyengo et al. 2009). A series of case control studies in Uruguay found that dietary phytosterol intakes were lower in people diagnosed with stomach, lung, or breast cancer than in cancer-free control groups (De Stefani et al. 2000; Mendilaharsu et al. 1998; Ronco et al. 1999). Case control studies in the USA found that women diagnosed with breast or uterine cancer had lower dietary phytosterol intakes than women who did not have cancer (McCann et al. 2000, 2003). In contrast, another case control study in the USA found that men diagnosed with prostate cancer had higher dietary campesterol intakes than cancer-free men, but total phytosterol consumption was not associated with prostate cancer risk (Strom et al. 1999). Finally, a meta-analysis aiming at a comprehensive synopsis on phytosterols intake and cancer risk highlighted a linear association for campesterol and a nonlinear association for total

phytosterol intake and cancer risk (Jiang et al. 2019). Such findings support that high phytosterol intake is inversely related to cancer risk, although further and more in-depth studies are needed, particularly of prospective design to more clearly establish the potential antitumoral properties of phytosterols.

12.2.4 Atherosclerosis and Cardiovascular Diseases

Over 20 years ago, Jones et al. reported in a 30-day trial that a 1.7-g/day dose of oil phytosterols was able to reduce LDL-C by 24.4% in hypercholesterolemic patients compared with 8.9% with the control diet (Jones et al. 1999). Furthermore, numerous meta-analyses studies validated that food supplemented with phytosterols significantly reduced the incidence of diseases related to hyperlipidemia (Abumweis et al. 2008; Demonty et al. 2009; Katan et al. 2003; Musa-Veloso et al. 2011). In a hyperlipidemic patient-based study, phytosterols (2 g/day) in combination with fish oil capsule for 3 weeks significantly reduced the serum levels of inflammatory mediators (i.e., CRP, TNF-α, IL-6, and leukotriene B4), and increased anti-inflammatory adiponectin levels by 29.5% (Micallef and Garg 2009). The formulation and composition of phytosterols enriched foods and supplements were also widely studied (Jones et al. 2018). The use of phytosterols was thus recommended as a non-pharmacological therapeutic approach and several different dietary guidelines were considered to reduce the risk of hypercholesterolemia (Ras et al. 2014; Trautwein et al. 2018; Gylling et al. 2014; Ying et al. 2019). However, despite their cholesterol-lowering impact, the use of phytosterols in the prevention of CVD was debated (Weingärtner et al. 2014). In the revision of the ESC/EAS dyslipidemia guideline 2019, plant sterols were recommended for the first time as an adjunct to lifestyle modification to lower blood cholesterol levels but limited in patients with high cholesterol who have intermediate and low cardiovascular risk, who cannot receive statins, and in patients with hypercholesterolemia. However, the German Cardiac Society was more critical on the use of phytosterols in food supplementation and called for controlled trials investigating adverse cardiovascular outcomes for food supplemented with phytosterols. In 2021, Makhmudova et al. discussed this controversy in retrospective cohort studies suggesting that the use of phytosterols may contribute directly to atherogenesis (Makhmudova et al. 2021). In addition, few adverse effects of phytosterols occur in a small group of individuals with phytosterolemia, an inherited lipid disorder described below. Although most of the data published showed that phytosterols play an important role in lowering serum cholesterol concentrations, more prospective studies are needed to validate their beneficial effects on cardiovascular events.

12.2.5 Phytosterolemia

Besides potential beneficial properties in several diseases, phytosterols have also been associated with potential harm in humans. Phytosterolemia also known as sitosterolemia, is a rare autosomal recessive sterol storage disease characterized by the accumulation of phytosterols in the circulation and tissues. The disease is caused by mutations in ABCG5 and ABCG8 genes. These mutations lead to intestinal hyperabsorption and reduced hepatic secretion of cholesterol and plant sterols with subsequent accumulation of phytosterols and cholesterol in plasma and deposition in tissue. The clinical manifestation of this disease includes xanthomas and premature coronary atherosclerosis similar and often misdiagnosed as familial hypercholesterolemia in the early stage (Escolà-Gil et al. 2014). The tardive diagnosis results in an inappropriate dietary intervention consisting of foods supplemented with phytosterol, leading to serious vascular complications. Ezetimibe, an inhibitor of NPC1L1 that mediates absorption of dietary cholesterol in the intestine, has been shown to be effective in reducing serum sitosterol as well as cholesterol in sitosterolemic patients (Tsubakio-Yamamoto et al. 2010). The original description

of the disease was reported by Bhattacharyya and Connor in 1974, who found significantly elevated plasma levels of plant sterols in the form of beta-sitosterol, campesterol, and stigmasterol in two sisters (Bhattacharyya and Connor 1974). In 2013 Ras et al., reported that phytosterolemia was correlated with the consumption of phytosterols. In meta-analysis-controlled studies, a dose–response type relationship is observed between consumption and percentage changes in plasma concentration of campesterol and sitosterol (Ras et al. 2013). Moreover, in patients with homologous ABCG mutations, the absorption of phytosterols is multiplied by a factor of 3–12 and phytosterolemia seems to be multiplied by a factor of 30–100 (Othman et al. 2013). Although phytosterols may be associated with several benefits, there are also a few downsides to consider. Further characterization of the influence of various phytosterol subcomponents on lipoprotein profiles in humans is required to maximize the usefulness of this non-pharmacological approach. Indeed, the potential harmful side effects of phytosterols underscore the need to be prudent with the use of plant sterols in humans.

12.3 Conclusion

Oxysterols and phytosterols have gained attention over the last decades and are now recognized as fully bioactive lipids involved in fine tuning the immune responses and contribute to the development of several chronic diseases including neurodegenerative disorders, intestinal diseases, cancer, and cardiovascular diseases. By their ability to act through the modulation of oxidative stress, inflammation and cell toxicity, oxysterols, and phytosterols are involved in various biological processes. In this chapter, we summarized and discussed the implications of oxysterols and phytosterols in human health and several diseases. However, despite recent and promising advances in the field, long-term prospective translational studies are mandatory to elucidate the use of oxysterols and phytosterols either as disease biomarkers or new pathways to be targeted to treat inflammatory and cardiovascular diseases as well as cancer.

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Sterols in Inflammatory Diseases: Implications and Clinical Utility

13

Ahmet Yalcinkaya, Yeşim Er Öztaş, and Suna Sabuncuoğlu

Abstract

The characteristic steroid skeleton, with its 4-ringed 17-carbon structure, is one of the most recognizable organic compounds in biochemistry. In the presence of a hydroxyl ion bound to the third carbon, this structure is defined as a "sterol" (chemical formula: $C_{17}H_{28}O$). The hydroxyl group provides a hydrophilic site for the otherwise hydrophobic molecule, yielding an amphipathic lipid, which is a vital property for cellular function. It is crucial to remark that the term "steroid" describes a larger group of compounds that often retain the hydroxyl group but are primarily characterized by methyl groups, double bonds in the rings, and an aliphatic side-chain extending from the 17th carbon. In addition to serving various structural roles in the cellular membrane, sterols and steroids contribute to cellular and systemic functions as messengers, hormones, and regulators of several critical metabolic pathways.

Sterol nomenclature is often confusing, partly due to structural complexity and partly due to the sheer number of different compounds that fall under the definition. Fortunately, the foremost sterols of interest in biochemistry are much fewer, and therefore, these lipids have been defined and studied vigorously. With the renaissance of lipid research during the 1990s and 2000s, many different metabolites of sterols, and more specifically phytosterols, were found to be associated with various diseases and conditions, including cardiovascular disease, hypercholesterolemia, cancer, obesity, inflammation, diabetes, and inborn errors of metabolism; thus, it is evident that the ever-evolving research in this field has been, and will continue to be, exceedingly productive.

With respect to inflammation and inflammatory diseases, plant-based sterols (i.e., phytosterols) have gained considerable fame due to their anti-inflammatory and cholesterol-lowering effects demonstrated by experimental and clinical research. Besides, the exceptional pharmacological benefits of these sterols, which operate as antioxidant, antidiabetic, and anti-atherosclerotic agents, have been the subject of various investigations. While the underlying mechanisms necessitate further research, the possible function of phytosterols in improving

A. Yalcinkaya
Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey

Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Y. E. Öztaş
Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey

S. Sabuncuoğlu (✉)
Department of Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

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health outcomes is an important topic to explore.

In this regard, the current review aims to offer comprehensive information on the therapeutic potential of plant-based sterols in the context of human health, with a focus on preclinical effects, bioavailability, and clinical use.

Keywords

Sterols · Phytosterols · Inflammation · Disease

Abbreviations

CVD	Cardiovascular disease
ERK	Extracellular signal-regulated protein kinase
FDA	Food and Drug Administration
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IFN	Interferon
IL	Interleukin
JNK	C-Jun amino-terminal kinase
LAL	Lysosomal acid lipase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-associated protein kinase
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
ROS	Reactive oxygen species
SREPB	Sterol regulatory element-binding proteins
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
TNF-α	Tumor necrosis factor-α
VLDL	Very-low-density lipoproteins

13.1 The First and Foremost: Cholesterol

Cholesterol is the best-known sterol, not only because it was the first sterol—and for that matter, lipid—to be identified (Endo 2010) but also because of its established role in human health, especially cardiovascular disease (CVD) (Goldstein and Brown 2015). More than two centuries after its identification and almost a century after the exact determination of its structure, cholesterol was presented as *the most highly decorated small molecule in biology* by Goldstein and Brown at their Nobel lecture in Stockholm in 1985 (Eastwood 2012; The Nobel Prize in Physiology or Medicine 1985 2023). Cholesterol contains one double bond and one hydroxyl (-OH) group in the 17-carbon structure. Its unique structural properties are critical to the functions of cholesterol, including its participation in cellular membrane organization, fluidity, and raft structure, as well as its other physiological roles (Ikonen 2008). However, cholesterol's insolubility in water also makes it a high-risk molecule for cardiovascular health (Soliman 2019).

13.1.1 History of Cholesterol Research

The history of cholesterol research is worthy of mention (Table 13.1). Although solid cholesterol was first identified at the end of the eighteenth century (Olson 1998), elucidation of its structure was made possible by the valuable work of many scientists during the first half of the twentieth century (Table 13.1). Researchers focused on cholesterol and sterol-related research have won many Nobel prizes.

Wieland's work on clarifying the structure of bile acids and sterols was awarded the Nobel Prize in chemistry in 1927. Windaus, who reported that plaques in the aortas of atherosclerosis patients contained 20 times more cholesterol than normal in 1910, was awarded the Nobel Prize in Chemistry in 1928 "for the services rendered through his research into the constitution of the sterols and their connection with the vitamins". Ruzicka synthesized the male sex hormones androsterone and testosterone from cholesterol and was awarded the Nobel Prize in 1939. Diels, who worked on elucidating the structure of cholesterol, received the Nobel Prize in Chemistry in 1950, and the title of his Nobel Lecture was "Description and Importance of the

Table 13.1 History of cholesterol research

1769	Solid cholesterol was identified in gallstones
1815	The name "cholesterin" was used for the first time
1833	Cholesterol was first reported in human blood
1856	Atherosclerotic plaque was described as a fundamental lesion of atherosclerosis
1888	Exact molecular formula of cholesterol was established
1889	An 11-year-old child with xanthomatosis and sudden cardiac death was reported
1913	Lipid hypothesis was put forward
1927	Nobel prize in chemistry was awarded for clarifying the structure of bile acids and sterols
1928	Nobel prize in chemistry was awarded for elucidating the structure of cholesterol
1929	Lipoproteins were discovered
1939	Nobel prize in chemistry was awarded for synthesis of androsterone and testosterone
1950	Low-density lipoprotein (LDL) was isolated from horse serum. The Framingham study was started
1964	Nobel prize in medicine or physiology was awarded for the discovering the mechanism and regulation of the cholesterol and fatty acid metabolism
1971	Serum cholesterol was associated with increased risk of cardiovascular disease in the Framingham prospective cohort study
1974	LDL receptor was elucidated
1975	Nobel prize in chemistry was awarded for establishing the orientation of all the hydrogen atoms in the cholesterol molecule
1976	The first statin group drug was produced
1985	Nobel prize in medicine was awarded for the discovery of the inhibition of cholesterol synthesis by LDL receptor mediated pathway
1987	Food and Drug Administration approved the use of statins for cholesterol lowering
1997	Sterol regulatory element-binding proteins pathway was elucidated

Aromatic Basic Skeleton of the Steroids" in 1950 (The Nobel Prize in Chemistry 1950 2023).

Gofman was named the father of clinical lipidology when he started the Framingham Heart Study in 1950. Ultracentrifugation was used to isolate lipoproteins, and results indicated that individuals with higher blood cholesterol levels at baseline were more likely to suffer from myocardial infarction in the follow-up (Kannel et al. 1961). In later studies, they reported direct associations between serum cholesterol levels and CVD (Kannel et al. 1971).

Bloch and Lynen were awarded the Nobel Prize in Medicine or Physiology in 1964 for their discoveries concerning the mechanism and regulation of cholesterol and fatty acid metabolism (The Nobel Prize in Physiology or Medicine 1964 2023). Their landmark studies deciphered the cholesterol biosynthetic pathway, a complex sequence involving at least 30 steps. The initial substrate discovered by Lynen was acetate, which is itself an intermediate in fat and carbohydrate metabolism (Bloch 1965). Woodward, who pioneered the stereochemical synthesis of cholesterol, received the Nobel Prize in Chemistry in 1965 "for his outstanding achievement in the art of organic synthesis. Their work led to the future development of statins. Barton and Hassel were awarded the Nobel Prize in Chemistry in 1969 "for developing and applying the principles of conformation in chemistry," which included establishing the all-chair conformation of cholesterol.

In 1976, Endo discovered and characterized compactin, the first statin from fungus (Endo et al. 1976). In 1978, an 18-year-old patient with severe familial hypercholesterolemia benefited significantly from compactin treatment, with a significant decline in cholesterol levels.

When Brown and Goldstein reported that lovastatin could raise low-density lipoprotein (LDL) receptors in dogs, leading to a significant decrease in plasma LDL, lovastatin was later tested in humans and resulted in a decrease in plasma LDL (Brown et al. 1974). The research carried out by Goldstein and Brown was a milestone in

cholesterol research after their discovery of LDL receptors in 1974, and they received the Nobel Prize in Medicine about a decade later in 1985 (The Nobel Prize in Physiology or Medicine, 1985–2023). They discovered the mechanism of inhibition of cholesterol synthesis, which resulted in new approaches for the treatment of atherosclerosis, particularly by statins.

Lovastatin was approved by the FDA in 1987 and became the first statin on the market (Vagelos 1991). A flurry of studies in the following years demonstrated the health benefits of reducing cholesterol levels, including the Scandinavian Simvastatin Survival Study (4S), which proposed that simvastatin treatment significantly reduced coronary mortality in patients with coronary heart disease (Pedersen et al. 1994).

The regulation of cholesterol synthesis by transcriptional regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and Scap and the sterol regulatory element-binding protein (SREBP) pathway was discovered in 1997. It was found that increased intracellular cholesterol-induced ubiquitination of HMG-CoA reductase and its degradation by the proteasome initiated the posttranslational control (Brown et al. 2018). The 1990s and 2000s saw a renaissance in lipid research with the development of better extraction methods and mass spectrometry technology that enabled high-throughput lipidome analysis.

13.2 Plant-Based Sterols in Inflammation and Inflammatory Diseases

Plant sterols (phytosterols) and stanols (phytostanols) have well-known inhibitory effects on intestinal cholesterol absorption, which decreases LDL cholesterol concentrations. These compounds are found in diets and in functional foods. The health effects of phytosterols are a growing topic in the scientific literature (Plat et al. 2019).

Fatty acid esters, hydroxycinnamic acid esters, and glycosides are the major forms of phytosterols (Moreau et al. 2018). All ester linkages are broken within the digestive tract by certain enzymes, releasing free phytosterols. In typical diets, sitosterol (66%), campesterol (22%), stigmasterol (8%), sitostanol, and campestanol (4%) are the most commonly detected phytosterols and phytostanols. These compounds can be found in bread, cereals, vegetables, fruits, vegetable oils, and food made from these oils (Klingberg et al. 2008). Following their incorporation into mixed micelles, phytosterols reduce the amount of cholesterol that can be packed into the micelles. Their decreasing effect on intestinal cholesterol absorption is partly explained by this mechanism. Similar to cholesterol, plant sterols are absorbed into enterocytes by the Niemann-Pick C1-like 1 receptor (Davis et al. 2004).

Phytosterols and phytostanols are characterized by methyl (ampesterol) or ethyl (-β-sitosterol and stigmasterol) groups in their side chains, and they do not have a double bond in the B ring, which are structural characteristics that distinguish phytosterols from cholesterol. The unsaturated forms (−sterols) of phytosterols are more prevalent in nature than the saturated forms (−stanols) (Ostlund 2002; Brufau et al. 2008; Aldini et al. 2014). β-sitosterol, has been demonstrated to have anti-inflammatory, anticancer, antispasmodic, antioxidant, and antidiabetic activities (Baskar et al. 2010, 2012; Liz et al. 2011; Plat et al. 2000). In both normo- and hypercholesterolemic patients, phytosterols cause cholesterol-reducing effects, often without affecting the levels of high-density lipoprotein cholesterol and triglycerides (Plat et al. 2000; Ostlund and Lin 2006). Experimental and clinical studies have revealed that phytosterols reduce inflammation. The ability of phytosterols to reduce inflammation has primarily been studied in relation to atherosclerosis. Yet, phytosterols' anti-inflammatory effects are distinct from their cholesterol-reducing effects (Shishodia et al. 2007; Medeiros et al. 2007; Holanda Pinto et al. 2008; Othman and Moghadasian 2011).

13.2.1 Inflammation and Phytosterols

The immune system responds to injury caused by microbial, chemical, and physical insults by activating inflammation (Brüll and Mensink 2009). The inflammatory response is mostly controlled by macrophages (Fessler 2016; Moore et al. 2013; Tall and Yvan-Charvet 2015). To enhance the inflammatory response, they can generate and secrete a number of bioactive chemicals, including reactive oxygen species (ROS), nitric oxide (NO), and tumor necrosis factor-α (TNF-α). Lipopolysaccharide (LPS), which is mostly found in the outer membrane of Gram-negative bacteria and is composed of lipid A, core sugars, and O-antigen, can strongly trigger an immunological response and is a powerful innate immune stimulator. LPS can precisely recognize Toll-like receptors on macrophages, enhance the release of proinflammatory cytokines, and increase the transcription of immune-related genes (Chu et al. 2018; Wang et al. 2017; Yuan et al. 2019). LPS can also trigger the stimulation of cytokine release from macrophages (Li et al. 2017; Herrera et al. 2000).

Research on the nuclear factor kappa B (NF-κB) and mitogen-associated protein kinase (MAPK) signaling pathways may explain the impact of phytosterols on inflammation. Regarding the NF-κB pathway, TNF-α suppresses NF-κB and induces inflammatory reactions (Martínez-Soto and Ruiz-Herrera 2017; Wazea et al. 2018; Yu et al. 2018). It has been shown that phytosterols reduce the amount of proinflammatory TNF-α that mice produce throughout the inflammatory response. Moreover, it was discovered that protein tyrosine phosphatase activation has an anti-inflammatory effect on macrophages, whereas β-sitosterol can prevent NF-κB translocation to the nucleus. Regarding the MAPK pathway, research revealed that ergosterol and ergosterol peroxide block LPS-induced DNA-binding activity of NF-κB, as well as impacting the phosphorylation of p38, c-Jun amino-terminal kinase, and extracellular signal-regulated protein kinase MAPKs (Shishodia et al. 2007). In RAW264.7 cells, ergosterol peroxide was found to inhibit the expression of the LDL receptor, which is controlled by C/EBP and the enzyme HMG-CoA, resulting in anti-inflammatory action (Yuan et al. 2019; Valerio and Awad 2011).

13.2.2 CVDs and Phytosterols

The most common cause of death in the world is CVD. Increased LDL levels are a significant risk factor for CVD, and available evidence shows that lowering LDL can reduce the incidence of CVD. Since the early 1950s, it has been known that consuming supplements containing phytosterols can significantly reduce serum levels of cholesterol and LDL. Phytosterols are consumed in amounts comparable to cholesterol (200–400 mg per day), but they are poorly absorbed, resulting in circulatory levels several orders of magnitude lower than cholesterol (around 0.1 mg/dL). However, increased phytosterol consumption leads to competitive obstruction of cholesterol absorption and impacts transcriptional stimulation of genes involved in cholesterol metabolism, which act in concert to lower blood lipid levels (Group WCPS et al. 1995; Sacks et al. 1994; Silbernagel and Maerz 2008; Genser et al. 2012). For instance, daily consumption of 1.6 g of phytosterols on average can lead to sitosterol and campesterol increases of about 31% and 37% in the circulation, respectively (Plat et al. 2019; Ras et al. 2013).

Whether or not phytosterols are atherogenic is still up for dispute. The current theory is that those with high phytosterol concentrations can actually be classified as subjects who have increased cholesterol absorption, and therefore, the higher phytosterol levels are a byproduct of this elevated intake. This is an explanation for conflicting findings on the subject (Silbernagel et al. 2013). According to observational data, the "plant sterol-atherogenicity" theory suggests that CVD risk and its severity are inversely correlated with higher cholesterol absorption rates and lower cholesterol synthesis rates (lathosterol or desmosterol), which may be assessed through

surrogate markers of absorption (plasma phytosterols) and low synthesis (Plat et al. 2019; Silbernagel et al. 2013). Elevated levels of desmosterol, a marker of cholesterol production, may also enhance the cardioprotective benefits of plant sterol and stanol esters, both of which mildly boost endogenous cholesterol synthesis. Unfortunately, the direct effects of phytosterols or phytostanols remain poorly researched topics (Rideout et al. 2010; Spann et al. 2012).

The overall impact of phytosterols can be influenced by a number of variables. It has been reported that men are slightly more sensitive to phytosterol intake than women. The initial plasma lipid levels are another variable that might affect plant sterol effectiveness. For instance, it has been demonstrated that patients with phytosterol levels corresponding to ideal therapeutic values experience greater decreases in LDL than subjects with high or very high baseline levels (Naumann et al. 2008; Mussner et al. 2002; Santas et al. 2013).

Another advantageous effect of phytosterols is based on their capacity to reduce triacylglycerol serum concentrations, particularly in individuals with high serum concentrations. This effect may be attributed to a reduction in the synthesis of very-low-density lipoproteins (VLDL), which are the main transporters of triglycerides (Rideout et al. 2010; Spann et al. 2012).

13.2.3 Hepatic and Intestinal Inflammatory Diseases and Phytosterols

It is well known that the liver is essential for maintaining healthy levels of cholesterol. The balance of intestinal absorption, synthesis, bile acid degradation, and excretion with the bile, and VLDL homeostasis, determine the concentration of cholesterol in the liver. Reduced cholesterol absorption alters the liver's lipid homeostasis in numerous ways (Santas et al. 2013).

Many disorders, including Niemann-Pick C, nonalcoholic steatohepatitis (NASH), and lysosomal acid lipase deficiency-D, are characterized by lysosomal cholesterol storage issues. It is of note that the liver is impacted by all of these disorders. This suggests that healthy hepatic cell activity depends on adequate cholesterol homeostasis. Decreased intestinal absorption of cholesterol also decreases the transit of cholesterol from the intestine to the liver. The consumption of diets high in phytosterols or phytostanols has been found to reduce hepatic inflammation; however, since steatosis was unaffected, the effect was probably associated with an inflammatory response. It is therefore questionable whether the hepatoprotective benefits of high-phytosterol diets are due to direct or indirect effects due to lower circulating cholesterol or reduced inflammation (Wouters et al. 2008; Plat et al. 2014). According to some theories, the flow of cholesterol from the intestine to the liver stimulates the buildup of lysosomal cholesterol in the liver, which then causes inflammation. Another study that employed the same NASH model to test this theory provided the animals with a high-fat, high-cholesterol diet that was supplemented with phytosterols, or phytostanols. In addition to boosting cellular cholesterol precursor concentrations and decreasing cholesterol fluxes to the liver, phytosterols may also have direct impacts on hepatic inflammation. Accordingly, it has been demonstrated that in vitro sitosterol and sitostanol led to reduced inflammation through their effects on macrophages from the bone marrow (Plat et al. 2014).

There are also several studies examining the impact of phytosterols on outcomes associated with NASH. In one study, a hyperlipidemic mouse model for NASH was administered a plant sterol extract for 25 weeks, which resulted in a considerable reduction in the CD4/CD8 ratio and an increase in the number of CD4+ CD25+ Treg cells. Moreover, a decline in serum interleukin (IL)-1 and transforming growth factor beta (TGF-β) levels was observed (Drori et al. 2017). Accordingly, a study administering phytosterols to a hyperlipidemic rat model for NASH showed a decrease in the inflammatory cytokines TGF-β, IL-6, IL-10, and also C-reactive protein in the liver. Taken together, available animal research in NASH models suggests that hepatic

inflammation is suppressed by phytosterols (Plat et al. 2019; Song et al. 2017).

Inflammatory bowel diseases, primarily Crohn's disease and ulcerative colitis, are multifactorial diseases in which different underlying mechanisms lead to the activation of mucosal immune and nonimmune responses, causing excessive inflammation and tissue destruction in the bowel. The prevalence of these disorders has increased in several countries. Few studies have assessed the effects of phytosterols in experimental models of colitis, and taking into consideration that the pathogenesis of both illnesses is unclear and appears to be distinct, it is evident that further studies are necessary on this topic. Nonetheless, research on experimental models of inflammatory bowel disease has demonstrated the anti-inflammatory benefits of phytosterols such as β-sitosterol, guggulsterone, and phytosteryl ferulates (Shi et al. 2011; Melgar et al. 2005; Lee et al. 2012; Islam et al. 2008).

13.2.4 Cancer and Phytosterols

Although there are many elements that contribute to the etiology of cancer, it has been proven that food intake and dietary habits greatly affect the risk of cancer. Recent meta-analyses have found that daily doses of 1–2 g of plant sterols, or stanols, can successfully lower LDL cholesterol levels by 8–12%, which could alter the metabolic background leading to cancer development. Despite the reasonably convincing evidence that these substances reduce the risk of CVD, their potential effects on cancer have been sparsely researched. Available research on phytosterols report encouraging findings, and it has been suggested that the growing body of information supporting their biochemical and molecular effects implicates these compounds as promising candidates in cancer treatment (Grattan 2013).

In cultured macrophage cells with oxidative stress induced by phorbol 12-myristate 13-acetate, it was found that β-sitosterol increased the activities of antioxidant enzymes, superoxide dismutase, and glutathione peroxidase, indicating that phytosterols can protect cells from damage caused by reactive oxygen species (Vivancos and Moreno 2005). In another study, campesterol and β-sitosterol were administered to lipopolysaccharide-activated macrophage cells at doses of 8 and 16 mM, respectively. The results revealed a 68 and 67% (for sitosterol) and 55 and 52% (for campesterol) reduction in prostaglandin E and prostaglandin I synthesis, respectively. According to these studies, phytosterols appear to reduce the growth of cancer cells (Awad et al. 2004).

Chronic inflammation has been identified as a key component of cancer development and progression, and the immune system plays a significant role in the genesis of cancer. For instance, immune system dysregulation contributes to the spread of cancer. In animal models of breast cancer, IL-2, and interferon (IFN)-γ have been shown to play key roles in limiting metastasis. Phytosterols control cytokine release, increasing both IL-2 and IFN-γ secretion. As such, liposomal administration of β-sitosterol was demonstrated to reduce metastasis in a murine melanoma model. While IL-2 levels and NK-κB cell activity were found to be increased after liposome injection, phytosterols had beneficial effects. Hence, their stimulatory effects on cytokine production may be a mechanism through which they inhibit cancer growth (Qin et al. 2011; Calpe-Berdiel et al. 2007; Grattan 2013).

Phytosterol consumption has been suggested to play a role in preventing the development of many types of cancer. In order to ascertain the impact of phytosterol intake on lung carcinogenesis, a study involving 463 individuals with newly-diagnosed primary lung cancer and 465 hospitalized controls was conducted in Uruguay. After accounting for known confounding factors such as smoking, intake of vegetables and fruits, and antioxidant levels, phytosterol consumption was linked to a 50% lower risk of the disease. In another study, the risk of lung cancer was found to have been lowered by 38% as a result of high consumption of phytosterols compared to consumption of carotene and flavonoids, which were also found to

lower the risk of disease (Mendilaharsu et al. 1998). In 120 patients with gastric cancer and 360 controls, plant sterol intake was found to have a negative correlation with gastric cancer (De Stefani et al. 2000). In a case-control study, McCann et al. (2003) found that larger intakes of stigmasterol (>23 mg per day) compared to smaller intakes (12 mg per day) were associated with a lower risk of developing ovarian cancer (odds ratio 0.42). In mice, the effects of phytosterols (9.8 g per kg of diet) on the proliferation of estrogen-dependent human breast cancer cells were studied. Despite having no effect on cancer cell development, 17-estradiol levels were found to be associated with slower tumor growth --by 38.9% (Ju et al. 2004).

The primary mechanisms by which phytosterols prevent the growth of different malignancies appear to be by preventing the proliferation of cancer cells and encouraging apoptosis by activating the caspase enzymes. The increased activity of caspase enzymes may be caused by changes in the structure and function of cell membranes brought on by the incorporation of phytosterols, which also increase the activities of proteins involved in extracellular and intracellular signal-transduction pathways that can activate caspase enzymes. Since high blood cholesterol levels (and consequently the concentration of cholesterol in lipid rafts) are linked to impaired apoptosis of cancer cells, phytosterols may also suppress the growth of cancer by lowering blood cholesterol which may remove the inhibition on apoptosis (Shahzad et al. 2017; Cioccoloni et al. 2022).

13.3 Clinical Utility of Sterols in Humans

The current review has established that the rich literature on sterols illustrates the importance of sterols and phytosterols in the maintenance of cell membranes, cholesterol metabolism, immune response, inflammation, and human disease. The discussion concerning “healthy” and “unhealthy” types of sterols and their impact on human health has been a topic of interest for the best part of the last 50 years, spearheaded by results from decades of longitudinal studies. Today, despite the need to exercise caution when interpreting the outcomes of any single study, research describing the health-related effects of various lipid classes is widely circulated by the health industry and media, fueling public attention. These discussions and controversies are not within the scope of the present review; rather, in this section, we will be focusing largely on the potential health impacts of the most common phytosterols and the direct associations that have been established between phytosterols and different pathologies.

13.3.1 Beta-Sitosterol, Campesterol, and Stigmasterol

Phytosterols are found in plant-based foods such as fruits, nuts, seeds, and vegetables and have been extensively studied for their cholesterol-lowering properties. As mentioned previously, long-term research shows that consumption of phytosterols significantly reduces LDL cholesterol levels, a key risk factor for CVD (Demonty et al. 2009). This effect is believed to be due to the ability of phytosterols to inhibit cholesterol absorption in the small intestine and its cellular effects (Plat and Mensink 2005). In addition to their cholesterol-lowering properties, phytosterols have been suggested to demonstrate a range of potential health benefits owing to their anti-inflammatory, antioxidant, and cancer-preventive effects (Salehi et al. 2020; Moreau et al. 2002).

Some of the most important phytosterols are β-sitosterol, campesterol, stigmasterol, and brassicasterol (Salehi et al. 2020; Poli et al. 2021). In the human diet, β-sitosterol and campesterol are the most common phytosterols (Poli et al. 2021; Weihrauch and Gardner 1978), which has led to extensive studies focusing on the effects of these molecules. Studies in murine models that extracted β-sitosterol from different sources have revealed anti-inflammatory (Nirmal et al. 2012; Paniagua-Pérez et al. 2017), antioxidative (Devaraj et al. 2020), antibacterial

(Ododo et al. 2016), and hepatoprotective (Devaraj et al. 2020; Abdou et al. 2019; Feng et al. 2018) effects that have been replicated in other species (Zhang et al. 2023). Additionally, in vitro research on β-sitosterol has demonstrated antiviral (Chen et al. 2022; Shokry et al. 2023) and wound healing (Abbas et al. 2019) properties. Furthermore, studies involving humans have also shown several beneficial effects in various conditions, including benign prostatic hyperplasia (Wilt et al. 2000; Klippel et al. 1997) and skin burns (Dalloul et al. 2020), as well as cholesterol-lowering properties (Poli et al. 2021; QuIlez et al. 2003). Despite these promising findings, it is critical to consider that, relative to cholesterol, the percentage-wise β-sitosterol uptake of the human body is very low (Salen et al. 1970), which is bound to have considerable effects on their use as supplements. However, even low intakes of β-sitosterol have been shown to be effective in reducing LDL cholesterol levels (Sadler 2014). Thus, even low levels of supplementation may improve health-related outcomes in patients with inflammation-related diseases and cancer.

Campesterol is the second-most abundant phytosterol in the human diet. Despite limited research concerning its potential impact on human disease states, it has been demonstrated to have a range of effects in *in vivo* and *in vitro* experimental studies, including antioxidant activity in ovarian cancer (Bae et al. 2021), reduced storage of visceral fat (Ikeda et al. 2006), and unclarified effects on lowering plasma cholesterol and triglyceride levels (Vissers et al. 2000). Also, interestingly, it was shown that oxidation products of phytosterols did not contribute to atherosclerosis development in rats, despite the known adverse effects of these oxyphytosterols on endothelial function (Tomoyori et al. 2004). This particular result demonstrates the need for further studies that aim to assess the outcomes of supplementation with campesterol, especially since this molecule could competitively limit the creation of compounds arising from cholesterol oxidation.

Stigmasterol is another commonly encountered phytosterol in plants; however, compared to β-sitosterol and campesterol, it is often at lower concentrations in human diet and edible oils (Yang et al. 2019). Considering its numerous positive effects demonstrated in the literature, this may be an important deficiency in the human diet and could indicate that it may be feasible to prefer oils that contain relatively higher concentrations of stigmasterol, including the likes of soybean, sesame, and rice bran oils (Yang et al. 2019). Stigmasterol's effects illustrated in experimental studies include anti-inflammatory (cytokine inhibition) (Khan et al. 2020), antidiabetic (Ramu et al. 2016), and cholesterol-lowering effects (Batta et al. 2006).

Despite the presence of conflicting findings concerning phytosterols and their impact on human disease (Assmann et al. 2006; Wilund et al. 2004), the established effects strengthen the notion that phytosterols may emerge as potential therapeutic options in various diseases, especially in the prevention or management of chronic conditions, due to their ease of use, widespread presence throughout the plant flora of the world, and vanishingly few side effects (Salehi et al. 2020; Cabral and Klein 2017). That said, it is important to note that adverse effects have also been reported by the use of phytosterol supplementation in humans, including gastrointestinal side effects, elevated liver enzymes, and male impotence (the latter in patients with benign prostate hyperplasia) (Ling and Jones 1995), as well as rare reports involving severe outcomes such as pancreatitis (Lorenze et al. 2020) and markedly reduced absorption of fat-soluble vitamins (QuIlez et al. 2003).

13.3.2 Clinical Use and Implications

Sitosterolemia is an extremely rare genetic disorder that causes the accumulation of phytosterols in the body. Excessive accumulation may lead to premature atherosclerosis and an increased risk of CVD (Yoo 2016). The diagnosis is based on the detection of phytosterol elevation from reference human levels (up to 100-fold) via advanced methods such as gas chromatography and liquid chromatography, often in tandem with mass

spectrometry (Kidambi and Patel 2008). It is treated by administering a low-cholesterol diet and bile acid sequestrants, which prevent the absorption of phytosterols in the intestines (Yoo 2016; Tsubakio-Yamamoto et al. 2010).

Phytosterol levels may also have diagnostic or prognostic roles in CVD. One large prospective study from Spain determined that patients who had been diagnosed with coronary heart disease had significantly lower levels of sitosterol intake as well as lower levels in their plasma, indicating that some phytosterols could be used as predictive markers for CVD (Escurriol et al. 2010). Paradoxically, high sitosterol levels were found to be associated with an increased likelihood of suffering from coronary events among men (Assmann et al. 2006), which could be explained by the different effects and metabolism of different phytosterols. Nonetheless, apart from a few studies showing adverse effects, the majority of research has shown benefits with phytosterol supplementation. As a result, phytosterols have been introduced as supplements, leading to studies describing that these interventions can counteract symptoms in benign prostate hyperplasia and disrupt the progression of prostate cancer (Lu and Zhang 2007; von Holtz et al. 1998).

There are also studies from other fields which have described benefits through various mechanisms. For instance, phytosterol supplementation is suggested to counteract the underlying biochemical triggers and pathways associated with cancer development and progression, potentially due to the intertwined relationships between dysfunctional cholesterol metabolism, oxidative stress, and inflammation—which are known to be impactful on cancer pathogenesis (Grattan 2013; Plat and Mensink 2005; Salehi et al. 2020; Bae et al. 2021).

In multiple sclerosis, which is a disease characterized by neuron loss due to immune activation against the myelin sheath of nerve cells (Tafti et al. 2023), phytosterols and their oxidation products are suggested to have dual effects. While phytosterols can alleviate the negative impact of oxidative stress in multiple sclerosis lesions, oxyphytosterols may cause cytotoxicity, thereby causing adverse effects (Vanmierlo et al. 2015). Considering these opposite effects, it may be feasible to suggest that phytosterol levels could potentially be utilized as disease markers in patients with multiple sclerosis. Further studies on this topic are necessary, and considering that phytosterols are capable of freely crossing the blood–brain barrier, these effects could be quantified by measuring circulatory levels of phytosterols and evaluating each compound with respect to its impact on outcomes.

13.3.3 Conclusion

Overall, the clinical utility of sterols, particularly phytosterols, is an area of ongoing research. While these compounds show promise for a range of health benefits, further studies are needed to fully understand their mechanisms of action and potential therapeutic applications. However, given their widespread availability in a range of foods, including fruits, vegetables, and nuts, increasing the intake of phytosterols and some zoosterols may be a simple and effective way to promote overall health and reduce the risk of chronic diseases.

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Role of Oxysterols in Ocular Degeneration Mechanisms and Involvement of P2X7 Receptor

14

Elodie Olivier and Patrice Rat

Abstract

Ocular degeneration, including cataracts, glaucoma, macular degeneration, and diabetic retinopathy, is a major public health challenge, as it affects the quality of life of millions of people worldwide and, in its advanced stages, leads to blindness. Ocular degeneration, although it can affect different parts of the eye, shares common characteristics such as oxysterols and the P2X7 receptor. Indeed, oxysterols, which are cholesterol derivatives, are associated with ocular degeneration pathogenesis and trigger inflammation and cell death pathways. Activation of the P2X7 receptor is also linked to ocular degeneration and triggers the same pathways. In age-related macular degeneration, these two key players have been associated, but further studies are needed to extrapolate this interrelationship to other ocular degenerations.

Keywords

P2X7 · Oxysterol · Ocular degeneration

Abbreviations

AMD Age-related macular degeneration
HO-1 Heme oxygenase-1
LXR Liver X receptor
MAPK Mitogen-activated protein kinase
VEGF Vascular endothelial-derived growth factor

14.1 Ocular Degeneration

The eye is a complex organ at the interface between the external environment and the human body. The eye is the major organ of the visual system. Its structure is briefly detailed in Fig. 14.1. Any alteration, such as degeneration of any part of the eye, can affect vision.

Thus, ocular degeneration includes several pathologies such as cataracts, glaucoma, macular degeneration, and diabetic retinopathy, which are the ophthalmological diseases with the highest impact in terms of prevalence and morbidity around the world, impairing the quality of life of millions of people (Nuzzi and Vitale 2021). Indeed, in 2020, the diseases leading to major causes of blindness for people 50 years and older worldwide were cataracts with 15.2 million cases, glaucoma with 3.6 million cases, age-related macular degeneration (AMD) with 1.8 million cases, and diabetic retinopathy with 0.86 million cases, for a total of 33.6 million adults who were blind (Steinmetz et al. 2021). These four diseases can be due to multiple factors, but for the first three, the most common one is age. The incidence of all these diseases increases

E. Olivier (✉) · P. Rat
Université Paris Cité, CNRS, CiTCoM, Paris, France
e-mail: elodie.olivier@u-paris.fr

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with age. Due to the lengthening of average life expectancy and thus the aging of the population, the prevalence of patients suffering from glaucoma was estimated to increase from 76 million people in 2020 to 111.8 million in 2040 (Tham et al. 2014). The same trend was predicted for AMD: depending on the studies and the world regions, its prevalence is predicted to increase by 15–50% (Colijn et al. 2017; Li et al. 2020; Keenan et al. 2021). In 2040, 288 million people could suffer from AMD worldwide (Keenan et al. 2021). Nowadays, AMD is the major cause of irreversible vision loss over the age of 55, particularly in industrialized countries (Keenan et al. 2021).

14.1.1 Cataract

Cataract corresponds to a clouding or an opacification of the normal clear lens, resulting in blurred vision as the lens allows the passage of light (Nizami and Gulani 2022). Symptoms vary, including blurred and cloudy vision, colors that seem faded, sensitivity to light, halos, and night vision disorders. At the physiopathological level, the opacification of the lens follows a process of degeneration of the proteins of the lens, which become denatured and coagulate. Severe cataracts are treated by surgery in which the clouded cornea is removed and replaced with a new, clear artificial lens (Thompson and Lakhani 2015).

14.1.2 Glaucoma

Glaucoma refers to a group of optic neuropathies that share the common characteristic of retinal ganglion cell and optic nerve degeneration leading to irreversible vision loss (Schlamp et al. 2006; Weinreb et al. 2014). At a pathophysiological level, elevated intraocular pressure is associated with retinal ganglion cell death. This increase in intraocular pressure is due to an imbalance between the secretion of aqueous humor and its outflow. Depending on the type of glaucoma, the resistance to aqueous outflow is increased in the case of open-angle glaucoma, or the outflow pathways are obstructed in angle-closure glaucoma. The use of medication to reduce intraocular pressure can slow down the disease. With technological advances, surgical techniques and lasers can provide less invasive and better results than before (Weinreb et al. 2014; Kerr 2022).

14.1.3 Macular Degeneration

Macular degeneration refers primarily to AMD, but there are also rarer conditions such as Stargardt's disease and Best's vitelliform macular dystrophy, which can be grouped as juvenile macular degeneration because they occur in childhood or adolescence (Nuzzi and Vitale 2021). At a cellular level, both juvenile and AMD lead to photoreceptor and retinal-pigmented epithelial cell degeneration. This progressive damage causes the impossibility of transforming a light stimulus into a nerve signal and, in fine, the loss of the central visual field. Contrary to AMD, whose main risk factor is age, juvenile macular degeneration's main risk factor is genetic. In this chapter, we will focus on the AMD. AMD at early stages is usually asymptomatic, but the hallmark is the presence of drusen, which accumulates in the macula (Forest et al. 2015). Drusen are extracellular proteolipidic deposits composed of cholesterol-containing lipids, amyloid-beta, apolipoproteins, for example, and also cellular debris (VanDenLangenberg and Carson 2022). The evolution of the disease leads to two clinical forms that can also coexist: dry AMD, the most common, also called non-exudative, which leads to geographic atrophy, and wet AMD, also called neovascular or exudative, which leads to macular neovascularization and induces the most severe vision loss (Ferris et al. 2013). In the advanced stages of the dry form of AMD, a degeneration of the retinal pigmented epithelium, the retina, and the choriocapillaris is observed. In the wet form of AMD, after the recruitment of immune cells, a secretion of proinflammatory cytokines and proangiogenic, factors, especially vascular endothelial-derived growth factor (VEGF), is observed, leading to endothelial cell proliferation

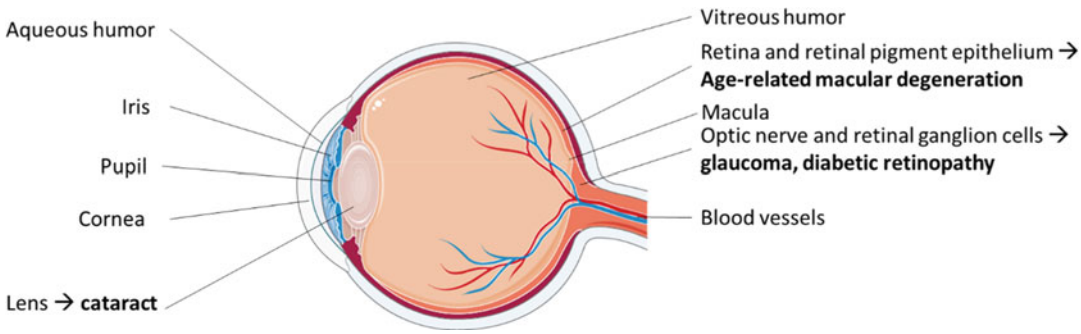

Fig. 14.1 Structure of the eye and target areas of ocular degenerations

and angiogenesis. This neovascularization disrupts and damages the retina, resulting in vision loss (Flores et al. 2021; Hadziahmetovic and Malek 2021; Keenan et al. 2021; Vyawahare et al. 2022). There is no curative treatment for the dry form of AMD; existing treatments consist of intravitreal anti-VEGF molecules to prevent neovascularization in the wet form of AMD (Flores et al. 2021; Nuzzi and Vitale 2021). These repetitive, invasive treatments can place a high burden on patients and be a source of discontinuation of the treatment (Adamis et al. 2020).

14.1.4 Diabetic Retinopathy

Diabetic retinopathy, triggered by hyperglycemia, constitutes the most common complication of diabetes mellitus (Wang and Lo 2018). Contrary to the three other types of ocular degeneration described above, aging is not a risk factor for the development of diabetic retinopathy. Indeed, diabetic retinopathy is a leading cause of visual loss in the working-age population in developed countries (Shukla and Tripathy 2023). At a cellular level, neuronal degeneration is observed from the beginning of diabetic retinopathy and is associated with vision loss (Oshitari 2021). At an early stage, diabetic retinopathy is nonproliferative, but the disease then progresses to a proliferative stage characterized by neovascularization (Wang and Lo 2018). In clinics, after the first stages of vascular permeability and capillary occlusion leading to microaneurysms, hemorrhages, and hard exudates, neovascularization leads to vitreous hemorrhage. Macular edema, which triggers visual loss, is a hallmark of diabetic retinopathy due to the breakdown of the blood–retinal barrier, leading to an accumulation of fluid in the macula. The management of diabetic retinopathy includes the general systemic control of diabetes to prevent hyperglycemia. In the eye, macular edema is treated mostly by laser therapy, while neovascularization is treated first with intravitreal injections of anti-VEGF molecules and then with intravitreal steroids (Heng et al. 2013; Wang and Lo 2018; Shukla and Tripathy 2023).

This chapter will focus on two key players in this ocular degeneration pathogenesis: oxidized derivatives of cholesterol, oxysterols, and the cell degeneration purinoreceptor P2X7.

14.2 Oxysterols in Ocular Degeneration

14.2.1 Oxysterols

Cholesterol is one of the most important molecules in the body. Cholesterol is present in all cells as a key component of the lipid bilayer of the cell membrane and regulates membrane fluidity. Cholesterol is also involved in the biosynthesis of many endogenous molecules, such as steroid hormones, bile acids, and vitamin D (Schade et al. 2020). Cholesterol can be synthesized endogenously but can also come from the diet. Cholesterol can be easily oxidized

on its side chain or on its ring system to form numerous oxidation products, such as oxysterols. Oxysterols can result from a nonenzymatic oxidation of cholesterol or from an enzymatically driven oxidation. Nonenzymatic oxidation, also called autoxidation, can be caused by different chemical and/or physical agents, such as reactive oxygen species, which can result from inflammatory conditions, ultraviolet light, ozone, and metal ions (Vejux et al. 2011; Poli et al. 2022). Enzymatic oxidation is mostly mediated by enzymes belonging to the cytochrome P450 family. The major oxysterols coming from nonenzymatic oxidation are 7-ketocholesterol, 7-β-hydroxycholesterol, 5α,6α-epoxide, 5β,6β-epoxide, and cholestan-3β,5α,6β-triol. 7-α-Hydroxycholesterol and 25-hydroxycholesterol can be partly due to a nonenzymatic oxidation, but they can also be provided by an enzymatic oxidation. 27-Hydroxycholesterol and 24-hydroxycholesterol come exclusively from enzymatic oxidation (Brown et al. 2021; Poli et al. 2022).

Oxysterols are related to a wide variety of diseases concerning many organs, such as ocular, neurological, cardiovascular, metabolic, and cancer diseases (Fig. 14.2).

Oxysterols are associated with neurological and, more precisely, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Björkhem et al. 2006, 2009, 2013; Leoni and Caccia 2014; Kreilaus et al. 2016; Doria et al. 2016; Abdel-Khalik et al. 2017; Vejux et al. 2018, 2021; Griffiths and Wang 2019; Fellows Maxwell et al. 2019; Griffiths et al. 2021). They have also been associated with neurodevelopmental disorders such as autism spectrum disorders (Grayaa et al. 2018). Oxysterols are also related to cardiovascular diseases such as atherosclerosis (Brown and Jessup 1999; Zmysłowski and Szterk 2017; Anderson et al. 2020; Samadi et al. 2021), diabetes mellitus (Yoshioka et al. 2005; Samadi et al. 2019), and osteoporosis (Liu et al. 2005; DuSell et al. 2010; Seo et al. 2023). Numerous cancers, such as breast, prostate, kidney, liver, or colon cancers, are also associated with oxysterols (Nelson et al. 2013; Javitt 2015; Marwarha et al. 2017; Nazih and Bard 2020; Samadi et al. 2021; Biasi et al. 2022). Ocular degeneration is also associated with oxysterols, as detailed below.

14.2.2 Oxysterols and Cataracts

The human lens membrane is known to contain a very high level of cholesterol compared with other membranes of the human body (Girão et al. 1998). The lens is exposed to many environmental stresses, including ultraviolet radiation or ozone, which can be a prooxidant. In clinics, studies showed higher amounts of cholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6-α-epoxycholestanol, 20α-hydroxycholesterol, and 25-hydroxycholesterol in cataractous human lenses when compared to normal lenses, showing an association between oxysterols and cataract (Feldman and Feldman 1965; Girão et al. 1998; Vejux et al. 2011). Another study also highlighted that pathologies characterized by defects in cholesterol metabolism, such as 7-dehydrocholesterol reductase or sterol 27-hydroxylase (CYP27A1), are associated with cataract development (Cenedella 1996). A recent study demonstrated that the oxysterol pathway and lanosterol synthase, the enzyme producing lanosterol, an oxysterol molecule, play a functional role in the pathogenesis of human cataracts (Reyes et al. 2023).

14.2.3 Oxysterols and Glaucoma

As for cataracts, high levels of cholesterol were associated with glaucoma (Posch-Pertl et al. 2022). The association between oxysterols and glaucoma needs to be further explored. Several studies were performed focusing on CYP46A1, also named cholesterol-24S-hydroxylase, which transforms cholesterol into 24S-hydroxycholesterol. Fourgeux et al. showed that the frequency of a polymorphism in intron 2 of the *CYP46A1* gene was higher in patients suffering from glaucoma, but another

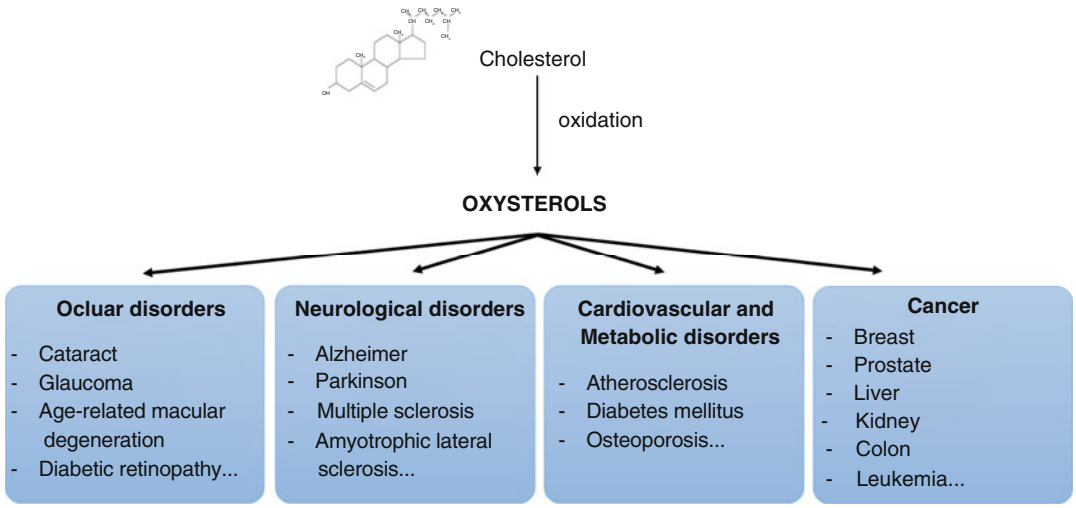

Fig. 14.2 Oxysterols are associated with a wide variety of diseases

epidemiologic study did not find the same result (Fourgeux et al. 2009; Mossböck et al. 2011). However, intraocular pressure, a hallmark of glaucoma, was associated with the transient induction of CYP46A1 and the increase of 24S-hydroxycholesterol levels (Fourgeux et al. 2012b; Ishikawa et al. 2016). According to Ishikawa et al. (2016), 24S-hydroxycholesterol may have neuroprotective properties in pathological conditions of glaucoma.

14.2.4 Oxysterols and AMD

Elevated levels of cholesterol were associated with AMD (Mares-Perlman et al. 1995; Dasari et al. 2011; Nordestgaard et al. 2021; Kananen et al. 2021). In clinics, some studies demonstrated the association between oxysterols and AMD either at the level of the oxysterols themselves or of the genes involved in their formation or binding. Indeed, 7-ketocholesterol has been found in significant amounts in the proteolipidic deposits characteristic of AMD called drusen (Javitt and Javitt 2009; Rodriguez et al. 2014). The role of some allelic variants of the oxysterol-binding-protein (*OSBP2*) gene, mainly expressed in the retinal pigmented epithelium, was highlighted in patients with AMD (Torrini et al. 2007). *OSBP2* gene products bind and transport oxysterols such as 7-ketocholesterol and may inhibit their cytotoxicity. According to another study, an allele in the cholesterol-24S-hydroxylase (*CYP46A1*) gene could confer a higher risk for the exudative form of AMD without any risk alleles in genes coding for members of the complement factor H, for example, Fourgeux et al. (2012a). Cholesterol-24S-hydroxylase, expressed in the neural retina, metabolizes the cholesterol of the neurons into 24S-hydroxycholesterol, an oxysterol that may be involved in the triggering of degenerative diseases such as AMD (Bretillon et al. 2007).

These clinical observations were completed by numerous in vivo and in vitro experiments to study the pathophysiological role of oxysterols in AMD.

Oxidative stress plays a key role in the pathogenesis of AMD (Datta et al. 2017; Ozawa 2020; Ruan et al. 2021). Oxysterols, which can be formed by oxidative stress, can also trigger oxidative stress. Indeed, in vitro studies performed in retinal epithelium pigmented cells demonstrated that oxysterols, such as 27-hydroxycholesterol, 25-hydroxycholesterol, 24-hydroxycholesterol, 7-ketocholesterol, or 7β-hydroxycholesterol induced oxidative stress via reactive oxygen species production, glutathione depletion, or induction of Heme oxygenase-1 (HO-1) as a sensitive marker of oxidative stress (Joffre et al. 2007;

Dasari et al. 2010; Dugas et al. 2010; Olivier et al. 2016; Xie et al. 2022). As oxysterols trigger oxidative stress, which can promote oxysterol formation, it can constitute a vicious circle, inducing retinal pigmented epithelium degeneration and worsening AMD. Mitochondria are an important source of reactive oxygen species, and thus, oxidative stress increases in cases of mitochondrial dysfunction. Oxysterols can damage mitochondria, resulting in mitochondrial dysfunction via altered calcium homeostasis, mitochondrial porin levels, loss of mitochondrial membrane potential, and alteration of mitochondrial DNA (Dasari et al. 2010; Gramajo et al. 2010; Xie et al. 2022). This loss of mitochondrial membrane potential can be closely linked to apoptosis. Apoptosis induced by oxysterols has been highlighted in vivo and in vitro. Indeed, in mice, 7-ketocholesterol induced apoptosis in retinal pigmented epithelial cells (Xie et al. 2022); the same oxysterol induced caspase-dependent apoptosis in human retinal pigmented epithelial cells (Luthra et al. 2008). Other oxysterols, such as 27-hydroxycholesterol, also trigger apoptosis in human retinal pigmented epithelial cells (Dasari et al. 2010). According to another study, 7-ketocholesterol and 7β-hydroxycholesterol only induced a slight apoptosis but rather induced a phospholipid accumulation called phospholipidosis, leading to cell death (Dugas et al. 2010).

Oxysterols were associated with inflammatory processes via the activation of several kinase signaling pathways, inducing among others, proinflammatory cytokine transcription (Vejux et al. 2008; Vejux and Lizard 2009; Pariente et al. 2019). More particularly concerning the AMD, in vitro studies conducted in primary porcine retinal pigmented epithelial cells and in human retinal pigmented epithelial cells demonstrated that 25-hydroxycholesterol, 24-hydroxycholesterol, and 7-ketocholesterol induced IL-8 gene expression and IL-8 protein secretion (Joffre et al. 2007; Dugas et al. 2010), while another study in human retinal pigmented epithelial cells demonstrated that 7-ketocholesterol activated the inflammasome and stimulated the secretion of the proinflammatory cytokine IL-18 (Shi et al. 2015). This production of proinflammatory cytokines leads to chronic inflammation related to AMD (Rodríguez and Larrayoz 2010). Oxysterols are also associated with neovascularization. Studies conducted in human retinal pigmented epithelial cells highlighted an increase in VEGF secretion after incubation with 7-ketocholesterol, 7β-hydroxycholesterol, or 25-hydroxycholesterol (Moreira et al. 2009; Dugas et al. 2010).

14.2.5 Oxysterols and Diabetic Retinopathy

As with the other three ocular degenerations described above, cholesterol levels are higher in the retina in cases of diabetic retinopathy (Jenkins et al. 2022). Regarding cholesterol circulating levels, the results of the performed studies are inconsistent; a meta-analysis concluded a slight association between LDL cholesterol and diabetic retinopathy (Zhou et al. 2018; Jenkins et al. 2022). However, lipid abnormalities, including cholesterol abnormalities and dysregulation, are associated with diabetic retinopathy (Busik 2021; Rao et al. 2021; Jenkins et al. 2022). In the diabetic retina, a decrease in both cytochromes P450 27A1 (which forms 27-hydrocholesterol) and 46A1 (which forms 24-hydroxycholesterol) was observed, leading to an oxysterol level decrease (Jenkins et al. 2022). This decrease in oxysterol levels was observed in a rat model, suggesting a decrease in Liver X Receptor (LXR) activation, as oxysterols are endogenous ligands of LXR and could lead to inflammatory processes as LXR controls inflammation (Hammer et al. 2017). However, further studies are needed to better understand the association between oxysterol and diabetic retinopathy, as other types of oxysterol could be involved. Indeed, 7-ketocholesterol, 7-β-hydroxycholesterol, cholesterol-α-epoxide, cholesterol-β-epoxide, and cholestane-3β,5α,6-β-triol levels are increased in the plasma of patients suffering from diabetes (Ferderbar et al. 2007; Samadi et al. 2019, 2020).

14.3 The P2X7 Receptor and Ocular Degeneration

14.3.1 The P2X7 Receptor

The P2X7 purinoreceptor, as its endogenous agonist is ATP, belongs to the ligand-gated cationic channel family P2X, composed of seven subtypes in mammals (P2X1–P2X7). The P2X7 receptor was identified by Surprenant's group in 1996 (Surprenant et al. 1996) but was previously known as the ATP^{4-} receptor or P2Z receptor (Cockcroft and Gomperts 1980; Ferrari et al. 1996). The P2X7 receptor is the longest of the P2X7 family, with 595 amino acids in length (Khakh et al. 2001). The P2X7 receptor is also the most ATP-insensitive of the P2X receptors, requiring 50 μM to 2.5 mM to be activated (Surprenant et al. 1996; Matyśniak et al. 2022). The human *P2X7* gene is located on chromosome 12 (Sluyter 2017). In addition to the common form of the *P2X7* gene, nine human splice variants exist (Sluyter 2017; Di Virgilio et al. 2017). The P2X7 receptor is composed of three subunits. A subunit is a polypeptide composed of two transmembrane regions (TM1 and TM2) with intracellular N- and C-termini and a bulky extracellular loop (Illes et al. 2021). The P2X7 purinoreceptor, as a cationic channel, allows the entry of Na^+ and Ca^{2+} ions into the cells and the exit of K^+ ions. The P2X7 can also form or promote the opening of a nonselective pore, allowing the passage of molecules up to 900 Da, as detailed in Fig. 14.3 (Di Virgilio et al. 2018). Indeed, the molecular mechanisms for this pore formation are still enigmatic. Several hypotheses exist, such as the dilation of the P2X7 receptor itself during prolonged stimulation, the recruitment of other P2X7 subunits during prolonged stimulation to form a large pore, or the involvement of other molecules such as the hemichannel pannexin-1 (Surprenant et al. 1996; Kim et al. 2001; Ferrari et al. 2004; Pelegrin and Surprenant 2006; Di Virgilio et al. 2017, 2018). The microenvironment in the membrane can play a key role by stimulating the pore opening (Robinson et al. 2014; Karasawa et al. 2017; Murrell-Lagnado 2017).

P2X7 receptors are found throughout the body as they are expressed by a wide variety of cells, such as immune cells (lymphocytes and macrophages; Di Virgilio et al. 2017), brain cells (microglia and oligodendrocytes; Sperlágh et al. 2006), skin cells (fibroblasts and keratinocytes; Solini et al. 1999; Inoue et al. 2005), and bone cells (osteoclasts and osteoblasts; Agrawal and Gartland 2015). P2X7 receptors are also expressed in ocular cells such as conjunctival, corneal, lens, retinal pigmented epithelial, and retinal ganglion cells (Suzuki-Kerr et al. 2008; Dutot et al. 2008; Mayo et al. 2008; Xue et al. 2016; Minns et al. 2016). P2X7 receptor activation triggers numerous biological processes such as cell survival, cell proliferation, and inflammation (Solini et al. 1999; Ferrari et al. 2006; Di Virgilio 2012; Di Virgilio et al. 2017). The P2X7 receptor has been associated with a wide variety of diseases, including infectious, inflammatory, neurodegenerative, cardiovascular, and cancer (Oliveira-Giacomelli et al. 2018; Adinolfi et al. 2018; Guerra Martinez 2019; Andrejew et al. 2020; Lara et al. 2020).

14.3.2 P2X7 Activation and Ocular Degeneration

The P2X7 receptor can be localized in membrane microdomains rich in cholesterol and phospholipids called lipid raft domains (Garcia-Marcos et al. 2006; Gonnord et al. 2009). Cholesterol has been associated with the activation and sensitization of the P2X7 receptor. Indeed, in cases of cholesterol depletion, the P2X7 pore dilatation is enhanced, and vice versa (Robinson et al. 2014). All of the ocular degenerations described above are linked to cholesterol dysregulation and thus could impact P2X7 receptor activation.

In the different ocular degenerations described above, the ATP levels, the natural endogenous agonist of the P2X7 receptor, are modified.

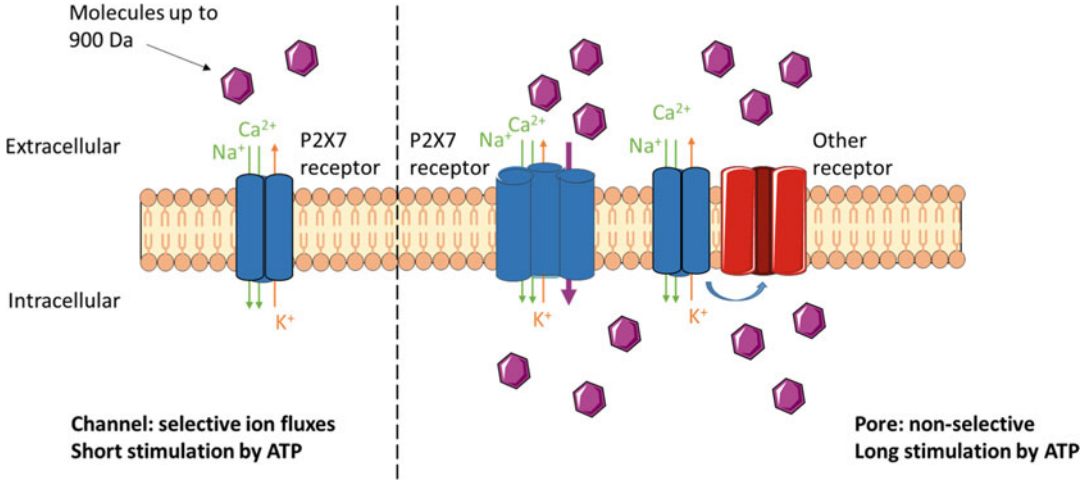

Fig. 14.3 Channel and pore P2X7 formation. Different hypotheses exist regarding P2X7 pore formation. P2X7 can form or promote the opening of a nonselective pore

Indeed, in cases of intraocular pressure elevation, a hallmark of glaucoma, ATP levels were increased in in vitro and in vivo animal models (Reigada et al. 2008; Lu et al. 2015; Shinozaki et al. 2023). The ATP level increase was also observed in a model of glaucomatous mice (Pérez de Lara et al. 2015) and, above all, in human patients suffering from glaucoma (Zhang et al. 2007; Li et al. 2011). Regarding AMD, the levels of ATP in patients suffering from AMD with vitreous hemorrhage are higher than in other patients (Notomi et al. 2013), and for diabetic retinopathy, the levels of ATP were higher in the vitreous of patients suffering from that disease (Loukovaara et al. 2015). This accumulation of ATP has also been demonstrated in in vitro models that mimic diabetic retinopathy with hyperglycemic conditions (Costa et al. 2009; Shivashankar et al. 2021). These increases in ATP were correlated with degeneration mechanisms such as retinal epithelial cells, photoreceptors, retinal ganglion cells, and microvascular cell death. ATP dysregulation was also observed in cataract pathogenesis, but contrary to the three other ocular degenerations we studied, lens ATP levels seemed to decrease (Deussen and Pau 1989; Nabekura et al. 2004; Kisic et al. 2012; Swarup et al. 2018).

The P2X7 receptor is closely linked to many ocular disorders, including ocular degeneration, and is being considered as a therapeutic target (Reichenbach and Bringmann 2016; Dutot et al. 2017; Platania et al. 2022).

Few data exist about the association between cataracts and the P2X7 receptor. Cotlier demonstrated that the stimulation of purinergic receptors, including P2X7, in animal and human crystalline lenses may initiate cataracts (Cotlier 2004). An eye morphology phenotypic assay performed in mice also demonstrated the association between P2X7 and cataracts, as they identified that mice with lens opacity are mutated for P2X7. The data are available at the International Mouse Phenotyping Consortium (IMPC) website (www.mousephenotype.org).

The P2X7 receptor is associated with glaucoma and its elevated ocular pressure. Indeed, studies including in vivo work with elevated intraocular pressure demonstrated the role of P2X7 in retinal ganglion cell dysfunction via an increase in P2X7 receptor expression and activation of this receptor (Mitchell et al. 2009; Sugiyama et al. 2013; Zhang et al. 2019; Wang et al. 2021). The reduction of blood flow to the optic nerve, leading to ischemia and retinal ganglion cell death, is part of the pathogenesis of glaucoma. Niyadurupola et al. (2013) highlighted

in a human organotypic retinal model of ischemic neurodegeneration that retinal ganglion cell death is mediated by P2X7 receptor activation. Pérez de Lara et al. (2019) more recently demonstrated using a mouse glaucoma model the association between the P2X7 receptor and the pathogenesis of glaucoma. Taken together, all of these studies showed the role of P2X7 in glaucoma pathogenesis through the activation of many cellular signaling pathways, such as the NLRP3 inflammasome, the MAPK signaling cascade, and the caspase pathway leading to retinal ganglion cell death.

The P2X7 receptor is also associated with AMD. A genetic study associated a combination of P2X7 and P2X4 variants with an increased susceptibility to AMD (Gu et al. 2013). Several studies performed on animals showed that P2X7-null mice developed the characteristics of early AMD (Vessey et al. 2017), P2X7 is involved in the pathogenesis of dry AMD through inflammasome activation in mice (Kerur et al. 2013), and in the pathogenesis of wet AMD through VEGF secretion (Mizutani et al. 2015). Moreover, P2X7 triggered photoreceptor apoptosis in a mouse model of subretinal hemorrhage, a related event of wet AMD (Notomi et al. 2013). In vitro studies, performed in human retinal pigmented epithelial and human retinal glial models mimicking AMD using amyloid β or oxysterols, demonstrated the role of the P2X7 receptor in the pathogenesis of AMD, especially the apoptosis of retinal cells (Wakx et al. 2016; Olivier et al. 2016). The P2X7 receptor triggered Ca^{2+} extracellular levels and caspase-dependent apoptosis in retinal pigmented epithelial cells, processes associated with AMD pathogenesis (Yang et al. 2011). P2X7 also plays a key role in immune cell phagocytosis, leading to the accumulation of subretinal deposits and, consequently, to retinal cell inflammation and death (Gu et al. 2013; Vessey et al. 2017). More recently, P2X7 has been associated with alterations of membrane fluidity in immune cells occurring during late AMD (Drysdale et al. 2022). P2X7's role in membrane fluidity was shown to be independent of its pore formation or its role in phagocytosis.

As with the other ocular degenerations, diabetic retinopathy is associated with P2X7. An in vivo study in mice showed an increase in P2X7 expression in the retinal tissues of diabetic mice compared to control mice (Kong et al. 2022). In addition, Sugiyama et al. (2004) demonstrated that diabetic retinal microvessels were more vulnerable to P2X7 receptor activation, leading to apoptosis in retinal capillaries, yet dysfunction of the retinal microvasculature is a feature of diabetic retinopathy. They also highlighted that retinal blood velocity was reduced by P2X7 receptor activation and that these effects are more marked in diabetic rabbits than in control rabbits (Sugiyama et al. 2006). According to Shibata et al. (2018), the vasotoxicity triggered by P2X7 receptor activation is associated with an increase in intracellular calcium concentration, an activation of K_{ATP} channels, and the production of pro-oxidant species such as reactive oxygen species. Furthermore, using an in vitro model of early diabetic retinopathy, Platania et al. (2017, 2019) showed the association between the P2X7 receptor and the release of the proinflammatory cytokine IL1-β from human retinal pericytes and human retinal endothelial cells, highlighting the role of P2X7 in the inflammatory process that occurs in diabetic retinopathy. This inflammatory environment is involved in the blood–retinal barrier breakage observed in diabetic retinopathy; the P2X7 receptor plays an important role in the regulation of this barrier integrity (Platania et al. 2019).

14.3.3 P2X7 Activation and Oxysterols

Oxysterols and P2X7 activation are both associated with ocular degeneration. In our previous work, we demonstrated that oxysterol toxicity is mediated by P2X7 receptor activation in a human retinal pigmented epithelial model (Olivier et al. 2016). Indeed, 7-ketocholesterol and 25-hydroxycholesterol, both associated with AMD as developed above (Dasari et al. 2010; Poli et al. 2013), activated the P2X7 receptor. Depending on the oxysterol, P2X7 receptor activation is dependent on pannexin-1 (for

25-hydroxycholesterol) or not (for 7-ketocholesterol). This pathway could be associated with the pathogenesis of AMD and constitute a target for future treatments. We also demonstrated the link between oxysterols and P2X7 activation in another tissue at the interface between the environment and the human body: the skin. Indeed, we demonstrated in a human skin model the involvement of P2X7 receptor activation and inflammasome activation induced by oxysterols (Olivier et al. 2017). More precisely, the 25-hydroxycholesterol triggered the activation of the P2X7 receptor, the activation of caspase 1, the key component of the inflammasome complex, and the release of several proinflammatory cytokines. This concomitant activation of the P2X7 receptor and the inflammasome corresponds to pyroptosis.

14.4 Conclusion

Ocular degeneration, which includes several pathologies such as cataracts, glaucoma, AMD, and diabetic retinopathy, is a major public health challenge. Indeed, these pathologies are the leading cause of blindness worldwide. Both oxysterols and the P2X7 receptor play a key role in the pathogenesis of these diseases, as both oxysterols and P2X7 receptor activation have been associated with mechanisms of inflammation and cell death. Oxysterols and the P2X7 receptor could be linked to the triggering of these mechanisms, as demonstrated in the case of AMD. Further studies are needed to better understand whether oxysterols and P2X7 receptor activation are linked to other ocular degenerations.

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15

24S-Hydroxycholesterol in Neuropsychiatric Diseases: Schizophrenia, Autism Spectrum Disorder, and Bipolar Disorder

Meriam Messedi and Fatma Makni-Ayadi

Abstract

Neuropsychiatric diseases (NPDs) are severe, debilitating psychiatric conditions that affect the nervous system. These are among the most challenging disorders in medicine. Some examples include Alzheimer's, anxiety disorders, autism spectrum disorder, bipolar disorder, and schizophrenia. NPDs represent an ever-increasing burden on public health and are prevalent throughout the world. For most of these diseases, the particular etiopathogeneses are still enigmatic. NPDs are also associated with structural and functional changes in the brain, along with altered neurotransmitter and neuroendocrine systems.

Approximately 25% of the total human body cholesterol is located in the brain. Its involvement in neuronal functions starts in the early growth stages and remains important throughout adulthood. It is also an integral part of the neuronal membrane, ensuring membrane lipid organization and regulating membrane fluidity. The main mechanism for removing cholesterol from the brain is cholesterol 24-hydroxylation by cytochrome P450 46A1 (CYP46A1), an enzyme specifically found in the central nervous system. Although research on 24S-OHC and its role in neuropsychiatric diseases is still in its early stages, this oxidized cholesterol metabolite is thought to play a crucial role in the etiology of NPDs. 24S-OHC can affect neurons, astrocytes, oligodendrocytes, and vascular cells. In addition to regulating the homeostasis of cholesterol in the brain, this oxysterol is involved in neurotransmission, oxidative stress, and inflammation. The role of 24S-OHC in NPDs has been found to be controversial in terms of the findings so far. There are several intriguing discrepancies in the data gathered so far regarding 24S-OHC and NPDs. In fact, 24S-OHC levels were reported to have decreased in a number of NPDs and increased in others.

Hence, in this chapter, we first summarize the available data regarding 24S-OHC as a biomarker in NPDs, including schizophrenia, autism spectrum disorder, and bipolar disorder. Then, we present a brief synopsis of the pharmacological targeting of 24S-OHC levels through the modulation of CYP46A1 activity.

M. Messedi (✉)
Research Laboratory "Molecular Basis of Human Diseases", LR19ES13, Sfax Medicine School, University of Sfax, Sfax, Tunisia

F. Makni-Ayadi
Research Laboratory "Molecular Basis of Human Diseases", LR19ES13, Sfax Medicine School, University of Sfax, Sfax, Tunisia

Department of Clinical biochemistry, Habib Bourguiba Hospital, Sfax, Tunisia

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Keywords

Neuropsychiatric diseases · Brain · 24S-hydroxycholesterol · CYP46A1 · Schizophrenia · Autism spectrum disorder · Bipolar disorder

Abbreviations

24S-OHC	24S-hydroxycholesterol
ABC	ATP-binding cassette
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ASD	Autism spectrum disorder
Aβ	β-amyloid
BD	Bipolar disorder
CNS	Central nervous system
DSM-V	5th edition of the Diagnostic and Statistical Manual of Mental Disorders
ICD-10	10th revision of International Classification of Diseases
L-Glu	L-glutamate
LRP1	Low-density lipoprotein receptor-related protein 1
LXR	Liver X receptor
NMDARs	*N*-methyl-D-aspartate receptors
NPDs	Neuropsychiatric diseases
ROC	Receiver operating characteristic

15.1 Introduction

Neuropsychiatric diseases (NPDs) are neurological and psychiatric disorders that affect the nervous system. Some examples include Alzheimer's, anxiety disorders, autism spectrum disorder, bipolar disorder, and schizophrenia. They range from mild to severe and can cause profound effects on several aspects of life, including physical health, relationships, and overall quality of life.

NPDs represent an ever-increasing burden on public health and are prevalent throughout the world. Indeed, the estimated number of instances of mental diseases increased by 48.1% from 654.8 million cases in 1990 to 970 million in 2019 (Collaborators 2022). Moreover, the World Health Organization estimates that the global burden of neuropsychiatric illnesses is 13% greater than that of diseases like cancer and cardiovascular disease (Organization 2008).

NPDs are complex and heterogeneous. Their exact etiopathogeneses are not fully understood. However, they are believed to be a complex interplay of genetic, epigenetic, environmental, and neurobiological factors. It is worth mentioning that NPDs are also related to structural and functional brain changes, as well as alterations in neurotransmitters and neuroendocrine systems. Research on neuropsychiatric diseases is ongoing to better understand the causes of these disorders. As an early diagnosis can improve outcomes for individuals living with these conditions, one of the major concerns of scientists is identifying putative biomarkers. Recent advances in omics techniques have opened new opportunities for diagnosing devastating diseases, including NPD. Recent lipidomics-based technologies contribute to highlighting the role of cholesterol metabolites in the pathogenesis of NPDs. Indeed, the brain holds about 20% of the body's total cholesterol, making it the most cholesterol-rich organ in the body (Björkhem and Meaney 2004). Disruption of cerebral cholesterol metabolism has been associated with negative outcomes that lead to diseases of the human brain, including NPDs (Sethi et al. 2017). Emerging evidence suggests that cholesterol oxidation products, called oxysterols, maybe the link between altered cholesterol metabolism and NPDs. Among the different brain oxysterols, 24S-hydroxycholesterol (24S-OHC) is prevalent, with a crucial function as a leading signaling molecule. The excess cholesterol in the brain is metabolized into more hydrophilic cerebrosterol, the 24S-OHC. Apolipoprotein E (ApoE) is combined with cholesterol synthesized by astrocytes to form lipoproteins. ATP-binding cassette (ABC) transporters on the astrocyte cell membrane transport these lipoproteins into the extracellular fluid. The apoE-containing lipoproteins deliver cholesterol from astrocytes to neurons. These lipoprotein particles containing cholesterol are internalized

into neurons after interacting with surface lipoprotein receptors such as the low-density lipoprotein receptor-related protein-1 (LRP1). A neuronal-specific and rate-limiting enzyme, the cytochrome P45046A1 (CYP46A1 or cholesterol 24-hydroxylase), catalyzes the hydroxylation of cholesterol to 24S-OHC, which can diffuse out of the neuron and pass over the blood–brain barrier (Fig. 15.1). But before this molecule crosses the blood–brain barrier, it can affect neurons, astrocytes, oligodendrocytes, and vascular cells locally, modulating their functioning. In addition to regulating the homeostasis of cholesterol in the brain, 24S-OHC is thought to influence the growth and operation of the nervous system.

It is important to note that further studies will be needed to fully understand how 24S-OHC is involved in NPDs. However, oxysterol seems to play a crucial role in the etiology of neuropsychiatric diseases, according to some research. Cyp46a1 knockout mice exhibited severe neurological defects in spatial learning and memory, demonstrating its critical role in neuronal function (Kotti et al. 2006; Djelti et al. 2015).

The role of 24S-OHC in NPDs has been found to be controversial in terms of the findings so far. However, it is not clear why the activity of CYP46A1 and consequently 24S-OHC levels would undergo a drastic change depending on the disease and the cellular context. For instance, the data gathered with respect to 24S-OHC in NPDs has thus far revealed several intriguing discrepancies. Alterations in 24S-OHC levels were reported in a number of NPDs, including Alzheimer's disease and Parkinson's disease (Roy et al. 2019). Withal, patients with cognitive impairment displayed increased 24S-OHC levels (Shi et al. 2021).

The purpose of this chapter is first to comprehensively examine the available data regarding the 24S-OHC as a biomarker in NPDs, including schizophrenia, autism spectrum disorder, and bipolar disorder. Then, we present a brief synopsis of the pharmacological targeting of 24S-OHC levels through the modulation of CYP46A1 activity.

15.2 NPDs Associated with High Levels of 24S-OHC

15.2.1 Schizophrenia

Schizophrenia is a psychiatric disorder with a lifetime prevalence of about 1% worldwide (Kahn et al. 2015). It has devastating effects on individuals and a huge financial burden on healtcare systems. It is a heterogeneous illness characterized by positive and negative symptoms and cognitive dysfunction. Hallucinations, delusions, and thought disorders are some of the positive symptoms of schizophrenia. Negative symptoms include social withdrawal, flattened affect, and amotivation. Despite the fact that the etiology of schizophrenia is still poorly understood and the biochemical focus has been on glutamate and dopamine, numerous neuromodulators, neurotransmitters, and neuroactive steroids, such as oxysterols, seem to be involved in this disorder.

To date, three studies have investigated the plasma levels of 24S-OHC in patients with schizophrenia (Table 15.1). The first one was conducted on schizophrenia patients and individuals with high clinical risk (Sun et al. 2021). The authors found a significantly increased level of 24S-OHC in either schizophrenic or high clinical-risk patients when compared to healthy controls. However, other researchers reported no significant difference in this oxidized cholesterol metabolite's plasma levels in schizophrenia patients (Guidara et al. 2022; Chiappelli et al. 2020). The dissimilarities in findings might be due to the differences in sample size, sex ratio, and antipsychotic drug use in the studied populations. Nonetheless, on the basis of statistical analyses and receiver operating characteristic (ROC) curves, all the studies failed to properly assess 24S-OHC as a potential biomarker in schizophrenia.

It has been shown that 24S-OHC may promote inflammation, autophagy, oxidative stress, and necroptosis, which are the main driving forces in schizophrenia (Nóbrega et al. 2020; Zarrouk et al. 2015). Furthermore, increased 24S-OHC levels in

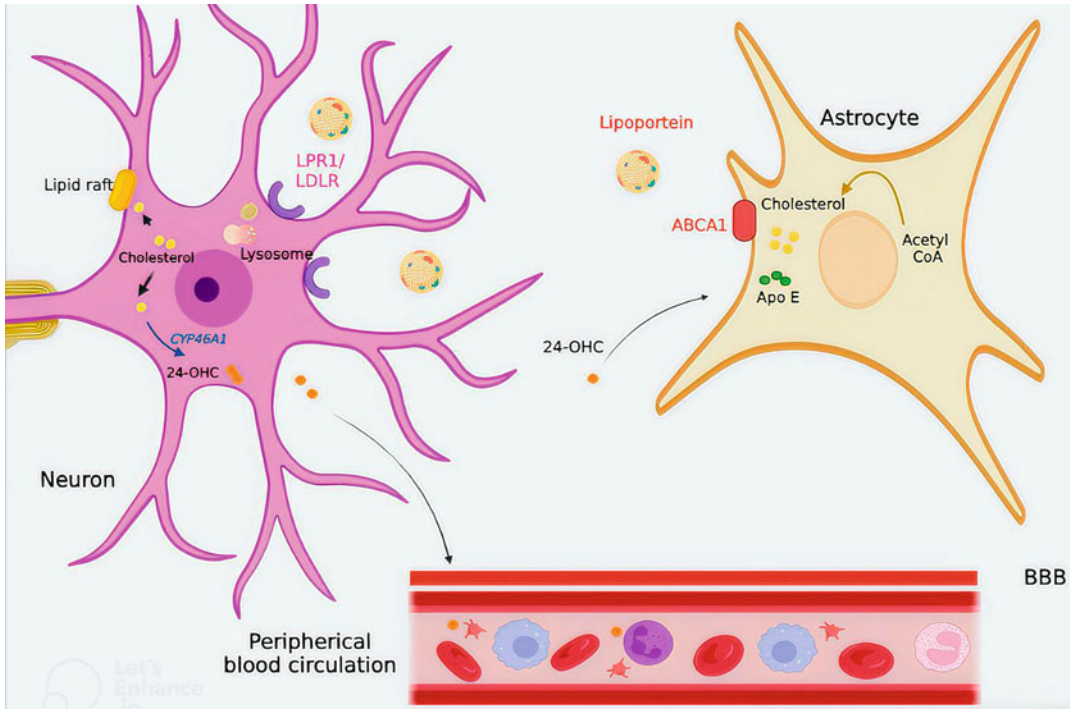

Fig. 15.1 24-OHC metabolism brain. The majority of the metabolite 24S-OHC (orange spheres) is formed in neurons by activating CYP46A1, which uses cholesterol as a substrate (yellow spheres). Because neurons have a limited rate of cholesterol production, cholesterol-containing lipoprotein particles are imported through the LRP1/LDLR pathway to meet the needs of the neurons for this lipid. Astocytic cholesterol is exported to the extracellular environment via ABC transporters, and these particles are extracellularly combined with cholesterol and apolipoproteins E (apoE) synthesized in astrocytes. In order to alter transcription, the produced 24S-OHC can stay inside the neurons, diffuse into the nucleus, and activate LXRs. The nuclei of the other integrants of the neurovascular unit can likewise be reached by this soluble oxysterol by traversing various cell membranes (astrocytes, pericytes, and vascular cells). *ABC* ATP-binding cassette transporter, *ApoE* apolipoprotein E, *LRP1* low-density lipoprotein receptor-related protein 1, *LDLR* low-density lipoprotein receptor, *CYP46A1* cytochrome P450 46A1, *24S-OHC* 24S-hydroxycholesterol, *BBB* blood–brain barrier

Table 15.1 Studies of 24S-hydroxycholesterol levels in schizophrenia patients

Study	Sample size	Age (years)	Sex ratio (M/F)	24- hydroxycholesterol levels (nmol/L)	*P*-value
Sun et al. (2021)	76 schizophrenia patients 39 clinical high-risk subjects 101 healthy controls	25.68 ± 6.85 24.28 ± 4.78 26.45 ± 4.57	34/42 24/15 62/39	69.25 ± 22.23 70.87 ± 30.24 68.21 ± 21.75	0.020
Guidara et al. (2022)	40 schizophrenia patients 40 healthy controls	36.50 (31–40.5) 35 (31–44)	40/0 40/0	112.30 (94.15–149.35) 127.20 (106.05–149.80)	0.22
Chiappelli et al. (2020)	226 schizophrenia patients 204 healthy controls	35.6 ± 12.9 34.7 ± 13.6	156/70 98/106	88.89 ± 35.26 94.36 ± 31.78	0.79

the brain have been shown to produce schizophrenia-like symptoms in animal models (Lu et al. 2021).

As 24S-OHC is one of the few ways for the brain to recycle excess cholesterol, it is believed that its plasma levels could reflect the amount of myelin remodeling processes and therefore might be related to brain structure. It has been reported that high levels of 24S-OHC in the brain can induce drastic cerebral changes in mice, such as a thinner cortex (Lu et al. 2021), which is associated with schizophrenia (Van Erp et al. 2018; Weerasekera et al. 2022).

Cyp46a1 (the mouse ortholog of CYP46A) upregulation results in increased brain production of 24S-OHC, leading to its elevation in the bloodstream as well. This upregulation may be the result of excitotoxicity since oxidative stress, a hallmark of schizophrenia (Pandolfo et al. 2022; Murray et al. 2021), is known to increase Cyp46A1 promoter activity (Sodero et al. 2011). There is also the possibility that 24S-OHC produced by upregulation of CYP46A1 may cause dysfunction of *N*-methyl-D-aspartate receptors (NMDARs), which has an outstanding impact on brain function and cognition and has been involved in many neuropsychiatric disorders pathophysiology, such as schizophrenia (Nakazawa and Sapkota 2020). NMDARs are a major subtype of glutamate receptors that mediate excitatory transmission throughout the central nervous system (CNS). The glutamate pathway disturbance could trigger dysfunctions in neurons (Green et al. 2021). It is believed that 24S-OHC can alter NMDAR's functions since it is a potent allosteric modulator (Sun et al. 2016). The use of NMDAR antagonists like ketamine can result in psychotic symptoms, memory loss, and cognitive deficiencies. The study of Paul et al. (2013a) reported that systemically administered 24S-OHC analogs corrected NMDAR channel blocker-induced behavioral abnormalities in social interactions.

15.2.2 Autism Spectrum Disorder

Autism spectrum disorder (ASD) is defined as a developmental disorder according to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) (American Psychiatric Association and Association 1994) and the International Classification of Diseases, 10th Revision (ICD-10) (Organization 2004). In ASD, significant impairment in social communication occurs along with repetitive and/or restrictive behaviors and interests, irrespective of socioeconomic class, race, or culture (Lord et al. 2018). ASD symptoms vary widely in severity, and significant comorbidities are often associated with them, including intellectual disability, epilepsy, anxiety, and gastrointestinal problems (Hirota and King 2023).

ASD arises generally in the early developmental period, before the age of 3 years, mainly in boys (Lord et al. 2018), and its global prevalence is about 100/10,000 (Zeidan et al. 2022).

The pathogenesis of ASD is not fully understood. Early neural reorganization and altered brain development likely contribute to ASD development. Several factors appear to contribute to the onset of ASD. These factors include epigenetic interactions involving genes and the environment. Research to date has highlighted the critical role of synapse architecture and functionality in ASD etiology. Indeed, during brain development, ASD seems to be caused by an imbalance between excitatory and inhibitory synapses and a dysfunction in synaptic plasticity, leading to a disruption of connectivity (Serati et al. 2019; Masini et al. 2020). As cholesterol was demonstrated to be involved in synaptic functioning, it was hypothesized that cholesterol as well as its oxidized metabolites might also be involved in ASD etiology. The study by Grayaa et al. (2018) reported significantly increased levels of 24S-OHC in ASD patients. The authors provided evidence that ASD is associated with increased levels of 24S-OHC. The latter can also be suggested as a potential biomarker for ASD diagnosis. The ROC curve showed an area under the curve of 92%, and the predictive threshold

value was about 103.89 nmol/L. The sensitivity and specificity of 24S-OHC as a biomarker of ASD were 96.3% and 73.7%, respectively.

In ASD patients, high levels of 24S-OHC may cause functional changes in the brain via multiple mechanisms, such as cytotoxicity and apoptosis (Sodero 2021; Nury et al. 2015; Mukhutdinova et al. 2019), which are known to play a role in the disease's pathophysiology (Jiang et al. 2022; Dong et al. 2018). Moreover, the increased levels of 24S-OHC may cause changes in the neuronal circuit function resulting from glutamate excitotoxicity in ASD (Bjorklund et al. 2020). Increased concentrations of 24S-OHC could exacerbate neurotoxicity through overactivation of NMDARs (Paul et al. 2013b). Excessive NMDAR activity causes the rapid opening of certain ion channels and a significant increase in glutamate release. These changes enhance mitochondrial dysfunction and oxidative stress up-regulation (Yildizhan and Naziroglu 2023). These features are closely linked to ASD (Thorsen 2020). It has also been reported that high 24S-OHC levels were associated with higher total brain volume (Koschack et al. 2009), which is typical of ASD (Stanfield et al. 2008; Weerasekera et al. 2022). Furthermore, high levels of 24S-OHC may be related to β-amyloid (Aβ) formation suppression through liver X receptor (LXR) activation, favoring an anabolic state and amyloid precursor protein (APP) accumulation (Urano et al. 2013; Noguchi et al. 2014). Indeed, APPα accumulation is accompanied by intracranial neuron overgrowth in both density and size, which promotes the enlargement of the brain in ASD (Sokol et al. 2019).

15.3 NPD Associated with Low Levels of 24S-OHC

15.3.1 Bipolar Disorder

Bipolar disorder (BD) is a chronic, severe mood disorder characterized by mood fluctuations that alternate between current episodes of depression, hypomania, or mania and mixed states (Carvalho et al. 2020). The oscillation between these phases is the main characteristic that separates BD from other affective disorders. As a lifelong illness, BD can have a significant impact on the mental and physical health of patients and their caregivers. Moreover, in terms of disability, morbidity, and mortality from suicide, BD continues to lead the world in terms of burden (Stanley et al. 2017; Plans et al. 2019). According to the longitudinal course of bipolar illness, which is frequently characterized by the presence of subthreshold symptoms, BD can be classified into types II and I. While bipolar II disorder has a high episode frequency, high rates of psychiatric comorbidities, and recurrent suicidal behaviors that negatively impact life quality, bipolar I disorder may appear to have a more difficult evolution and a more dire prognosis due to the severity of the cross-sectional symptoms (Carvalho et al. 2020).

Although the disorder can affect people of all ages, the most typical age of onset seems to be between 15 and 19. It was estimated that bipolar disorder prevalence was 2.4% over the course of a lifetime and 1.5% over the course of a year, according to the World Mental Health Survey Initiative (Merikangas et al. 2011). Bipolar I disorder has similar rates in males and females, whereas bipolar II disorder could be diagnosed more often among females.

The pathophysiology of BD is complex and involves numerous molecular and morphological changes, suggesting impaired cell plasticity and resilience. Several studies of recurring episodes in patients with BD have found progressive alterations in brain structure and cellular function, called neuroprogression (Berk et al. 2011). It appears that long-term illness is associated with reduced cortical thickness in areas of the brain involved in stress regulation, such as the prefrontal cortex (Hibar et al. 2018). Neuroprogression in BD is attributed to epigenetic mechanisms, deregulation of mitochondrial function, neuroplasticity pathways, inflammation, and increased oxidative and nitrosative stress (Berk et al. 2011; Moylan et al. 2013; Scaini et al. 2020). The pathogenesis and development of BD are also thought to be significantly influenced by abnormalities in the hypothalamic–pituitary–adrenal axis. The

deterioration of cognitive and functional impairments in BD patients may be caused by neuroprogression. Cholesterol and its oxidized metabolites, including oxysterols, have been reported to be involved in the molecular aspects of neuroprogression (Morris et al. 2021; Muthuraman et al. 2020). Over the past decade, oxysterol has been extensively studied in neurobiology in relation to many NPDs, such as Alzheimer's disease. Nevertheless, only one study conducted with patients with BD investigated plasma levels of hydroxycholesterol (Guidara et al. 2021). Guidara et al. revealed that BD patients displayed lower levels of 24S-OHC than controls ($P = <0.001$), and the latter could be a biomarker of this disorder ($P = 0.002$; OR = 0.966, 95% CI [0.945–0.987]). Additionally, 24S-OHC seems to be important when evaluating disease activity and progression related to different mood states.

The decrease in 24S-OHC levels in plasma is mostly due to a reduction in neural 24-hydroxylase activity (Meljon et al. 2014). Indeed, the CYP46A1 in the neurons produces about 90% of the 24S-OHC present in the plasma. At least two receptors, liver X receptors (LXRs) and NMDARs, are known to be activated or modulated by 24S-OHC (Mutemberezi et al. 2016; Courtney and Landreth 2016). Synthetic LXR agonists have been found to activate LXR in mouse models of neurodegenerative disorders. This activation is both neuroprotective and anti-inflammatory (Courtney and Landreth 2016; Mouzat et al. 2019). Moreover, 24S-OHC is a positive allosteric modulator of NMDARs, which are essential for synaptic plasticity and learning and mediate excitatory neurotransmission throughout the CNS (Linsenbardt et al. 2014; Paul et al. 2013b; Wei et al. 2019). The specific endogenous enhancement of NMDARs, which is crucial to cortical plasticity, may be disrupted by decreased concentrations of 24S-OHC in patients with BD, especially during manic episodes (Reiner and Levitz 2018).

Surprisingly, cholesterol 24-hydroxylase knockout mice do not exhibit an increase in brain cholesterol but rather a decrease in cholesterol synthesis (Meljon et al. 2014). Cholesterol and 24S-OHC metabolism impairments can adversely affect neuronal homeostasis by different mechanisms and methods, including cytotoxicity, oxidative stress, apoptosis, and synaptic dysfunction (Gambert et al. 2017; Sun et al. 2017; Vejux and Lizard 2009), all of which have been implicated in BD pathology (Kim et al. 2010; Lee et al. 2018). The decreased cholesterol level is likely correlated with citric acid cycle inhibition (Rui 2014) and altered fatty acid synthesis (Ye and DeBose-Boyd 2011), both commonly observed in BD. Abnormalities of cholesterol synthesis were observed and were related to mitochondrial dysfunction (Leoni et al. 2017). In agreement with current evidence, mitochondrial dysfunction may be a contributing factor to BD. (Campbell and Campbell 2019). CYP46A1 is involved in memory and cognitive functions. A major deficiency in spatial, associative, and motor learning was observed in Cyp46a1 knockout mice, as well as delays in long-lasting potential (Gamba et al. 2021). Such alterations were described in BD patients (Chrobak et al. 2021; Sagheer et al. 2018). Moreover, Cyp46a1 downregulation has deleterious consequences; mice exhibited cognitive impairments and abnormal phosphorylation of tau protein (Djelti et al. 2015), which were reported in BD (Douglas et al. 2018; Jakobsson et al. 2016).

15.4 Pharmacologic Targeting of 24S-OHC Levels

There is evidence to suggest that treatment with drugs that modulate 24S-OHC levels may be effective in improving the NPD's outcomes. The ongoing clinical pharmacological strategies developed to use CYP46A1 as a therapeutic target to treat these diseases.

Besides cholesterol, CYP46A1 was found to metabolize structurally diverse C21- and C27-steroids and some marketed drugs (Petrov and Pikuleva 2019). Therefore, the active site of CYP46A1 is plastic and can fit molecules of different polarities, sizes, and shapes (Mast et al. 2003; Liao et al. 2009). Crystal structural and

biochemical data confirmed the plasticity of the enzyme's active site. Numerous drugs belonging to different classes have been shown to act on CYP46A1, including antidepressants (tranylcypromine), anticonvulsants (thioperamide), anticancer drugs (bicalutamide), antifungals (voriconazole and clotrimazole), and steroids (diclofenac, testosterone, and turinabol) (Pikuleva and Cartier 2021).

Accumulating evidence indicates the joint relationship between CYP46A1 activity and neurotransmission, which is important for brain plasticity and, interestingly, for both conditions of NMDAR hypofunction and hyperfunction. Hence, upregulation or mimicry of CYP46A1 activity along with 24HC signaling and downregulation or antagonism may be beneficial for treating many NPDs.

15.4.1 Pharmacological Inhibition of CYP46A1

CYP46A1 inhibition was demonstrated to be effective in vivo within a relatively short period of time. It did not adversely affect brain cholesterol biosynthesis or elimination. CYP46A1 inhibition was found to regulate glutamate excitotoxicity, a stirring mechanism in schizophrenia and ASD (Howes and Shatalina 2022; Bjorklund et al. 2021).

The first CYP46A1 inhibitor tested in vivo on wild-type mice was the antifungal medicine voriconazole (Shafaati et al. 2010). Intraperitoneal voriconazole injections (once a day for five consecutive days) of a clinically relevant drug dose (60 mg/kg of body weight) were used and resulted in reduced brain 24S-OHC levels.

Soticlestat, a first-in-class inhibitor of CYP46A1, was administered to an animal model of amyloidogenesis and analyzed for its pharmacodynamics, pharmacokinetics, and functional effects (Hawkins et al. 2021). After a single oral dose of 10 mg/kg of body weight, this small molecule inhibited Cyp46a1 at the first measured time point (8 h), while three daily administrations reduced the brain 24S-OHC levels to steady levels.

A recent clinical trial (ClinicalTrials.gov identifier: NCT02539134) showed that a daily dose of Soticlestat in the dose range 100–400 mg/day would lower plasma levels of 24S-OHC by 46.8% to 62.7% (Wang et al. 2022).

15.4.2 Pharmacologic Activation of CYP46A1

CYP46A1 activation could be beneficial for BD patients in whom 24S-OHC levels are decreased (Boussicault et al. 2016; Testa et al. 2016; Nóbrega et al. 2020) and, hence, can affect the NMDARs function (Zhou and Sheng 2013).

An anti-HIV drug, Efavirenz, can pharmacologically activate Cyp46a1 in mice at a very low daily dose of 0.1 mg/kg body weight (Mast et al. 2020). However, high concentrations of Efavirenz induce Cyp46a1 inhibition. It is likely that the drug binds to both the allosteric and active sites of Cyp46a1 and inhibits it by competing with cholesterol for the active site.

In vitro experiments with purified recombinant Cyp46a1 showed that the enzyme is activated by L-glutamate (L-Glu), L-aspartate, γ-aminobutyric acid, and acetylcholine, notably by L-Glu, which caused a threefold increase in Cyp46a1-mediated 24-hydroxylation of cholesterol. In mice, a loss of membrane cholesterol was demonstrated due to 24-hydroxylation as a result of excessive stimulation of glutamate receptors (Petrov and Pikuleva 2019). Cyp46a1 knockdown inhibited Glu-mediated cholesterol loss in cultured neurons, which is consistent with this cause-and-effect relationship (Sodero et al. 2012).

15.5 Conclusion

The role of 24S-OHC is still somewhat puzzling, but recent reports have shed new light on the implication of 24S-OHC in NPDs. 24S-OHC plays a key role in the pathophysiology of brain disorders and seems to be critical in the regulation of neurotransmitter mechanisms, such as NMDARs, which are known to be involved in mood and behavior. However, further research is

needed to fully understand the role of 24S-OHC as well as the significance of CYP46A1 for brain functions and as a therapeutic target for NPDs.

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Part IV

Biomarkers, Therapeutic and Industrial Applications

Oxysterols as Biomarkers of Aging and Disease

16

Irundika H. K. Dias and Hala Shokr

Abstract

Oxysterols derive from either enzymatic or non-enzymatic oxidation of cholesterol. Even though they are produced as intermediates of bile acid synthesis pathway, they are recognised as bioactive compounds in cellular processes. Therefore, their absence or accumulation have been shown to be associated with disease phenotypes. This chapter discusses the contribution of oxysterol to ageing, age-related diseases such as neurodegeneration and various disorders such as cancer, cardiovascular disease, diabetes, metabolic and ocular disorders. It is clear that oxysterols play a significant role in development and progression of these diseases. As a result, oxysterols are being investigated as suitable markers for disease diagnosis purposes and some drug targets are in development targeting oxysterol pathways. However, further research will be needed to confirm the suitability of these potentials.

Keywords

Oxysterols · Biomarkers · Ageing · Health · Disease

Abbreviations

22R-OHC	22R-hydroxycholesterol
24,25EC	24S,25-epoxycholesterol
24-OHC	24-Hydroxycholesterol
25-OHC	25-Hydroxycholesterol
26-OHC	26-Hydroxycholesterol
27-OHC	27-Hydroxycholesterol
4β-OHC	4β-Hydroxycholesterol
5,6 β-EC	5β,6β-Epoxycholesterol
5,6-EC	5,6-Epoxycholesterol
5,6α-EC	5α,6α-Epoxycholesterol
7-KC	7-Ketocholesterol
7α-OHC	7α-Hydroxycholesterol
7β-OHC	7β-Hydroxycholesterol
ABC	ATP-binding cassette
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
AD	Alzheimer's disease
AEBS	Anti-oestrogen-binding sites
ALS	Amyotrophic lateral sclerosis
AMD	Age-related macular degeneration
ApoE	Apolipoprotein E
BMI	Body mass index
CAD	Coronary artery diseases

I. H. K. Dias (✉)
Aston Medical School, College of Health and Life Sciences, Aston University, Birmingham, UK
e-mail: diashki1@aston.ac.uk

H. Shokr
Manchester Pharmacy School, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK

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cGMP	Cyclic guanosine monophosphate
ChEH	Cholesterol epoxide hydrolase
Chol	Cholesterol
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CTX	Cerebro tendinous in xanthomatosis
DM	Diabetes mellitus
ER	Oestrogen receptors
GC-MS	Gas chromatography-mass spectrometry
GSK3	Glycogen synthase kinase 3
HbA1c	Glycohemoglobin
HBV	Hepatitis B
HCV	Hepatitis C
HDL	High-density lipoprotein
IC50	Half inhibitory concentration
IL-1β	Interlukin-1β
IL-8	Interlukin-8
IOP	Intraocular pressure
IR	Insulin resistance
LDLR	Low-density lipoprotein receptor
LC-MS	Liquid chromatography-mass spectrometry
LXR	Liver-X-receptor
MCI	Mild cognitive impairment
MCP-1	Monocyte chemoattractant protein-1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PD	Parkinson's disease
ROS	Reactive oxygen species
RPE	Retinal pigment epithelial
SERM	Selective oestrogen receptor modulator
TGF-β1	Transforming growth factor beta 1
TLR-4	Toll-like receptor 4
TNF-α	Tumour necrosis factor α
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
α-triol	Cholestane-3β,5α,6β-triol

16.1 Introduction

The oxidation–reduction reactions that involve the transfer of electrons between chemical species have a significantly important physiological role in health and disease. Oxidation to cholesterol is a physiological reaction that plays a vital role in cholesterol homeostasis and represents key signalling mechanisms to metabolic variations and environmental changes or stressors in all living systems. Changes to such complex network of communication could lead to acute and chronic disease conditions, and thus, it is a matter of important area to investigate in health and disease (Fig. 16.1). Since cholesterol and oxysterol metabolism underpins major metabolic pathways, their involvement in ageing is also inevitable.

Observational studies provide information to the levels of oxysterols in biological fluids or tissue samples collected from humans. Most commonly reported oxysterols in association with human diseases include: 25-hydroxycholesterol (25-OHC), 24-hydroxycholesterol (24-OHC), 5,6-epoxycholesterol isomers (5,6-EC), cholestane-3β,5α,6β-triol (α-triol), 27-hydroxycholesterol (27-OHC), 7-Ketocholesterol (7-KC), 7-α-hydroxycholesterol (7α-OHC) and 7-β-hydroxycholesterol (7β-OHC) (Sottero et al. 2019; Nury et al. 2013). Concentrations of 7-oxycholesterols (7-KC and 7β-OHC) where found to increase in many pathologies such as diabetes (Abo et al. 2000), dyslipidaemia (Arca et al. 2007), hypercholesterolaemia (Szuchman et al. 2008) and neurodegenerative diseases (Seet et al. 2010).

Cholesterol is also enzymatically converted to 27-hydroxycholesterol (27-OHC, also known as 26-hydroxycholesterol) in most cell types including neurones by the action of 27-hydroxylase (CYP27A1) enzyme that is located the inner membrane of the mitochondria. Mutation to the CYP27A1 gene has been associated with cerebrotendinous in xanthomatosis (CTX) (Nie et al. 2014; Dotti et al. 2001). These patients have extremely low serum 27-OHC levels and present with progressive upper motor neuron defects, accumulation of cholestanol in the body tissues triggering atherosclerosis, neurological dysfunction and ophthalmological pathologies such as cataract (Nie et al. 2014; Dotti et al. 2001). Advances in chromatography techniques

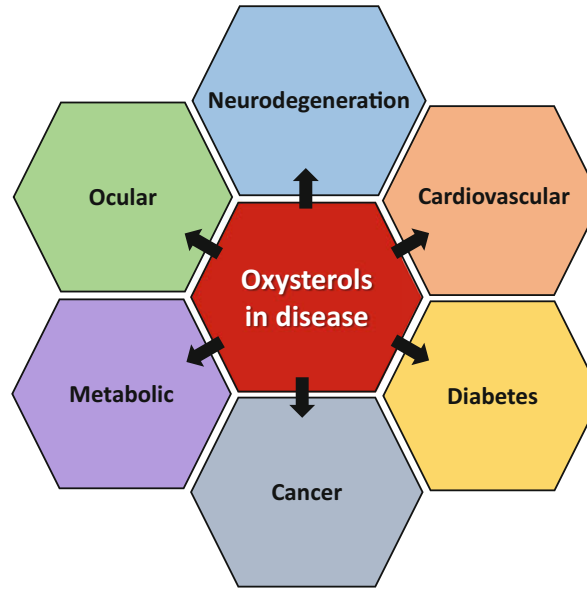

Fig. 16.1 Oxysterols in disease

facilitated the recognition of other oxysterols disturbances such as absence of 27-OHC and 3-β-hydroxycholest-5-enoic acid and the accumulation of bile acid intermediates such as 7α-hydroxy-4-cholesten-3-one and 7α,12-α-dihydroxy-4-cholesten-3-one in the plasma of CTX patients (Crick et al. 2015). Currently, an isotope dilution mass spectrometry method is utilized clinically to diagnose CTX by the identification of bile acid precursors in the patients' plasma (Sottero et al. 2019).

Recent advancement in analytical techniques enabled identification of oxysterol metabolites in wide range of sample types and to measure these very low abundant metabolites with high precision. Although in vivo and in vitro evaluation of oxysterols in metabolic disorders are strongly supporting their role in the aetiology of disease, the lack of consistency in the available data makes its clinical relevance as a biomarker for metabolic disorders still debateable.

16.2 Oxysterols and Ageing

Ageing is a gradual and irreversible process resulted by multiple factors including environmental, genetic and epigenetic factors and lifestyle conditions, which collectively cause progressive alterations in the homeostatic mechanisms of an organism. The involvement of oxysterols in the ageing process is well established and linked to many age-related diseases such as neurodegenerative diseases, cardiovascular, cancer, macular degeneration and diabetes (Negre-Salvayre et al. 2010; Shokr et al. 2021; Dias et al. 2022). This link was mainly explained by oxysterols contribution to (i) RedOX homeostasis imbalance (Titorenko and Terlecky 2011; Terlecky et al. 2006), (ii) cholesterol metabolism (Smiljanic et al. 2013; Fiorenza et al. 2013) and (iii) chronic inflammation (Fulop et al. 2018; López-Otín et al. 2013). It is now well known that many oxysterols including 7-KC, 27-OHC and 7β-OHC have cytotoxic effects leading to cellular dysfunction in many body tissues (Lemaire-Ewing et al. 2005; Zarrouk et al. 2014).

In vitro studies showed that concentrations of 7-KC, 27-OHC and 7β-OHC in the range of 25–50 μM were found lethal (half inhibitory concentration (IC50)) to many types of cells such as vascular endothelial, retinal and colon epithelial, monocytes and different neuronal cells (Nury et al. 2013; Ragot et al. 2013; Lizard et al. 1996; Vurusaner et al. 2014).These concentrations were

also found to be associated with less or more provocation of cytokine secretion, oxidative stress and autophagy-dependent cell death (Vejux and Lizard 2009; He et al. 2013; Nury et al. 2014) which are all identified as a main precipitating factors of ageing in the human body (López-Otín et al. 2013; Jenny 2012; Anik et al. 2022). Additionally, 7-KC and 7β-OHC were found to have a huge impact on the mitochondrial carbonylation of many enzymes that in turn can impair mitochondrial metabolism and cause bioenergetic deficits (Galea et al. 2012). These two mitochondrial-mediated imbalances were also identified as a hallmark of various age-related pathologies (Wallace 2011). Likewise, both 7-KC and 7β-OHC were found to depolarize the mitochondria which consequently impair the protein kinase B/Akt via glycogen synthase kinase 3 metabolic pathways (PDK1/PKB (Akt)/GSK3) (Ragot et al. 2013, 2011), which is similarly found to be disturbed in advanced ages (Petit-Paitel 2010) and in some age-linked diseases such as diabetes and Alzheimer's disease (Gao et al. 2012).

The second mechanism of oxysterol involvement in the ageing process is the manipulation of cholesterol metabolism which was mainly reported in neuro and macular degenerative diseases (Dias et al. 2022; Martin et al. 2010; Pikuleva and Curcio 2014). In this regard, 24 s-OHC the main cholesterol oxidation product by the CYP46A1 enzyme in the neurons, central nervous system and the retina was found to impact cholesterol homeostasis (Björkhem 2006, 2009; Fourgeux et al. 2014), in a similar way to ageing induced abnormal cholesterol catabolism to 24 s-OHC (Shi et al. 2021; Gamba et al. 2021). Furthermore, various types of oxysterols appear to induce inflammation through the overexpression of cytokines, chemokines and adhesion molecules (de Medina et al. 2022; Gargiulo et al. 2016). 7β-OHC and 7-KC were found to induce the secretion of interlukin-1β (IL-1β) (Lemaire-Ewing et al. 2009). Similarly, 27-OHC is able to up-regulate interlukin-8 (IL-8), IL-1β, tumour necrosis factor α (TNF-α) and matrix metalloproteinase-9 (Gargiulo et al. 2011). Likewise, 7β-OHC, 7-KC and 25-OHC can induce monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-8 and macrophage inflammatory protein-1β (Prunet et al. 2006), while 25-OHC enhances the vascular cell adhesion protein 1 (VCAM-1) (Naito et al. 2005). These low and sustained stimulation of inflammatory pathways contribute to biological cell ageing and can lead to senescence (de Medina et al. 2022).

In conclusion, although further investigations are warranted, current data support the important role oxysterols play in the ageing process and in the pathophysiology of age-related diseases; however, their exact mechanism needs further understanding.

16.3 Oxysterols in Neurodegenerative Diseases

It is estimated that 25% of the body cholesterol is located in the brain where 70% are present in myelin sheaths and 20% in glial cells such as microglia and astrocytes (Dietschy and Turley 2004). During brain development, most of the cholesterol are being utilised for myelin sheath production. The high demand for cholesterol in early brain development is maintained by efficient de novo synthesis. Since the blood–brain barrier; vascular endothelial cells that contain tight junctions to control transport of molecules, efficiently prevents cholesterol uptake from the circulation, systemic fluctuation of cholesterol levels does not affect brain development. In mature brain, neurones contain about 10% of total cholesterol pool and heavily rely on astrocytic transfer via the lipid carrier, apolipoprotein E (ApoE) (Fig. 16.2). ApoE-containing lipoproteins are being taken up by neurones with aid of prototypic low-density lipoprotein receptor (LDLR) and the LDL receptor-related protein 1 (William Rebeck et al. 1993). Following receptor-mediated endocytosis, cholesterol is used for various cellular functions such as cell membrane repair, myelin formation, synaptogenesis, neurotransmitter release and ApoE is recycled back to the plasma membrane.

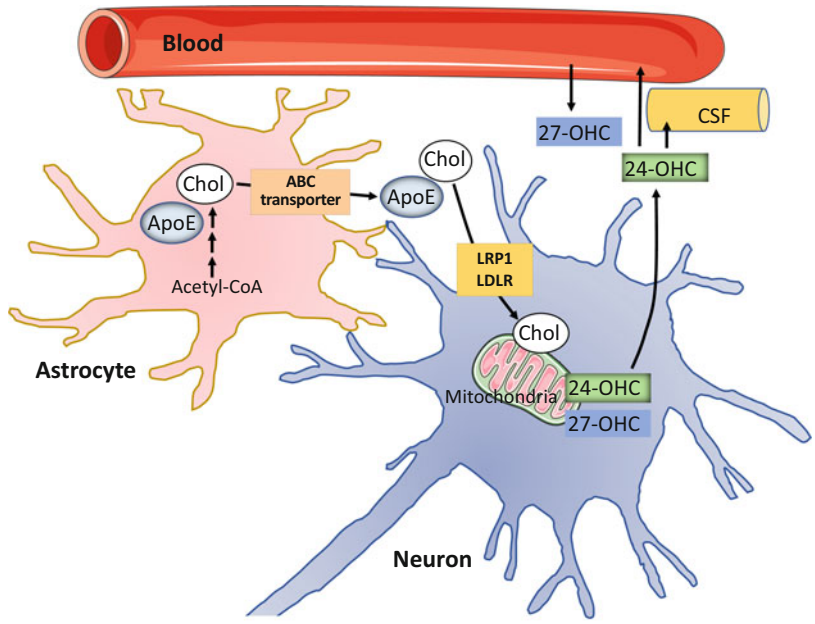

Fig. 16.2 Cholesterol metabolism and transport between neurones and astrocytes. Mature neurons have a lower rate of cholesterol (Chol) synthesis in comparison to glial cells. Hence, neurones relay on cholesterol supply from astrocytes. Starting from acetyl-coenzyme A and going through multiple steps, cholesterol is synthesised in endoplasmic reticulum of astrocytes via Bloch pathway. Cholesterol is then bound to ApoE proteins and transported out from astrocytes via membrane bound ABC transporters (ABCA1 /ABCG1). ApoE particles containing cholesterol are being uptake by low-density lipoprotein receptors (LDLR) with aid of lipoprotein-related protein 1. Excess cholesterol is converted mainly to 24sOHC via mitochondria-driven acidic pathway and excreted to the cerebrospinal fluid (CSF) or through blood–brain barrier into circulation

In order to maintain the cholesterol homeostasis, most of the excess cholesterol (about 6–8 mg/24 h) is converted into the more polar 24-OHC by a specific cholesterol 24-hydroxylase (CYP46A1), a cytochrome P-450 enzyme, expressed in metabolically active neurons. The mRNA levels of CYP46A1 were found to be associated with grey matter where expression levels are found to be high in neurons of the cerebral cortex, hippocampus, dentate gyrus, amygdale, putamen and thalamus (Lund et al. 1999). 24-OHC is then released into the circulation to be metabolise in the liver for excretion (Dias et al. 2014; Lütjohann et al. 1996). Accumulation of 24-OHC is known to act as a suppressor for astrocytic cholesterol biosynthesis through liver-X-receptor (LXR) activation and subsequent inhibition of the sterol regulatory element binding protein (Han et al. 2020). LXR activation by 24-OHC is also favour cholesterol transport from astrocytes to neurons by the expression and synthesis of ApoE and ATP binding cassette (ABC) transporter A1 and G1 ABCA1/ABCG1 in astrocytes (Abildayeva et al. 2006; Czuba et al. 2017).

Even though CYP27A1 is ubiquitously expressed, neurones show lower expression levels. Similar to 24-OHC, brain-derived 27-OHC is later transported to the blood and catabolized towards bile acids in the liver by oxysterol 7α-hydroxylase (CYP7B1) (Mignarri et al. 2016). There is evidence that peripheral 27-OHC can also cross blood–brain barrier to enter brain compartment.

Among many factors that contribute to progression of neurodegeneration, oxidative stress

and inflammation play an important role. Increased oxidative stress results generation of reactive oxygen species (ROS) that favour production of auto oxidised oxysterols such as 7-KC and 7β-OHC. Levels of free radical-derived oxysterols are found to be increased in neurodegenerative conditions (Mignarri et al. 2016; Ademowo and Dias 2022).

Owing to its great important in brain heath, cholesterols and oxysterol metabolism have been considered as one of the important contributors in many neurodegenerative conditions including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) Friedreich ataxia, Huntington's disease and Lewy body disease.

There are mounting evidence arising from both invitro and in vivo work to support that oxysterol metabolism and transport is impaired in AD brain (Shokr et al. 2021). Due to variable results, it is not yet possible to come to a conclusion if oxysterols can be suitable biomarkers for AD. Meta-analysis by Ademowo and Dias (2022) compared 14 published observational studies consist of 957 controls, 469 mild cognitive impairment (MCI) and 509 AD cases describing levels of 24-OHC, 27-OHC and 7-oxycholesterols. This paper showed no association between serum oxysterol levels and AD.

16.4 Oxysterols in Cardiovascular Diseases

16.4.1 Atherosclerosis

Atherosclerosis is a progressive multifactorial disease of the large arteries. It is considered the main cause of cardiac/cardiovascular and vascular diseases worldwide (Katta et al. 2021; Rubin and Borden 2012). The condition is characterized by the subendothelial accumulation of fatty streaks containing high levels of cholesterol and triglycerides in the inner arterial walls that is later forms an atheromatous plaque. Over time, the formed plaque could partially or completely obstruct or occlude the arterial intima and in some cases it can progress to vascular thrombosis (Rafieian-Kopaei et al. 2014).

Epidemiological studies have shown that dyslipidaemia, genetic predisposition, smoking, obesity, insulin resistance, diabetes and inflammation are the main risk factors behind the initiation and progression of atherosclerosis (Rafieian-Kopaei et al. 2014; Artinian et al. 2010). However, recently, considerable number of research advocated the pro-atherogenic activity of oxysterols which was found to be higher than that of their parent precursor in the atherosclerotic plaque (Sottero et al. 2009). This evidence prompted the investigation of vascular modulations induced by oxidized sterols detected in atherosclerotic plaques, plasma lipoproteins and plasma of dyslipidaemic patients (Vaya et al. 2001; Chang et al. 1997; Brown and Jessup 1999). In this regard, it has been found that oxysterols can stimulate atherogenesis by enhancing the activity of matrix metallopeptidase 9 for atherothrombotic events in the human macrophage cells (Blankenberg et al. 2003). This effect is mediated by the induction of the NADPH oxidase 2 and consequently increases ROS production and endothelial injury (Gargiulo et al. 2011; Rosenblat and Aviram 2002). Furthermore, in the late stages of atherosclerosis, oxysterols mediate special atherosclerotic pathways by up-regulating inflammatory cytokines such as interleukins, MCP and the CD36 scavenger receptor (Poli et al. 2013a; Sato et al. 2012). These up-regulations increase vascular endothelial apoptosis (Panini and Sinensky 2001) extracellular matrix degradation, diminish endothelial permeability (Durán et al. 2010) and increase the vulnerability of the formed atherosclerotic plaque (Gargiulo et al. 2011).

Research has also proved that oxysterols play a vital role in controlling vascular wall endothelial flexibility after incorporation into the cell membrane (Shentu et al. 2012). It has been shown to increase cell membrane stiffness and consequently increase vascular wall contractility and sensitivity to shear stress which could be a disease initiation trigger (Shentu et al. 2010; Oh et al. 2016; Luchetti et al. 2017).

Human atherosclerotic plaques are known to have increased levels of 25-OHC, 24-OHC, 5,6-EC, α-triol, 27-OHC and 7-oxycholesterols; 7-KC, 7α-OHC and 7β-OHC (Brown and Jessup 1999; Griffiths et al. 2016). Of them, 27-OHC, 7-KC were the most abundant, followed by the 7α-OHC, 7β-OHC (Shibata and Glass 2010; Garcia-Cruset et al. 2009; Kuver 2012) and together they present approximately 80% of total oxysterol content in the atherosclerotic plaques (Brown and Jessup 1999). While the highest concentrations of all the 7-oxygenated oxysterols are usually found in the foam cells (Arca et al. 2007; Poli et al. 2013a). Increased levels of 7-KC and 7β-OHC were found to induce oxidative stress by stimulating ROS production, suppressing cellular antioxidant load and they also exhibited proapoptotic properties (Larsson et al. 2006). On the other hand, 7α-OHC, 7β-OHC and 7-KC were proved to induce a clear inflammatory phenotype in the endothelial cells and mediate endothelial stiffness (Shentu et al. 2012). 7-KC molecules can also induce the creation of the foam cells (Poli et al. 2013a).

Owing to blood vessels shear stress, atherosclerotic lesions are usually initiated in the subendothelial of the aortic artery in the first decade of life, the coronaries in the second decade and the cerebral arteries thereafter (Lusis 2000; Wentzel et al. 2012; DeBakey et al. 1985). Atheroma lesions then mature with a noticeable increase in 27-OHC concentration compared to 7β-OHC and 7-KC (Brown and Jessup 1999). Higher concentration of 27-OHC was also found to be more abundant in the macrophage-rich environments hypercholesterolaemia (Szuchman et al. 2008) and neurodegenerative diseases (Seet et al. 2010) compared to the fibrous parts of the lesions with its level proportionally related to the severity of the condition (Brown and Jessup 1999). These 27-OHC-induced atherogenic effects are explained by its role as an endogenous selective oestrogen receptor modulator (SERM) (DuSell et al. 2008). In early stages of atherosclerosis, oestrogen prevents plaque formation and protects the vascular system from ischemia and endothelial destruction via mediating nitric oxide (NO) and cyclic guanosine monophosphate (cGMP), which contributes to smooth muscles relaxation and vasodilation (Nofer 2012). On the other hand, in the late stages of atherosclerosis, oestrogen stimulates inflammation, increase clotting, thrombosis and destabilize formed atheroma's cap which enhance the progression of the lesion (Lenfant et al. 2011). Elevated levels of 27-OHC were also found to attenuate the atheroprotective effects of oestrogen and enhance its proinflammatory properties (Umetani et al. 2014; Lee et al. 2014). Based on these findings, monitoring 27-OHC level is currently used to determine the maturity of the atherosclerotic plaques in different clinical sittings.

Another unique feature of oxysterols in atherosclerotic plaques is their selective distribution according to the lesion site, for example, carotid artery lesions contain higher concentrations of 27-OHC compared to lesions formed in the aorta and femoral artery, while coronary lesions contain mainly 7β-OHC and 7-KC (Hultén et al. 1996). These findings highlighted the sensitivity and specificity of oxysterols to the lesion formation site, which prompted the idea of their use as an early diagnostic biomarker for cardiovascular diseases (Shokr et al. 2021; Myoishi et al. 2007). Consequently, numerous antibodies against different oxysterols types have been developed to clinically identify the type of oxysterol in the atherosclerotic plaques (Myoishi et al. 2007). Likewise, since advances in chromatography techniques gas chromatography-mass spectrometry (GC–MS) and liquid chromatography (LC–MS) allowed precise quantification and identification of oxysterols in human tissue, serum and fluids samples (Schött and Lütjohann 2015; Griffiths and Wang 2019), these techniques are currently adapted to clinically measure and monitor oxysterols levels in many diseases (Poli et al. 2022).

16.4.2 Coronary Artery Diseases (CAD)

For decades, CAD was merely linked to dyslipidaemia especially hypercholesterolaemia, elevated serum low-density lipoprotein (LDL) and low concentrations of high-density

lipoprotein (HDL) (Malakar et al. 2019). However, with new clinical evidence unambiguously proving the direct link between atherosclerosis and oxysterols, it was important to assess oxysterols influence in both the development and progression of CAD (Zmysłowski and Szterk 2017). Numerous studies demonstrated the pro-apoptotic and necrotic effect of autoxidation-derived oxysterols in the vascular endothelial (Lizard et al. 1996; Ares et al. 2000). In the coronaries, atherosclerotic lesions had specifically high concentrations of 7β-OHC and 7-KC (Rimner et al. 2005). Similarly, 7β-OHC and 7-KC serum concentrations were found to be twice as high in CAD patients compared to individuals with CAD risk factors without an established disease (Rimner et al. 2005).

Despite the identified high concentration of 7-KC in the coronary lesions, epidemiological and clinical studies acknowledged 7β-OHC as the most abundant and physiologically active oxysterol in CAD lesions. Research has also shown that 7β-OHC serum concentrations are directly associated with not only the CAD progression but also the future risk of developing the disease (Khatib and Vaya 2014). In addition, 7β-OHC was found to be a highly sensitive single predictor of carotid atherosclerosis progression (Salonen et al. 1997) and carotid wall thickness in healthy individuals (Ziedén et al. 1999). Moreover, trivial increases in 7β-OHC plasma concentration were shown to be a strong predictor of various cardiovascular diseases including acute coronary heart diseases events (Ziedén et al. 1999; Fuhrmann et al. 2018; Meisinger et al. 2005).

Unlike assessing atherosclerotic lesions in CAD, there is scarce amount of evidence evaluating oxysterols in stenotic patients. Oxysterols levels in stenotic patients were found to be higher in patients undergoing coronary angiography versus angiographically normal subjects (Yasunobu et al. 2001). Similarly, plasma levels of oxysterols in coronary angiography patients were proved to be higher than healthy asymptomatic control subjects (Liu et al. 1992). In addition, blood analysis studies showed higher plasma levels of free oxysterols among vascular catheterized patients compared to control subjects; however, it did not differ between stable and unstable angina patients (Yasunobu et al. 2001; Zhou et al. 2000).

In conclusion, 7β-OHC plasma concentrations has a strong potential as a diagnostic, predictive and monitoring biomarker for CAD; however, it was not found suitable to distinguish between disease subtypes. Additionally, its efficacy to clinically assess the stability of coronary plaques and carotid occlusion should be further investigated.

16.4.3 Heart Failure

Heart failure is a progressive condition where the cardiac myocytes are unable to pump sufficient amount of blood to meet the needs of the body organs (Groenewegen et al. 2020). Calcium ions (Ca^{2+}) release from sarcoplasmic reticulum plays a principal role in the cardiac myocytes function (Eisner et al. 2000). Defective release and uptake of Ca^{2+} ions result in faulty contraction, relaxation and excitation in the heart muscles (Schmidt et al. 1998; Haghighi et al. 2001) and can contribute to several cardiac dysfunctions such as arrhythmia and heart failure (Lehnart et al. 2004, 2023).

While oxysterols' role in heart failure is not well investigated, preclinical research highlighted their effect on cardiac conduction and myocardial function. Oxysterol concentrations above 1 ug/ml were found to increase intracellular calcium which can mediate cardiac cells cytotoxicity and apoptosis (Rusiñol et al. 2000). Additionally, 25-OHC has the ability to inhibit the activity of the sodium/potassium ATPase pump, which is responsible for potassium influx to maintain cardiac cells action potential and sustain normal conduction between the cells (Zhou et al. 1991). 25-OHC can also affect the sarcoendoplasmic reticulum calcium ATPase pump which transport calcium back to the sarcoplasmic reticulum after muscle contraction (Lukyanenko and Lukyanenko 2009). 25-OHC-induced cell apoptosis was found to be completely antagonised by calcium channel blockers such as verapamil

and nifedipine, which confirms the involvement of calcium channels in the induced toxicity (Ares et al. 1997). Similar effect was observed with 7β-OHC; however, its effect was not reversible by verapamil (Ares et al. 2000).

Although the physiological effect of oxysterols on the cardiac muscles has been known for decades, their clinical implications have not clearly investigated. Further investigation of the effect of oxysterols could open the doors for pharmaceutical/medical innervations that can modulate cardiomyocytes dysfunction and would have a huge impact on heart failure treatment.

16.5 Oxysterols and Cancer

In addition to its physiological role in the human body, cholesterol plays a multifaceted role in the pathogenesis of cancer (Fritsche 2015). In various tumor cells, cholesterol accumulation was found to occur either via increased cellular uptake or biosynthesis upregulation (Sharma et al. 2019; Yue et al. 2014). This phenomena was found to increase cancer cells aggressiveness and stimulate tumor progression (Yue et al. 2014; Wang et al. 2017a). Compared to non-cancerous cells, malignant cells can overexpress cellular LDL receptors (LDLR) and evade cholesterol uptake negative feedback mechanism (Chen and Hughes-Fulford 2001). This disruption in cholesterol uptake and feedback regulation were found to trigger rapid proliferation and altered circulatory cholesterol concentrations in different types of malignancies (Halimi and Farjadian 2022). Additionally, in recent research, cholesterol has been shown to be a prognostic factor in many types of cancer (Yan et al. 2020), which was evident by total cholesterol and HDL concentrations being identified as protective factors of overall survival and relapse rate in cancer patients (Zhou et al. 2018; Guillaumond et al. 2015).

Similar to cholesterol, oxysterols were found to clinically associate with different types of malignancies including colon, prostate, breast, lung and skin cancers (Jusakul et al. 2011). While cholesterol carcinogenesis was mainly attributed to immune-mediated inflammation (Grivennikov et al. 2010), oxysterols pro-cancerous effect was elaborated by their ability to modulate specific cell proliferation/differentiation signalling pathways such as the hedgehog, ERK and Wnt pathways (de Weille et al. 2013; Olkkonen et al. 2012). Additionally, oxysterols were found to induce inflammatory signals by stimulating the overexpression of the cyclooxygenase-2 (COX-2) enzyme (Yoon et al. 2004) and the toll-like receptor 4 (TLR-4) receptors in the endothelial cells (Huang et al. 2014). Moreover, oxysterol-binding proteins stimulate cell proliferation were found to play a crucial role in oxysterols-mediated carcinogenesis (Li et al. 2016a; Nagano et al. 2015). The role of oxysterols in the aetiology of cancers has long been studied. However, recent preclinical data have also shown a potent mutagenic/genotoxic attitude of various oxysterols molecules (Zarrouk et al. 2014; Guardiola et al. 1996). Additionally, most of cancer research found that many oxysterols have both cancer triggering and cancer protective effects. For example, carcinogenic oxysterols were also found to exert a cytotoxic effect on malignant cells. This effect is mediated by either a mitochondrial pathway (Kang et al. 2005a; Appukuttan et al. 2013) or the Fas/Fas ligand-mediated death receptor pathway (Lee and Chau 2001). Both pathways can either overstimulate ROS production, mediate cellular calcium modifications or induce mitochondrial membrane modulations that can stimulate cell apoptosis (Kulig et al. 2016). Additionally, 7α-OHC, 7β-OHC and 25-OHC were found to have an antiproliferative, antimigration and antiinvasion effect on cancer cells (Lin et al. 2013).

Another interesting target of oxysterol-mediated pro-apoptotic effect is the *LXR*, which is responsible for cholesterol haemostasis and metabolism. Various tumor types are highly sensitive to LXR activation, which can contribute to the amelioration and progression of the disease (Zarrouk et al. 2014; Bovenga et al. 2015; De Boussac et al. 2013; Kloudova et al. 2017). On the other hand, LXR modulation inhibits cell proliferation by reducing cellular cholesterol content which hinder cell membrane biogenesis and

cause cell death. Congruently, oxysterols deprivation or inactivation by sulfation was found to decrease LXR expression in cancer cells which can either promote or protect against malignancy, depending on the condition and the type of the involved tumor tissues (Jusakul et al. 2011).

16.5.1 Oxysterols and Breast Cancer

27-OHC is the first identified SERM. It acts as a partial oestrogen receptor agonist that produces different physiological effects depending on the type of the affected cells (Nelson 2018; Raza et al. 2017). In malignant cells, oxysterols activation of the oestrogen receptors (ER) leads to the induction of different cell proliferations genes such as the cyclin d1 oncogene and the E2F transcription factor which prompts rapid tumour growth (DuSell et al. 2008). Despite, the limited availability of clinical data on this topic, preclinical evidence confirmed the crucial role 27-OHC plays as a SERM in the development and progression of breast cancer (DuSell et al. 2008; Lee et al. 2014; Javitt 2015). Treating breast cancer cell lines, e.g., MCF-7 and MDA-MB-231 with 27-OHC was found to stimulate not only proliferation (Wu et al. 2013; Raza et al. 2015) but also cell detachment and migration from the parent tumour through the modulation of the LXR proteins (Silva et al. 2003, 2006; Molostvov et al. 2023). These findings proposed the role of 27-OHC as a migration-inducing factor that can stimulate metastasis in breast cancer patients (Silva et al. 2003, 2006; Nelson et al. 2013). 27-OHC effects on breast cancer cells were further investigated by comparing mRNA expression of mitochondrial oxysterol biosynthesis pathway enzymes, e.g., CYP27a1 and CYP7b1 in oestrogen receptor positive cancer tissues (ER-positive) and normal breast tissues. CYP27a1 levels were similar in both tissues, while CYP7b1 expression was decreased in the ER-positive tumours (DuSell et al. 2008; Nelson et al. 2013). Elevated 27-OHC and decrease CYP7b1 expression were found to significantly correlate with increased tumor aggressiveness and metastasis in breast carcinoma (Lee et al. 2014; Nelson et al. 2013). Furthermore, lower survival rates were observed in cancer patients with low tumor CYP7b1 expression compared to patients with high or normal expression of the enzyme (Nelson et al. 2013). Beside the mitochondrial pathway, MCF-7 cells exposure to 27OHC was also found to significantly lower the expression of the E-Cadherin/β-catenin complex which plays an important role in the formation and the maintenance of the cellular epithelial barrier (Torres et al. 2011). Additionally, cell culture assays showed that 27-OHC has a pro-estrogenic effect on breast cancer cells in the absence of oestrogen, while exerting a suppressive ER activity in the presence of oestrogen (Umetani et al. 2007).

25-OHC was also found to stimulate MCF-7 breast cancer cells proliferation via the stimulation of oestrogen receptor α (ER-α) and its target genes (Lappano et al. 2011). However, while 27-OHC is a SERM, 25-OHC acts only as an agonist ligand of ER in cancerous cells (Lappano et al. 2011). Both 25-OHC and 27-OHC can substitute oestrogen role in the activation of ER-mediated target genes expression and proliferation signal (Simigdala et al. 2016). Cancer cells aggressiveness was also linked to increased 25-OHC blood concentration in breast cancer patients (Dalenc et al. 2017).

B-ring oxysterols such as 7-KC and 6-KC were also found to participate in the aetiology of breast carcinoma. They exert their action through high affinity binding to intercellular sites called the anti-oestrogen-binding sites (AEBS), which do not bind to oestrogens and their function is still unknown (Hwang and Matin 1989; Lazier and Bapat 1988). B-ring oxysterols binding to AEBS legends results in increased intermediate cholesterol production (Gylling et al. 1995), which mediates cell arrest in the G0-G1 phase of the cell cycle and in turn induces apoptosis (Payré et al. 2008; de Medina et al. 2009). In addition to its effect on AEBS, 7-KC was also found to be a weak activator of ER subtype alpha (ER-α) in breast cancer cells (Wang et al. 2017b).

Clinical studies have also highlighted the role 5-epoxide cholesterol (5-EC) as a tumor-promoter cholesterol metabolite in breast cancer

patients (Voisin et al. 2017; Samadi et al. 2021). Additionally, a main secondary metabolite of 5-EC, 6-oxo-cholestan-3β,5α-diol was recently found to stimulate the pathogenesis of breast cancer by binding to certain glucocorticoid receptors (Dumolt et al. 2018). This was further confirmed by the association between high 6-oxo-cholestan-3β,5α-diol concentrations in breast tumours and poor prognosis and low overall survival rate in breast cancer patients (Voisin et al. 2017).

Owing to their effect on ER activity and its target genes, oxysterols modulation of tumours response to hormonal and chemotherapeutic agents grabbed scientises attention for decades. Tamoxifen a SERM molecule was one of the most investigated drugs owing to its central role in the management of ER-positive breast cancer patients (Osborne 1998). Tamoxifen clinical use is always limited by the development of acquired or de-novo resistance to the drug in an initially responsive breast tumours (Nelson et al. 2013) and patients' tendency to develop tamoxifen resistance is not fully understood until now (Chang 2012). Recent pre-clinical research found that in tamoxifen resistance MCF-7 cells, 27-OHC stimulates cell proliferation more than oestradiol which has a more potent effect on tamoxifen-naïve MCF-7 cells (Kloudova-Spalenkova et al. 2021). These findings suggested 27-OHC contribution to tamoxifen treatment failure and the development of resistance by promoting the growth of tumor cells that acquired SERMs resistance. Consequently, scientists recommended routine evaluation of 27-OHC levels in early stages of malignancies to help personalize hormonal therapy offered to the patients; however, this is still not clinically implemented (Nelson et al. 2013).

Building the connection between oxysterols and hormonal therapy in breast cancer was not limited to the effect 27-OHC and oestrogen. Tamoxifen was also found to block the activity of an AEBS called cholesterol epoxide hydrolase (ChEH). ChEH catalyses the hydration of 5,6-EC into α-triol (Silvente-Poirot and Poirot 2012). Blocking ChEH activity increases cellular concentrations of 5α,6α-epoxycholesterol (5,6α-EC) and 5β,6β-epoxycholesterol (5,6β-EC) which in turn induces cell death and was linked to tamoxifen induced anti-cancer properties (Segala et al. 2013; Cheng et al. 2005). Congruently, breast carcinoma exposure to 27-OHC and 25-OHC was also found to increase the resistance to a group of drugs that inhibit oestrogen formation by blocking the aromatase enzyme activity, i.e., aromatase inhibitors (Kloudova et al. 2017). In line with these findings, clinical studies showed that breast cancer patients had an increased blood levels of 27-OHC and 25-OHC after 28 days of aromatase inhibitors treatment which again supports their forceful role as a new surrogate marker for diseases progression and treatment response in ER-positive patients (Dalenc et al. 2017).

Beside their impact on hormonal therapy, oxysterols were also found to modulate tumor responses to various types of breast cancer chemotherapeutic agents including doxorubicin, 5-fluorouracil and etoposide. Doxorubicin is anthracycline agent that exerts its antineoplastic effect via inhibiting the topoisomerase II enzyme and stimulating the generation of ROS which mediate biomolecules and chromatin damage in breast cancer cells (Taymaz-Nikerel et al. 2018). In ER-positive cells, co-exposure to doxorubicin and 4β-OHC, 7α-OHC or 27-OHC increased doxorubicin cytotoxicity, while 7-KC decreased its cytotoxic effects and co-exposure to 25-OHC didn't employ any effects (Wang et al. 2017b). On the other hand, co-exposure to doxorubicin and 7-KC in ER-negative cells (MDA-MB-231) augmented doxorubicin antineoplastic activity (Wang et al. 2017b). 7-KC effect on ER-negative cells was explained by its ability to increase doxorubicin accumulation in cancerous cells; however, other oxysterols such as 4β-OHC, 7α-OHC, 25-OHC and 27-OHC could not exert the same action (Wang et al. 2017b; Kloudova-Spalenkova et al. 2021). These observations suggested the presence of various mechanisms by which oxysterols can affect the response to doxorubicin in breast cancer cells; however, no research was conducted to further investigate this theory.

Aside from doxorubicin, potential effects of co-exposure to oxysterols especially 7β-OHC

and other breast cancer chemotherapeutic agents such as 5-fluorouracil and etoposide did not show any effect of these agents (Hyun et al. 2002).

Regardless the strong evidence of oxysterols effect on chemotherapy, limitations in the conducted studies, e.g., the use of non-breast cancer cells such as hepatocytes, pro-monocytic or leukaemia cell lines, lack of comparison between premenopausal and postmenopausal women and the limited number of conducted clinical research hinder the proper utilization of this data in practice. In summary, exploring the effect of oxysterols on breast cancer is far from straightforward, hormonal therapy remains the main explored area, with 27-OHC being the main focus of the conducted research, while other oxysterols have received less attention.

16.5.2 Oxysterols and Gastrointestinal Tract Malignancies

Dietary oxysterols are one of the main sources of oxysterols in the human body. Similar to cholesterol they are absorbed in the intestine, and thus can influence gastrointestinal tract malignancies (Kovač et al. 2019). Additionally, some oxysterols were found as intermediates in the bile acid synthesis pathway which suggested their role in bile duct and gallbladder carcinomas (Kloudova et al. 2017).

Hepatocellular carcinoma, colorectal and pancreatic cancer are the major identified malignancies in the gastrointestinal tract in the human body.

16.5.2.1 Hepatocellular Carcinoma

Hepatocellular carcinoma is one of the most aggressive types of cancer, and the most common primary malignancy in the hepatic system. Hepatocellular carcinoma is predominantly associated with chronic hepatitis B (HBV), hepatitis C (HCV) infections and alcohol-induced hepatic cirrhosis (Bartosch 2010).

In HCV patients, cholesterol transport is affected, and serum cholesterol levels are usually lower than the population average. Interestingly, 4β-OHC, 7α-OHC and 25-OHC serum levels were found elevated despite serum cholesterol concentration (Arciello et al. 2012). Clinical analysis showed that the increased levels of these oxysterols are not attributed to the activity of the cytochrome p450 enzymes family as CYP7A1 expression did not change in HCV patients (Ikegami et al. 2014) and CYP3A4 was downregulated (Morcos et al. 2013).

Potential effect of oxysterols on hepatocellular carcinoma was investigated in animal models where elevated levels of 24S-OHC, 25-OHC, 27-OHC and 24S,25-epoxycholesterol (24,25EC) were identified in rats with induced hepatoma (Hirayama et al. 2006). Similarly, animal models indicated that 25-OHC has a pro-apoptotic effect in liver tumours and can also mediate sub gastrointestinal apoptosis (Yokoyama et al. 2000). Research has also revealed the cytotoxic effect of oxysterols on human hepatoma cells (HepG2), and their ability to suppress hepatocellular carcinoma cell growth by inhibiting the function of acetyl-coenzyme A: cholesterol acyltransferases enzyme which in turn results in intercellular accumulation of unesterified oxysterols and cell death (Li et al. 2016b). Moreover, it was also found that treatment of overexpressed oxysterol-binding protein-related protein 8 HepG2 and hepatocellular carcinoma cell lines with 25-OHC promotes endoplasmic reticulum stress (Li et al. 2016b) and can increase the sensitivity to Fas-mediated apoptosis (Li et al. 2016b; Zhong et al. 2015). While lower expression of LXR was linked to poor survival in post-operative hepatocellular carcinoma patients (Long et al. 2018).

16.5.2.2 Colorectal Cancer

Colorectal cancer is identified as the third commonly diagnosed cancer, the second cause of mortality in all malignancies and one of the major public health burdens worldwide (Sung et al. 2021).

Studies on colorectal cancer cell lines (mainly Caco2 and SW620) reveiled that oxysterols can exert various types of effects on colrectal carcinomas. Treating human colonic epithelial cells with a representative mixture of dietary oxysterols (7α-OHC, 7β-OHC, 7-KC, α-epoxy

and β-epoxy) enhanced the production of ROS and upregulated mitochondrial-mediated programmed cell death (Biasi et al. 2009, 2013). 7α-OHC, 7β-OHC, 7β-OHC and 5,6α-EC were also able to induce apoptosis in the Caco-2 cell line (Biasi et al. 2013; Roussi et al. 2005). While 27-OHC was found to decrease proliferation in Caco2 and SW620 cells without inducing apoptosis, or interfering with the LXRs and ERs (Rossin et al. 2019; Warns et al. 2018). Similarly, 7-KC and 25-OHC were found to reduce intestinal epithelial barrier function and induce apoptosis in Caco-2 cell line (Chalubinski et al. n.d.). Beside decreasing intestinal barrier function, 25-OHC was also able to induce programmed cell death by detaching cells from the correct extracellular matrix (anoikis) in *DLD-1* colon cancer cell lines (Tanaka et al. 2013), while 7-KC induces endoplasmic reticulum stress (ER-stress) in HT-29 colon cancer cell line (Lee et al. 2009) and decreases mitochondrial functionality in Caco-2 cells (Alemany et al. 2012).

Oxysterols' role in the development of colorectal cancer was also found to be mediated by the overexpression of inflammatory mediators and oxidative stress in the colorectal tract. 7β-OHC was found to induce the production of inflammatory and chemotactic cytokines such as IL-8 in colonic epithelial cells (Mascia et al. 2010). Similarly, Caco-2 treatment with 25-OHC induced IL-8 production (Bai et al. 2005) and treatment with a mixture of oxysterols increased oxidative imbalance that was followed by induced production of IL-8 and 6 (Serra et al. 2018). These findings were further established by the proven link between serum IL-8, tumor growth and colorectal cancer progression (Biasi et al. 2012).

Similar to hepatocellular carcinoma, expression of the key oxysterol metabolizing enzymes was tested in colorectal cancer patients. CYP-P450 ezymes such as CYP2R1, CYP7B1, CYP8B1, CYP46A1, CYP51A1 were found to be overexpressed in primary colorectal cancer tissues while no change was noticed in CYP27A1 and CYP39A1 (Swan et al. 2016). Changes in CYP family expression were associated with patient prognosis and survival rate, this was evident by good prognosis in patients with increased CYP7B1 and poor prognosis in patients with high expression of CYP8B1, CYP2R1, CYP27A1 and CYP46A1. Both CYP27A1 and CYP7B1 are key enzymes in the metabolism of 27-OHC which suggest its role in the development and prognosis of colorectal cancer.

Moreover, it was also found that oxysterols exert their anti-proliferative effect in colorectal cancer cells via the activation of the LXR protein in the tumour tissues (De Boussac et al. 2013). Oxysterols were also able to modulate the transforming growth factor beta 1 pathway (TGF-β1) in the intestinal wall macrophages and fibroblasts which mediates the dedifferentiation and the expansion of neoplastic cells in the colon (Biasi et al. 2008). Additionally, oxysterol-binding protein-related protein 1A downregulation was linked to poor prognosis and low survival rate in colorectal cancer patients (Abdul Aziz et al. 2016; Thorsen et al. 2011).

16.5.2.3 Pancreatic and Bile Duct Cancer

Very little is known about the role of oxysterols in pancreatic and bile duct cancers; however, different types of oxysterols were identified in fresh bile and stone samples and was associated with bacterial infection in this area (Kloudova et al. 2017). It was suggested that bacterial-induced leukocyte activation is the main reason behind the formation of oxysterols in these cases, which directly associated oxysterols to the pathogenesis of gallstones and biliary tract cancers (Haigh and Lee 2001; Haigh et al. 2006). Overexpression of COX-2 enzyme by 22(R)-hydroxycholesterol (22R-OHC) was also shown to increase bile duct epithelial carcinoma progression (Yoon et al. 2004). Moreover, higher expression of oxysterol-binding protein isoforms was directly linked to cholangiocarcinoma and was found to play an important role as a tumor metastasis biomarker (Loilome et al. 2012). Oxysterol-binding protein-related protein 5 (ORP-5) has also been shown to be involved in the aetiology of pancreatic cancer, and its overexpression was associated with poor prognosis in pancreatic cancer patients (Koga et al. 2008). Similarly, activation of LXR had an anti-proliferative effect in pancreatic ductal adenocarcinoma patients (Candelaria et al. 2014).

Research evaluating effect of oxysterols on gastrointestinal tract chemotherapy is also limited. However, 7β-OHC was found to decrease the half-maximal inhibitory concentration (IC_{50}) of bleomycin in HepG2 cell line (Hyun et al. 2002). Similarly, in vitro and in vivo analysis showed that 25-OHC decrease the sensitivity of gastric cancer cells to 5-fluorouracil (Wang et al. 2019a).

16.5.3 Oxysterols and Lung Cancer

The association between several oxysterols and lung cancer was evaluated in several studies. Of all tested oxysterols, 7β-OHC concentration was found to positively associate with increased risk of lung cancer and thus was proposed as a possible predictive factor of the disease (Linseisen et al. 2002). Pre-clinical studies showed that 7β-OHC had an antiproliferative effect mediated by the activation of caspases 9 and 3 and the disruption of the transmembrane potential in the mitochondria in human lung cancer cells (NCI-H460) (Kang et al. 2005b). LXR receptors were also found to play an important role in lung cancer pathogenesis. Stage II non-small cell lung cancer patients had higher LXRα compared to stage III patients. LXR prognostic potential was further suggested by findings from animal studies showing suppression of LXR coding genes leading to chronic inflammation and consequently the progression of squamous cell carcinoma like lesions in the lung tissues (Dai et al. 2016). OSBPs were also found to play a role in the metastasis of lung cancer, as overexpression of ORP-5 was identified in metastatic cases compared to normal expression in localized tumours (Nagano et al. 2015). Recently, two LXR agonists were found to increase the radiosensitivity of lung cancer cells, which suggest the same potential in oxysterols (Liang and Shen 2020).

16.5.4 Oxysterols and Prostate Cancer

Prostate cancer belongs to the hormonal sensitive tumours group. Prostate cancer is dependent on androgens mediated actions via its receptor protein; androgen receptor. Androgen receptor activation stimulates the proliferation of prostate cancer cells and was linked to decreased disease free and overall survival in prostate cancer patients (Lee 2003). The crosstalk between androgen receptor and LXR was proved in many studies, with androgen receptor downregulates LXR activity, which proposed the modulatory role of oxysterols in prostate cancer (Krycer and Brown 2011). Of all the examined oxysterols, 27-OHC was found to increase the transcriptional activity of androgen receptor in prostate epithelial cells and increase their proliferation (Raza et al. 2016). It was also found to decrease docetaxel effect on prostate cancer cells, thus contribute to drug resistance in prostate epithelial cells (Raza et al. 2016).

16.5.5 Oxysterols as Antineoplastic Agents

As discussed above, some oxysterols were found to exert anti-neoplastic activities such as 22R-OHC, 7β-OHC, 7α-OHC, 25-OHC, 24-OHC, *5,6α-EC and* 3β,5α,6β-3OH-cholesterl in colon, lung, prostate and breast cancer (Lin et al. 2013). These findings led to the idea of using oxysterols as a therapeutic agents in malignancies (Bischoff et al. 2000). The first step in this direction was the analysis of the cytotoxic effects of a library of oxysterols on cancerous and non-cancerous cell cultures to identify potential candidates for therapeutic use (Carvalho et al. 2011). Eight naturally occurring oxysterols and 22 synthetic oxysterol derivatives were included in this analysis. Of them α-triol, 7β-OHC, synthetic 3β,6β-diol and 5,6-epoxy-7-keto derivatives were identified as the most potent cytotoxic agents (Carvalho et al. 2010, 2011).

Later, new semi-synthetic oxysterol derivative molecules were developed, for example, OXY186, which is a 20α,22(R)-dihydroxycholesterol analogue and a hedgehog signalling inhibitor was tested in lung, pancreatic cancer cells and in mouse fibroblasts (Wang et al. 2017c, 2019b) and Oxy210 which effectively decreased hedgehog and TGFβ

signalling pathways in mice (Wang et al. 2019b). Later, the cytotoxicity of more oxysterols metabolites was tested, e.g., DDA and 6-oxo-cholestan-3β,5α-diol, where some of them showed highly selective cytotoxic activity, which made them excellent candidates for new cancer therapeutics (Poirot and Silvente-Poirot 2018).

16.6 Oxysterol and Metabolic Diseases

Metabolic disorder is one of the main evolving medical concerns worldwide. It is characterized by abnormal cholesterol metabolism in the blood and tissues of affected individuals (Guillemot-Legris et al. 2016a). Oxidized lipids including oxysterols are considered key players in the pathogenesis of many metabolic disorders such as type II diabetes mellitus (DM-II), insulin resistance (IR) and obesity (Samadi et al. 2021; Sharpe et al. 2014). Clinical research showed that oxidative stress-induced lipid abnormalities are linked to adipose tissue damage, IR and higher risk of developing DM (Hutcheson and Rocic 2012). In this context, elevated levels of oxysterols were found to intensify the inflammatory effect of oxidative stress on the adipose tissue leading to IR. In agreement with these findings, two free radical derived oxysterols were found to significantly accumulate in the adipocytes of DM-II obese patients (7-KC and 7β-OHC). Similarly, non-enzymatically produced oxysterols such as 7β-OCH and epoxycholesterols were elevated in type I diabetes mellitus (DM-I) and II patients (Ferderbar et al. 2007). 7-KC was also characterized as an adipokine modulator in undifferentiated adipose precursor cells in DM-II patients (Murdolo et al. 2016). Likewise, serum levels of 7-KC were found to raise in type II DM patients and were also positively associated with increased risk of developing coronary complications in the same cohort (Endo et al. 2008). Moreover, 7-KC and α-triol were positively linked to poor glycaemic control in DM-II, and this was evident by a higher plasma concentrations of glycohaemoglobin (HbA1c) in comparison to DM-I and non-diabetic individuals (Samadi et al. 2019). Elevated levels of 7-KC were also observed in the erythrocytes of DM-II patients (Abo et al. 2000) and were reported as a biomarker of erythrocytes peroxidation in this population (Inouye et al. 1999). Likewise, clinical studies confirmed oxysterols role as a surrogate biomarkers of plasma oxidizability in the clinical course of DM (Szuchman et al. 2008; Murakami et al. 2000), where 7-KC, 25-OHC and 27-OHC concentrations were found to elevate in both types of DM compared to controls, while 7β-OHC and 5,6-EC were only high in the dyslipidaemia patients with the highest concentrations in DM patients with dyslipidaemia (Murakami et al. 2000). Similarly, quantitative *GC–MS* analysis of f-prostaglandin endothelial cells and neonatal placenta and *umbilical* cord blood revealed excessive presence of 7-KC, 7α-OHC, 7β-OHC and 27-OHC in women with gestational diabetes with all the identified oxysterol are suggested to be modulated by LXR activation (Sun et al. 2018).

Animal studies also confirmed the accumulation of oxysterols in the hepatic, cardiac and renal tissues of diabetic rats (Yoshioka et al. 2005; Matsui et al. 1997). Based on oxysterols measured levels in each tissue, these studies proposed the kidneys as the main target of DM-induced target organ complications (Yoshioka et al. 2005; Matsui et al. 1997).

In addition to DM, disruption of RedOx homeostasis was also evident in obesity animal studies. While little is known about the role of oxysterols in obesity, accumulation of 11 non-enzymatic oxysterols, e.g., 7α-OHC, 7β-OHC, 4α-OHC, 4β-OHC and 5,6-EC, was observed in the liver, adipocytes and the plasma tissues of diet-induced obesity mice (DIO) (Wooten et al. 2014; Mutemberezi et al. 2016; Guillemot-Legris et al. 2016b). Moreover, other studies highlighted oxysterols' ability to influence several sterol oxidation pathways leading to a significant increase in the bile acid synthesis intermediates such as 7α-OHC and 27-OHC and a significant reduction in other oxysterol concentrations, e.g., 4β-OHC and 7α,25-

dihydroxycholesterol (7α,25-diHC) (Mutemberezi et al. 2016).

Similarly, significant decrease in serum levels of 4β-OHC was observed in obese and metabolic syndrome patients compared to healthy individuals. While significantly higher concentrations of 7β-OHC and 7-KC were identified in the female patients with metabolic syndrome (Tremblay-Franco et al. 2015). Comparison between adult female individuals based on their IR and obesity status also showed higher plasma levels 7α-OHC, 7β-OHC and 7-KC in obese individuals compared to lean adults (Alkazemi et al. 2008). However, normalization to total cholesterol levels diluted these differences to non-significant values, which suggests the biological link between serum concentrations of these oxysterols and blood cholesterol level (Alkazemi et al. 2008). Similarly, no differences were highlighted between IR patients and healthy individuals (Alkazemi et al. 2008).

Similar studies also showed no correlation between body mass index (BMI) and levels of 7α-OHC, 24S-OHC, 25-OHC and 27-OHC in postmenopausal women (Lu et al. 2018).While serum concentrations of 7-OHC and 7-KC showed a positive correlation with BMI, LDL and insulin concentrations in teenage girls (Alkazemi et al. 2008).

In the same context and in line with sexual dimorphism of human lipids kinetics (Palmisano et al. 2018), obese males with metabolic syndrome showed significant differences in 4α- and 4β-hydroxycholesterol production, where female patients showed higher concentrations of enzymatically and non-enzymatically generated oxysterols such as 7-oxocholesterol, 4α-OHC, 25-OHC and triol compared healthy or obese patients without metabolic syndrome (Tremblay-Franco et al. 2015).

16.7 Oxysterols and Degenerative Diseases of the Eye

Like other organs in the human body, the retina can regulate cholesterol haemostasis via lipids uptake from the systemic circulation through the LDL receptor (Tserentsoodol et al. 2006; Biswas et al. 2017; Fliesler and Bretillon 2010) and in situ cholesterol biosynthesis which accounts for approximately 70% of cholesterol concentration in the retina (Lin et al. 2016). Excess cholesterol in the retinal tissues is either metabolized to oxysterols via the CYP enzyme family or removed through the reverse transport pathway (Fliesler and Bretillon 2010). In line with these findings, oxysterol- producing enzymes such as LXR-α and β, CYP11A1, CYP27A1 and CYP46A1 were found to be excessively expressed in the retinal tissues (Zheng et al. 2012; Fu et al. 2001; Lee et al. 2006; Bretillon et al. 2007) abundantly in the retinal pigment epithelial (RPE) cells and the choroidal endothelial cells (Zheng et al. 2012; Heo et al. 2011; Liao et al. 2011). RPE cells were also found to be exposed to excessive amount of oxidative stress which stimulates free radical formation of oxysterols in the retinal tissues (Datta et al. 2017). Of all the identified oxysterols, 7-KC, 24-OHC and 27-OHC have been highlighted as the most abundant oxysterols in the retina (Zhang et al. 2021), and their potential implications in degenerative retinal diseases grabbed a lot of attention in the past decade (Poli et al. 2013b).

16.7.1 Age-Related Macular Degeneration (AMD)

AMD is an acquired degenerative disease of the retina that causes visual impairment and severe vision loss though a combination of non-neovascular and neovascular derangement abnormalities (Mitchell et al. 2018). The neovascular changes include the choroidal neovascular membrane formation abnormalities, while non-neovascular includes retinal drusen and retinal pigment epithelium abnormalities which lies posterior to the photoreceptors (Jager et al. 2008).

The first clinical hallmark of AMD is the presence of drusen, while advanced disease involves loss of some areas of the RPE, sub-RPE or sub-retinal haemorrhage, fluid accumulation and

fibrosis (Mitchell et al. 2018; Jager et al. 2008; de Jong 2006).

AMD-centred pre-clinical research showed that 7-KC has strong pro-inflammatory and pro-apoptotic properties, with high levels identified in aged and photodamaged RPE cells and in the retinal drusen (Moreira et al. 2009; Rodriguez et al. 2014). 7-KC was also found to increase ROS production in the RPE cells leading to decreased cellular viability and premature senescence (Ong et al. 2003; Olivier et al. 2016; Dugas et al. 2010). Additionally, it was proved to simulate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein complex and the MEK/ERK, Akt/PKB, PKC signalling pathways which induces the over-production of several inflammatory cytokines such as IL-1,6 and 8 (Dugas et al. 2010; Huang et al. 2012; Shi et al. 2015; Yang et al. 2019) which in turn promote retinal cells death. Moreover, it was also found to mediate endoplasmic reticulum stress, inhibit choroidal neovascularization (Huang et al. 2012) and disturb phagocytosis in the outer segment photoreceptor (Yang et al. 2019). In addition to the above, it was also proved that 7-KC can activate retinal microglia leading to pro-inflammatory cytokine release which aggravate neuroinflammation and impair retinal function (Karlstetter et al. 2015). Additionally, 7-KC was reported to induce the expression of various proangiogenic factors such as platelet-derived growth factor subunit B, vascular endothelial growth factor and the intercellular adhesion molecule 1 (Indaram et al. 2015).

In line with 7-KC findings, high plasma levels of 24S-OHC were also suggested as a candidate biomarker for macrophage aging in AMD patients (Lin et al. 2018). 24S-OHC was found to suppress the production of amyloid-β in the retinal drusen of age-related macular degeneration patients (Urano et al. 2013). This anti-amyloid effect was proved to suppress the progression of AMD and delay vision loss by inhibiting peptide induced neurotoxicity in numerous animal models (Ding et al. 2011; Biswas et al. 2018). Other oxysterols of interest in AMD are the 7β-OH and 25-OH, whoever their exact role in the pathogenesis of the disease is yet to be confirmed (Dugas et al. 2010).

Following the discovery of the various functional properties of oxysterols in the retinal tissues, scientists' interest was directed towards the evaluation of the effectiveness of different oxysterol-targeted therapy in the management of AMD. Of these agents, sterculic acid, a natural cyclopropene fatty acid, has been shown to inhibit 7-KC-mediated cell death and inflammatory effects in the RPE and choroidal cells (Huang et al. 2012). Similarly, Resveratrol, a natural polyphenol antioxidant, was found to have a cytoprotective effect against 7β-OH-, 7KC-, and 25-OH-mediated cytotoxic effects on cultured human RPE cells (ARPE-19) and reduced vascular endothelial growth factor (VEGF) secretion (Dugas et al. 2010).

Efavirenz, an anti-HIV drug, and translocator protein ligands, such as XBD173, were found to increase retinal concentrations of cytoprotective oxysterols such as 24S-HC and 27-HC (Biswas et al. 2018; Petrov et al. 2019; Mast et al. 2017; Papadopoulos et al. 2015); however, their role in the management of AMD and Glaucoma is still under investigation.

16.7.2 Glaucoma

Glaucoma is an eye condition characterized by increased intraocular pressure (IOP), gradual destruction of the optic nerve and loss of retinal ganglion cells leading to irreversible blindness (Almasieh et al. 2012). Association between oxysterols and glaucoma was first proposed when a single-nucleotide gene polymorphism of cholesterol 24S-Hydroxylase (CYP46A1) was linked to the increased risk of development of open-angle glaucoma in human subjects (Fourgeux et al. 2009). Later, animal research proved that the inhibition of CYP46A1 is associated with decreased retinal 24S-OHC concentrations and impaired retinal function (Fourgeux et al. 2014), while increased expression of CYP46A1 leads to increased IOP (Fourgeux et al. 2012). Similarly, elevated IOP was found to increase CYP46A1 gene expression

and 24S-OHC concentrations in glaucoma rat models (Ishikawa et al. 2016) and 24S-OHC was found to play a neuroprotective role under glaucomatous conditions (Ishikawa et al. 2016).

Finally, and as discussed in the metabolic disorders section, mutation of CYP27A1 was found to play a crucial role in the development of CTX which characterized by several ocular pathologies such as cataract, retinal vascular stenosis, and premature senescence (Samadi et al. 2021).

16.8 Conclusion

There is strong evidence gained from in vitro experiments, animal models and clinical studies for both auto oxidised and enzymatically oxidised cholesterol species are associated with ageing and disease. In cellular level, most of the oxysterol metabolic pathways (PI3-K/PDK-1/PKB (Akt)/GSK3, MEK/ERK) are being compromised. Changes to oxysterol metabolism and trafficking also affected mitochondria, lysosomes and peroxisomal activities and enzymes such as NADPH oxidase and COX-2. Levels of 27-OHC and 7-oxycholesterols were found to be increased in atherosclerotic plaques and coronary disease. During neurodegeneration, balance between 24-OHC and 27-OHC found to be compromised. As an endogenous selective oestrogen receptor modulator, 27-OHC and 25-OHC have been investigated for their involvement in cancer progression. These investigations warrant oxysterols and oxysterol pathways as a possible drug target for disease. Some recent work on oxysterol derivatives shows promising results at in vitro and in vivo experiments. The research field into oxysterol is an emerging research avenue to investigate oxysterol involvement and to develop future drug targets.

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17 The Diagnostic Use of the Plasma Quantification of 24S-Hydroxycholesterol and Other Oxysterols in Neurodegenerative Disease

Domenico Tripodi, Federica Vitarelli, Simona Spiti, and Valerio Leoni

Abstract

Cholesterol regulates fluidity and structure of cellular membranes. The brain is involved in signal transduction, synaptogenesis, and membrane trafficking. An impairment of its metabolism was observed in different neurodegenerative diseases, such as Multiple Sclerosis, Alzheimer, and Huntington diseases. Because of the blood–brain barrier, cholesterol cannot be uptaken from the circulation and all the cholesterol is locally synthetized. The excess cholesterol in neurons is converted into 24S-hydroxycholesterol (24OHC) by the cholesterol 24-hydroxylase (CYP46A1). The plasmatic concentration of 24OHC results in the balance between cerebral production and liver elimination. It is related to the number of metabolically active neurons in the brain. Several factors that affect the brain cholesterol turnover and the liver elimination of oxysterols, the genetic background, nutrition, and lifestyle habits were found to significantly affect plasma levels of 24OHC. Reduced levels of 24OHC were found related to the loss of metabolically active cells and the degree of brain atrophy. The dysfunction of the blood–brain barrier, inflammation, and increased cholesterol turnover might overlap with this progressive reduction giving temporary increased levels of 24OHC.

The study of plasma 24OHC is likely to offer an insight into brain cholesterol turnover with a limited diagnostic power.

Keywords

Cholesterol · 24S-Hydroxycholesterols · Oxysterols · Neurodegenerative diseases · Mass spectrometry · Lipidomic · Alzheimer disease · Huntington disease · Parkinson disease · Multiple sclerosis · Biomarkers

Abbreviations

24OHC	24S-Hydroxycholesterol
25OHC	25-Hydroxycholesterol
ABC-transporter family	ATP binding cassettes transporter family
ACAT	Acyl-Coa:cholesterol acyltransferase
AD	Alzheimer disease
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein E
BBB	Blood–brain barrier
BP	Bipolar disorders
CNS	Central nervous system
CSF	Cerebrospinal fluid

D. Tripodi · F. VitarelliS. SpitiV. Leoni (✉)
Laboratory of Clinical Pathology, Hospital Pio XI of Desio, ASST-Brianza and Department of Medicine and Surgery, University of Milano Bicocca, Desio, MB, Italy
e-mail: valerio.leoni@unimib.it

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G. Lizard (ed.), *Implication of Oxysterols and Phytosterols in Aging and Human Diseases*, Advances in Experimental Medicine and Biology 1440, https://doi.org/10.1007/978-3-031-43883-7_17

CTX	Cerebrotendinous xantomatosis
CYP46A1	Cholesterol 24-hydroxylase
CYP51	Lanosterol 14-a demethylase
DHCR24	24-Dehydrocholesterol reductase or seladin-1
DHCR7	7-Dehydrocholesterol reductase
ER	Endoplasmic reticulum
HD	Huntington disease
HDL	High-density lipoprotein
HMGCoA	3-Hydroxy-3-methylglutaril-CoA
HMGCR	HMGCoA reductase
LDL	Low-density lipoprotein
LXR	Liver X receptor
MBP	Myelin basic protein
MMSE	Mini-mental State Examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NPC	Niemann–Pick type C
PD	Parkinson's disease
PLP	Proteo-lipid protein
ROS	Radical oxygen species
SCA	Spinocerebellar ataxias
SREBPs	Sterol responsive element (SRE) binding proteins
VD	Vascular dementia
WHMs	White Matter Hyperintensities

17.1 Cholesterol

Cholesterol is a structural element of the cellular membrane in mammal cells. It regulates the fluidity of lipid bilayers. It is the precursor of bile acids, steroid hormones, and oxysterols. In humans (and in mice too), the cellular requirements are covered by de novo synthesis or by the uptake from circulating lipoproteins. All the cells in mammals are able to synthesize, release or uptake cholesterol to maintain their homeostasis: in some cases, cells are able to produce an excess of cholesterol to provide other cells via lipoproteins, some others, such as neurons, rely on exogenous cholesterol because of limited synthetic capacity. In humans, under normal conditions, about 60% of the body's cholesterol is synthesized (about 700 mg/day) and the remaining is provided by the diet.

Cholesterol is synthesized from acetyl-CoA and converted into 3-hydroxy-3-methylglutaril-CoA (HMGCoA) through two condensation steps. Microsomal HMGCoA reductase (HMGCR) catalyzes the reduction of HMGCoA into mevalonate in the endoplasmic reticulum (ER). Mevalonate is phosphorylated into isopentyl-pyrophosphate and other isoprenoids and then by condensation of six units is formed squalene which is cyclized into the parental steroid, lanosterol. Two alternative ways proceed with cholesterol synthesis: the Block pathway (desmosterol as the main intermediate) and the Kandutsch–Russell pathway (lathosterol and 7-dehydrocholesterol, the two main intermediates).

The quantification of the cholesterol precursors lanosterol, lathosterol, and desmosterol is considered as surrogate marker for tissue or whole-body cholesterol synthesis (Kempen et al. 1988; Bloch et al. 1957; Matthan et al. 2000; Mashnafi et al. 2022).

The HMGCR activity is the rate-limiting step of cholesterol synthesis and it is under tight regulation (Rodwell et al. 1976). Cholesterol itself and oxysterols act a negative feedback mechanism on the enzyme at the protein and the transcriptional levels. Oxysterols modulate lipid synthesis by binding the sterol responsive element (SRE) binding proteins (SREBPs). These transcription factors regulate lipid homeostasis in vertebrate cells by activation of more than 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids as well as NADPH (Horton et al. 2002) (Fig. 17.1).

17.2 Brain Cholesterol

In the brain, cholesterol is mainly in unesterified form (about the 99.5%) (Martins et al. 2009) and

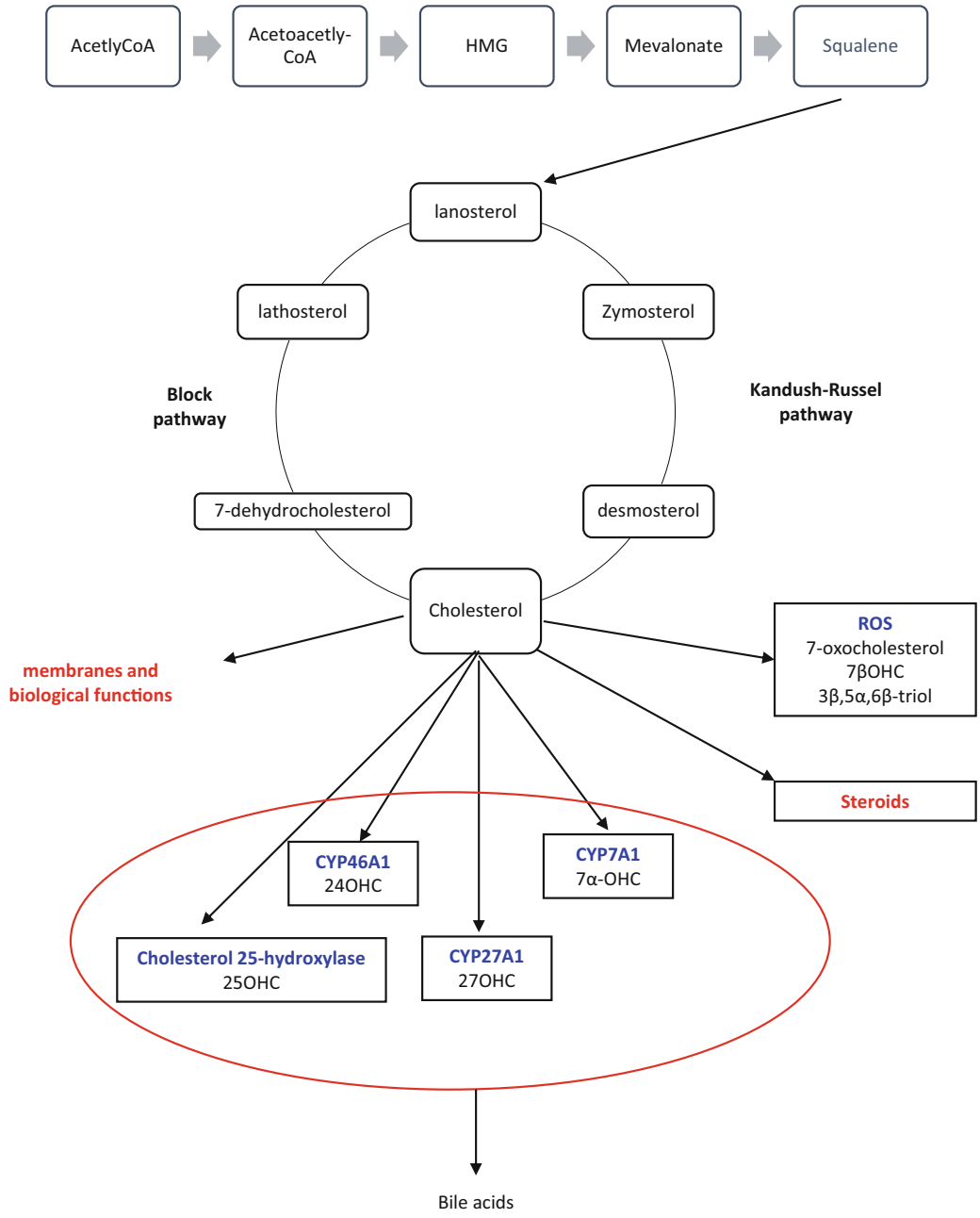

Fig. 17.1 Cholesterol and oxysterols metabolism. Cholesterol synthesis begins with the transport of acetyl-CoA from mitochondria into the cytosol. The rate-limiting step of the pathway is the 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase activity. HMG is followed by mevalonate formation. Pyrophosphate isoprenoids are condensed and cyclized by squalene synthetase then the first sterol, lanosterol is formed. Two alternative pathways (Block and Kandush-Russel) proceed to the cholesterol formation. Cholesterol is involved in the structure, the organization, and the function of cellular membranes (biological functions) and is the precursor of oxysterols, bile acids, and steroids. Liver cholesterol 7a-hydroxylase (CYP7A1) converts cholesterol into 7a-hydroxycholesterol (7aOHC), the main precursor of the neutral bile acid pathway. Cholesterol 27-hydroxylase (27OHC) expressed in different cell types oxidizes cholesterol into 27-hydroxycholesterol (27OHC), precursor of the acidic bile acid pathway. The neuronal specific cholesterol 24-hydroxylase (CYP46A1) forms 24S-hydroxycholesterol (24OHC). Cholesterol 25-hydroxylase produces 25-hydroxycholesterol (25OHC), involved in immunoresponse. Cholesterol autoxidation in the presence of lipid peroxidation and reactive oxygen species (ROS) results in the formation of several oxysterols: 7β-hydroxycholesterol (7βOHC), 7-oxocholesterol (7oxoC), and 3β,5α,6β-triol.

distributed between myelin (~70%), glial cells (~20%), and neurons (~10%) (Maxfield and Tabas 2005). It plays a structural role in the cellular membranes. About 25% of the total body cholesterol is located in the brain (which represents just 2% of the total body weight) (Maxfield and Tabas 2005).

Cholesterol is organized in microdomains called lipid rafts, involved in the maintenance of the properties of membrane proteins such as receptors and ion channels (Allen et al. 2007). In the brain, the cholesterol turnover was estimated to be about 4–6 months in rodents and more than 5 years in humans, confirming the importance of the structural role of this molecule. The presence of the blood–brain barrier (BBB) formed by the tight junction attachments between adjacent capillary endothelial cells prevents the passage of lipoprotein-bound cholesterol from the circulation into the brain (Dietschy and Turley 2004; Snipes and Suter 1997; Björkhem and Meaney 2004). No transvesicular movement of lipids across the capillaries was observed. One or more members of the ABC-transporter family seem to be involved in the exclusion of circulating cholesterol from the brain. Local constant synthesis is responsible for almost all the cholesterol present in the brain.

Cholesterol with its structural function, regulates permeability, fluidity, and functions of membrane-bound proteins. It is involved in synaptogenesis, synaptic function, axonal growth, dendrite outgrowth, and microtubule stability and is a limiting factor for the outgrowth of neuritis being involved in vesicle transport and exocytosis at synaptic levels (Koudinov and Koudinova 2001; Pfrieger 2003a, b; Pfrieger and Ungerer 2011; Hussain et al. 2019). Since cholesterol appears as a major regulator of neuronal activity, metabolic dysfunctions have severe consequences on brain function. Converging evidence in cellular and animal models and observations supports the importance of disturbance of cholesterol metabolism for the pathogenesis of neurodegenerative diseases (Table 17.1).

A dynamic equilibrium between de novo synthesis, transport, storage, and removal maintains the cholesterol homeostasis. In the brain, cholesterol synthesis via the 7-dehydrodesmosterol pathway seems to be preferred over the 7-dehydrocholesterol pathway (Wechsler et al. 2003). In neurons, the main sterols found belong to the Kandutsch–Russel pathway, whereas in astrocytes to the Bloch pathway. Since very low levels of lanosterol-converting enzymes, 24-dehydrocholesterol reductase (seladin-1, DHCR24) and lanosterol 14-alpha demethylase (cytochrome P450, family 51, CYP51) were detected, the cholesterol synthesis is likely to end at lanosterol step in neurons (Pfrieger and Ungerer 2011).

The myelin is formed by sections of oligodendrocyte plasma membrane repeatedly wrapped around an axon, with the extrusion of virtually all of the cytoplasm. Myelin allows the saltatory conduction of the action potential. Together with lipids, myelin also contains specific proteins such as the proteo-lipid protein (PLP) (about 50% of the total myelin proteins) and the MBP (about 30%) and they are essential to regulate the proper assembly of myelin (Dietschy and Turley 2004; Snipes and Suter 1997).

Oligodendrocytes differentiate after birth: the process of myelination in rodents and in humans increases constantly during the initial weeks (or months) giving an organized accumulation of cholesterol and myelin basic protein (MBP) (Dietschy and Turley 2004). Thus, the rate of cholesterol synthesis is very high during brain maturation and gradually decreases over time. Also, in regions with a higher fraction of myelin and white matter over gray matter, like midbrain and spinal cord, it is higher compared to the cortex. The process of myelin formation and the consequent accumulation of cholesterol is concluded in the adult age.

Under in vitro conditions, the oligodendrocytes have the highest capacity to synthesize cholesterol, followed by the astrocytes which synthesize about five times more cholesterol than neurons (Björkhem and Meaney 2004; Pitas et al. 1987).

The cholesterol synthesis by neurons seems to be adequate for cellular survival, axonal formation, and to form few synapses. However, the

Table 17.1 Cholesterol metabolism abnormalities in neurodegenerative diseases

Disorder	Pathogenesis	Cholesterol metabolism abnormalities	Oxysterols in plasma
Shmit-Lemli-Opitz syndrome	Loss of function of 7-dehydrocholesterol reductase	Accumulation of 7-dehydrocholesterol: developmental deformities, incomplete myelination, and mental retardation	Accumulation of 7-dehydrocholesterol
Niemann–Pick type C disease	Loss of function of NPC1 endosome and lysosome	Cholesterol ester accumulation	Increased 7-oxocholesterol and 3β,5α,6β-triol
Cerebrotendinous xantomatose	Loss of function of sterol 27-hydroxylase CYP27A1	Brain and tendon accumulation of cholesterol	Increased cholestanol, 7α,12α-dihydroxycholest-4-en-3-one and 7α-hydroxy-4-cholesten-3-one; very low 27OHC
Spastic paraplegia SPG5	Loss of function of sterol 7-hydroxylase (CYP7B1); upper motor neuron degenerative diseases characterized by selective axonal loss in the corticospinal tracts and dorsal columns	Accumulation of 25OHC and 27OHC	5 up to 10-fold increase of plasma and CSF 25OHC and 27OHC
Parkinson disease	Degeneration of dopamine neurons in substantia nigra	Cholesterol and membrane lipids are involved in the polymerization of alpha-synuclein high total cholesterol risk factor for PD, high HDL-cholesterol is associated with longer disease duration, and evidence for low LDL-cholesterol in PD	Reduced 24OHC and precursor sterols
Alzheimer disease	Aβ accumulation aggregation of tau protein and formation of neurofibrillary tangles (NFTs)	ApoE4 risk factor related to a less efficient cholesterol transport high midlife cholesterol as a risk factor for late-onset AD; imbalance of cholesterol homeostasis increases amyloid generation and tau aggregation; oxysterols 24OHC reduces while 27OHC increases Aβ deposition	Increased 24OHC in an early stage of disease (MCI) Reduction of 24OHC with disease progression proportionally to MRI brain atrophy Reduced precursor sterols (lathosterol, lanosterol, and desmosterol) Reduced 27OHC in advanced stages of disease
Multiple sclerosis	Auto-immune demyelination by T helper cells against myelin antigens	Inconsistent findings about beneficial effect of statins as therapy; oligodendrocytes cholesterol metabolism is under LXR control and is increased in presence of LXR agonist	24OHC reduced in MS patients proportionally to brain atrophy In RR patients plasma 24OHC correlated with the space and time disease burden (T2 volumes at MRI) Reduced precursor sterols in RR and PP patients Reduced 24OHC and 27OHC in late stage

(continued)

Table 17.1 (continued)

Disorder	Pathogenesis	Cholesterol metabolism abnormalities	Oxysterols in plasma
Huntington disease	Toxic gain of function and loss of function by muted Huntingtin transcriptional dysregulation (LXR and PGC1α) reduced activation of SREBP mitochondria abnormalities	Reduced cholesterol biosynthesis (reduced expression of HMGCR, HMGCoAS, CYP51) Reduced cholesterol transporter genes (ABC-A1, −G1, −G4, APOE) and MBP Reduced cholesterol synthesis in HD primary neurons, oligodendrocytes and astrocytes, and animal models Accumulation of cholesterol in lipid droplets, caveolae, and lipid rafts in neuronal HD cells reduced total cholesterol, lanosterol, lathosterol, and 24OHC in cortex, striatum, and whole brain from HD rodent models Reduced lipidation of ApoE Reduced production of 24OHC	Reduced plasma 24OHC together with lathosterol, lanosterol, 27OHC proportionally to disease progression

massive formation of synapses requires a significant amount of cholesterol which is provided by astrocytes via apolipoprotein E (ApoE) (Pfrieger 2003a, b; Pfrieger and Ungerer 2011). The oligodendrocytes are involved in cerebral cholesterol homeostasis supplying cholesterol to neurons and to astrocytes by ApoE (Fünfschilling et al. 2007; Vance et al. 2000).

17.3 Brain Cholesterol Transport

In adulthood, at the conclusion of the myelination and synaptogenesis process, the neurons downregulate their cholesterol synthesis and rely on the delivery of cholesterol from astrocytes which differentiate post-natally and release cholesterol-rich lipoproteins (the "outsourcing" hypothesis of brain cholesterol metabolism) (Pfrieger and Ungerer 2011). With this strategy, the neurons metabolically switch from structural synthesis to the maintenance of electrical activity rather than dispense energy on costly cholesterol synthesis: more than 100 mol of ATP are required for the synthesis of one mole of cholesterol (Poirier et al. 1993).

ApoE acts as a lipid carrier protein in CNS. It is released by astrocytes to supply neurons and synaptogenesis with lipids and cholesterol (Bu 2009; de Chaves and Narayanaswami 2008; Björkhem et al. 2010). The cholesterol transported by ApoE could be originated by synthesis or by recycling of cholesterol released from degenerating axons (van den Kommer et al. 2009; Jeong et al. 2019).

Cholesterol is released from astrocytes on HDL-like lipoproteins with ApoE via ABC-A1 and ABC-G1 transporters (Babiker and Diczfalusy 1998; Meng et al. 1997). The ApoE lipoproteins are internalized into neurons by the LDL-receptor (LDL-R) and the LDL-related protein 1 (LRP1) (Hudry et al. 2010; Kanekiyo et al. 2014; Raulin et al. 2022). Thus, the complex receptor—ApoE—cholesterol (and lipids) is delivered to the late endosomes/lysosomes where acid lipase hydrolyses the cholesterol esters followed by the intracellular release of free cholesterol. A Niemann–Pick type C (NPC) 1 and NPC 2 protein-dependent mechanism allow the unesterified cholesterol to leave the late endosomes/lysosomes and to be distributed to the membranes as well as to the

ER. HMGCoAR and LDL-R genes are the negative feedback sensors for cholesterol homeostasis. In case of an excess of cholesterol, this is esterified in the ER by acyl-Coa:cholesterol acyltransferase (ACAT) and stored in cytoplasmic lipid droplets as a reserve pool (Koudinov and Koudinova 2001; Seet et al. 2011a, b; Serrano-Pozo et al. 2010). There is also an exchange of cholesterol from the mature ApoE particles and the oligodendrocytes (Orth and Bellosta 2012).

17.4 Turnover of Brain Cholesterol and Conversion into 24S-Hydroxycholesterol

In the brain, the cholesterol synthesis is balanced by hydroxylation in position 24 catalyzed by the cholesterol 24-hydroxylase (CYP46A1) resulting in the formation of 24S-hydroxycholesterol (24OHC), which is the main elimination product of cholesterol from brain neurons. The rate of translocation of 24OHC across the blood–brain barrier is thousand times higher than cholesterol and enters the circulation. CYP46A1 is expressed almost only in neurons and it is localized at the level of the neuronal cell body and dendrites, in the cerebral cortex, hippocampus, dentate gyrus, amygdala, putamen, and thalamus, i.e., associated with gray matter. About 6–8 mg/24 h of cholesterol are released as 24OHC by the brain into the circulation (Meaney et al. 2002). In addition, there is a small efflux of cholesterol from the brain in the form of ApoE-containing lipoproteins via the cerebrospinal fluid (CSF). Since no regulative factors have been observed for cholesterol 24-hydroxylase, it is likely that the substrate availability is an important regulatory factor for the enzymatic activity in vivo. Under this prospective, the amount of free cholesterol in neurons is balanced by the conversion into 24OHC which is involved in the regulation of cholesterol synthesis and in the secretion of ApoE from astrocytes (Björkhem 2006).

Changes in CYP46A1 expression or activity in a number of neurodegenerative diseases [Alzheimer (AD), Huntington (HD), Niemann-Pick type C, spinocerebellar ataxias (SCA), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS)], in epilepsy, autism, Rett syndrome, glioblastoma, and prion infection (Pikuleva and Cartier 2021; Alavi et al. 2023; Leoni and Caccia 2015). CYP46A1 is now considered as a therapeutic target because of its key role in cerebral cholesterol elimination in several neurodegenerative diseases.

In the brain of cyp46a1 knockout mice, the synthesis rate of cerebral cholesterol was lower but the total cholesterol amount was unchanged. These mice presented severe deficiencies in spatial, associative, and motor learning associated with a delay of long-lasting potential and alterations in synaptic maturation (Lund et al. 2003; Kotti et al. 2006). As expected, the amount of plasma 24OHC was reduced (Meljon et al. 2014). When the CYP46A1 was overexpressed in transgenic mouse models, cerebral and plasmatic 24OHC and brain lathosterol and lanosterol were significantly increased without a significant increase in cholesterol amount (Alavi et al. 2023). In an ACAT knockout murine model were observed a 13% reduction of the total brain cholesterol, a 32% increase of 24OHC, lathosterol, and lanosterol unchanged. Since less cholesterol may be esterified, more cholesterol was oxidized into 24OHC and removed from neurons (Lund et al. 2003; Russell et al. 2009).

24OHC acts as an endogenous ligand of the nuclear receptor liver X receptor (LXR). In this way, it is an endogenous regulator of cholesterol and fatty acid synthesis. In the adult brain, it is the cholesterol supply via ApoE that covers almost all the cholesterol requirements of neurons (Pfrieger 2003a). Via LXR, 24OHC upregulates the expression, synthesis, and secretion of ApoE and the expression of the sterol transporters ABC-A1, -G1, and -G4 on the astrocyte membranes, involved in the transport of cholesterol from glia to ApoE particles (Abildayeva et al. 2006; Jansen et al. 2009). In such a way, the turnover product of neuronal cholesterol acts as a feedback regulator of astrocyte cholesterol metabolism (Pfrieger 2003b).

17.5 Plasma Oxysterols in Neurodegenerative Diseases

Almost all the 24OHC in the human circulation is formed by cerebral neurons. Its plasmatic concentration results from the balance between brain secretion, liver clearance, and lipoprotein metabolism (Leoni and Caccia 2011, 2013; Björkhem 2006).

The plasmatic 24OHC was observed decreased in AD, vascular dementia (VD), MS, Parkinson's Disease (PD), Huntington Disease (HD), and Amyotrphic Lateral Sclerosis (ALS) as a reflection of disease burden, the loss of metabolically active neurons and the degree of structural atrophy (Solomon et al. 2009; Bretillon et al. 2000a, b; Kölsch et al. 2004; Teunissen et al. 2003a, b; Leoni et al. 2002, 2008, 2011; Karrenbauer et al. 2006; Qureischie et al. 2008; Besga et al. 2012; Zuliani et al. 2011; Di Natale et al. 2018; Di Natale et al. 2023) (see Table 17.1). In case of brain tumors and severe CNS infections, reduced levels of 24OHC in the circulation were observed. In patients with diagnosis of cerebral death, plasma 24OHC was approximately 50% lower compared to controls. Under this prospectives, the plasmatic concentration of 24OHC may be considered as a surrogate marker for the number of metabolically active neurons located in the brain gray matter (Leoni and Caccia 2011, 2013; Björkhem et al. 2002; Hughes et al. 2013; Roy et al. 2019).

In case of the AD, some studies reported a correlation with the degree of brain atrophy measured by MRI and in others with the MMSE. However, in other studies, it was found that in patients with cognitive decline, plasmatic 24OHC was increased compared to controls. An explanation could be that neuronal damage could be associated with a higher turnover of neuronal membranes, providing higher levels of cholesterol to be converted into 24OHC (Zuliani et al. 2011; Lütjohann et al. 2000; Popp et al. 2012, 2013; Hughes et al. 2012). Recently, 24OHC was found to be reversed correlated with the quantification of agitation in AD patients: agitations and anxiety are predictors of a more rapid evolution and disease progression (Ruthirakuhan et al. 2019).

Autopsy-based studies confirmed decreased levels of 24OHC in various regions of the AD brain compared to that in controls (Heverin et al. 2004). A recent study with quantification of different oxysterols in post-mortem AD brains clearly demonstrated a significant decrease in brain 24OHC along with decreased mRNA expression of cholesterol-24-hydroxylase at different stages of the disease (Testa et al. 2016).

Moreover, 26-hydroxycholesterol, 25-hydroxycholesterol (25-OHC), and 7-oxycholesterol levels were higher in brain tissue and mitochondria extracted from late-stage AD brain tissue while 24OHC was decreased in late AD.

The inconsistent results about the plasma 24OHC concentration in AD patients may result by the interaction of different issues: the selection of patients with different degrees of disease severity, the differences in assay procedure, theraphy, dietary habits, APOE4 genotype, male:female ratio of the population under study. Moreover, a big issue depends upon the definition of Alzheimer's Disease: only in the last few years patients were defined on the basis of cerebrospinal fluid biomarkers. Finally, other comorbidities in the patients could affect plasma 24OHC. The different rates of hepatic clearance of 24-hydroxycholesterol from the peripheral circulation, polymorphism of 24-hydroxylase, and possibly other confounders may have added to the variability of the published results (Leoni and Caccia 2013).

Cholesterol metabolism was significantly dysfunctional: decreased levels of enzymes involved in cholesterol biosynthesis, such as HMGCR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase), CYP51 (lanosterol 14-a demethylase), and DHCR7 (7-dehydrocholesterol reductase) along with decreased levels of cholesterol precursors lanosterol and lathosterol have been observed in HD patients, animal models and cell lines (Leoni and Caccia 2014; Kreilaus et al. 2016; Shankaran et al. 2017). The observed decrease of plasma 24OHC was proportional to the degree of caudate atrophy and to the motor impairment (Leoni et al. 2008). In three

progression groups (Low, Medium, and High, evaluated by the CAG-Age product or CAP score, there was a progressive reduction of plasma 24OHC in relation to the HD progression. The group with higher progression had the most substantial difference relative to the controls and to the group with the lower progression (Leoni et al. 2011). The progression gradient was consistent with the findings in other studies using several psychological and neurological tests or MRI measurements of structural atrophy. The reduction of 24OHC was positively correlated with striatal volumes and thus to the progression of striatum atrophy (Leoni et al. 2008).

Along the course of a neurodegenerative disease such as AD, MS, or HD, there might be a phase of increased cholesterol turnover which is associated with increased plasma 24OHC, then followed by a phase of reduced turnover and neuronal loss associated with reduced plasma 24OHC. According to this hypothesis, in stroke patients, 24OHC was found to increase in the first week after the ischemic damage and thus progressively decreased proportionally to the size of the ischemic damage (Seet et al. 2011a, b). Also, 24OHC predicts long-term brain structural and functional burden in mice models of neonatal hypoxia-ischemia (Lu et al. 2021). These two phases may largely overlap, resulting in a complex profile for 24OHC. The progression of a patient through this hypothetical time course is individual and could be determinant for the apparently contradictory findings in some neurodegenerative diseases (i.e., AD) (Leoni and Caccia 2013; Hughes et al. 2013).

In the case of psychiatric diseases, children with Autism Spectrum Disorders showed a significantly higher plasma level of 24OHC which was also inversely correlated with age. The high plasma levels of 24OHC were found to be independent risk factors for Autism Spectrum Disorders. In Bipolar Disorders (BD) described disturbances of cholesterol metabolism: a reduction of 24OHC was found in patients with BD was found together with higher levels of cholestane-3β,5α,6β-triol, 27-hydroxycholesterol (27-OHC) and Cholestanol in patients with BD. 24OHC was inversely correlated with age independently related to BD acute decompensation (Guidara et al. 2021).

Anyway, a recent study on 226 individuals with schizophrenia compared to ag-matched healthy volunteers, missed to find a significant difference of 24OHC between patients and controls. Levels of 24OHC were not related to average fractional anisotropy of white matter or cortical thickness, or to cognitive deficits in schizophrenia (Guidara et al. 2022).

17.6 Other Oxysterols

In patients with Niemann–Pick type C1 (NPC1) disease, a rare progressive neurodegenerative disorder characterized by accumulation of free lysosomal cholesterol, high levels of plasma 7-oxocholesterol and 3β,5α,6β-triol were identified, suggesting a potential use of these markers for the diagnosis (Porter et al. 2010; Jiang et al. 2011).

The autoxidation oxysterols 7-β-hydroxycholesterol and 7-oxocholesterol were increased in brain samples from patients with peroxisomal disturbances such as AD, PD, X-linked adrenoleukodystrophy (Nury et al. 2020).

In MS no increases in 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-oxocholesterol, 24OHC, and 27OHC were reported (Farez et al. 2009; Björkhem et al. 2010; Björkhem et al. 2011).

In hereditary spastic paraplegia, SPG5, 27OHC, and 25OHC were increased in plasma (5–9-fold) and CSF (30-fold) due to mutations in CYP7B1 gene (Goizet et al. 2009, Tsaousidou et al. 2008; Schüle et al. 2010).

Cerebrotendinous xantomatosis (CTX), is an autosomal recessive neurometabolic disease leading to progressive spastic-ataxic gait disorder, cognitive decline, cataracts, and xanthomas in the tendons and in the nervous system, and frequently premature death due to abnormal bile acid and cholesterol metabolism due to the mutations in CYP27A1 gene. Increased plasma cholestanol and very low levels of 27OHC were reported in these patients. It is likely that most of the cholestanol derives from 7α-hydroxylated

intermediates during the synthesis of bile acids. CYP27A1 is required for oxidation of the steroid side chain in the normal biosynthesis of bile acids. In this disease, there is an accumulation of 7α-hydroxylated precursors such as, 7α,12-α-dihydroxycholest-4-en-3-one and 7α-hydroxy-4-cholesten-3-one (Höflinger et al. 2021). This accumulation is increased as a consequence of the markedly reduced formation of chenodeoxycholic acid. The latter bile acid is the most effective suppressor of the rate-limiting enzyme (CYP7A1) in humans (Ellis et al. 2003). In CTX, there is a marked upregulation of CYP7A1 and the levels of 7α-hydroxy-4-cholesten-3-one in the circulation are increased up to 100-fold. This oxysterol is able to cross the BBB and can be converted into cholestanol (Panzenboeck et al. 2007).

The 27OHC is produced in the whole body by the mitochondrial cholesterol 27-hydroxylase (CYP27A1). Circulating 27OHC can enter into the brain in presence of BBB dysfunction (Leoni et al. 2004). 27OHC favors the formation of Amyloid β, its accumulation and deposition (Wu et al. 2022). Moreover, 27OHC promotes oligodendrocyte maturation (Alanko et al. 2023). Thus, 27OHC is considered the link between mid-age hypercholesterolemia and later development of AD (Wu et al. 2022).

However, in patients with defined neurodegenerative diseases (such as HD, PD, VD, MS, carriers of PANK 2 mutation, in MCI and AD patients), the plasmatic 27OHC was found reduced. In AD, the decreases in 27OHC were associated with reduced lathosterol, desmosterol, and lanosterol in advanced disease stages (Popp et al. 2012, 2013). High-grade White Matter Hyperintensities (WHMs) and Infarcts were associated with a higher amount of 27OHC 4 years before blood draw. Individuals with AD progression were those with lower 27OHC (Hughes et al. 2012). Higher levels of 27OHC were correlated with lower cognitive performance in an aging population after 6 years of study (Teunissen et al. 2003a, b; van den Kommer et al. 2009). This reduction of 27OHC should be related to changes in cholesterol and lipoprotein metabolism, as supported by the observation that also lathosterol and lanosterol were reduced proportionally to the degree of brain atrophy (Solomon et al. 2009; Besga et al. 2012).

17.7 Conclusion

The upregulation of CYP46A1, cholesterol 24-hydroxylase, seemed to be a neuroprotective process to normalize brain cholesterol homeostasis by removing damaged cell membranes or eliminating various aggregates via autophagy induction. ROS may enhance CYP46A1 expression to reduce cholesterol in aged or stressed neurons. Changing CYP46A1 activity not only changes 24OHC levels but also changes the mevalonate pathway of cholesterol synthesis and thereby changes many intermediates that participate in a broad range of biological actions, including autophagy, endocytosis, and synaptic transmission (Alavi et al. 2023). CYP46A1 may act as a key player in neuronal responses to stresses, and oxidative stress enhances CYP46A1 expression (Sodero et al. 2011). Recent results in research showed that CYP46A1 has a remarkable role in neurodegenerative disorders such as AD, PD, HD, SCA, MS, ALS, and other neurological disorders such as seizure, Hypoxia-Ischemia, and Traumatic Brain Injury (Alavi et al. 2023). Moreover, CYP46A1 has been linked to many more neurological diseases such as Nieman-Pick type C, autism, Rett syndrome, schizophrenia, bipolar disorder, and depression (Beasley et al. 2005; Patel et al. 2017; Sun et al. 2021).

Under this prospective, the quantification of plasma 24OHC seems to be a biomarker of the metabolic integrity of cerebral and cortical neurons and the effect of therapeutic intervention over CYP46A1.

The plasma 24OHC is very high in children, decreases in teenagers, and becomes rather constant between the third to the sixth decade of life tending to increase constantly in more advanced age. The concentration of 24OHC resulted in being reversely correlated to the body surface area and the liver dimension. Diet, common forms of dyslipidemia (like familial combined

hypercholesterolemia or polygenic hypercholesterolemia), metabolic syndrome, and diabetes are associated with increased LDL and decreased HDL-cholesterol, increased triglycerides, increased sterol precursors, decreased phytosterols and significant changes in plasma oxysterol. Even the collection time (after overnight fasting vs post-prandial blood collection) significantly affects the levels of oxysterols (as 24OHC) and sterols (Leoni and Caccia 2013, 2014). Moreover, CYP46A1 is expressed by neurons but non-neuronal expression of CYP46A1 was found in the astrocytes of AD brains, after metabol injury and after brain trauma, in a model of MS (Leoni and Caccia 2014). Finally, the selection criteria of the reference population is very important in biomarker studies. The power of 24OHC determination of biomarkers in neurodegenerative diseases seems to be limited.

Conflict of Interest The authors do not have any conflict of interest.

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Oxy- and Phytosterols as Biomarkers: Current Status and Future Perspectives

18

Vesa M. Olkkonen and Helena Gylling

Abstract

Oxysterols and phytosterols are sterol compounds present at markedly low levels in tissues and serum of healthy individuals. A wealth of evidence suggests that they could be employed as biomarkers for human diseases or for cholesterol absorption.

An increasing number of reports suggest circulating or tissue oxysterols as putative biomarkers for cardiovascular and neurodegenerative diseases or cancers. Thus far most of the studies have been carried out on small study populations. To achieve routine biomarker use, large prospective cohort studies are absolutely required. This, again, would necessitate thorough standardization of the oxysterol analytical methodology across the different laboratories, which now employ different technologies resulting in inconsistencies in the measured oxysterol levels. Routine use of oxysterol biomarkers would also necessitate the development of a new targeted analytical methodology suitable for high-throughput platforms.

The most important use of phytosterols as biomarkers involves their use as markers for cholesterol absorption. For this to be achieved, (1) their quantitative analyses should be available in routine lipid laboratories, (2) it should be generally acknowledgment that the profile of cholesterol metabolism can reveal the risk of the development of atherosclerotic cardiovascular diseases (ASCVD), and (3) screening of the profile of cholesterol metabolism should be included in the ASCVD risk surveys. This should be done e.g. in families with a history of early onset or frequent ASCVD and in young adults aged 18–20 years, to exclude the presence of high cholesterol absorption. Individuals in high cholesterol absorption families need preventive measures from young adulthood to inhibit the possible development and progression of atherosclerosis.

Keywords

Cancer · Cardiovascular disease · Cholesterol absorption · Neurologic disease · Oxysterol · Phytosterol

Abbreviations

ABC	ATP-binding cassette (transporter)
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis

V. M. Olkkonen (✉)
Minerva Foundation Institute for Medical Research, Biomedicum 2U, Helsinki, Finland

Department of Anatomy, Faculty of Medicine, University of Helsinki, Helsinki, Finland
e-mail: vesa.olkkonen@helsinki.fi

H. Gylling
Heart and Lung Center, Cardiology, Helsinki University Hospital and University of Helsinki, Helsinki, Finland

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ASCVD	Atherosclerotic cardiovascular disease
BC	Breast cancer
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
CYP	Cytochrome
DC	Dehydrocholesterol
DDA	Dendrogenin A
EPOXC	Epoxycholesterol
GC	Gas chromatography
HC	Hydroxycholesterol
HD	Huntington's disease
HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
KC	Ketocholesterol
LDL	Low-density lipoprotein
MS	Mass spectrometry
MuS	Multiple sclerosis
NPC	Niemann-Pick C
NPC1L1	Nieman-Pick C1-like 1
OCDO	6-Oxo-cholestan-3β,5α-diol
PD	Parkinson's disease
SLOS	Smith-Lemli-Opitz syndrome
SPG5	Spastic paraplegia 5
TriolC	Cholestane-3β,5α,6β-triol
TRL	Triglyceride-rich lipoprotein
VLDL	Very-low-density lipoprotein

18.1 Oxysterol Analytics Causes Challenges in Their Use as Biomarkers

The use of oxysterols and diagnostic biomarkers is complicated by a number of challenges. The first one involves the fact that oxysterols are in biological materials present in very low concentrations and accompanied by >1000-fold excess of cholesterol, the autoxidation of which during sample collection and preparation will readily result in the formation of oxysterols not initially present in the sample. Thus, preferable for the diagnostic use of oxysterols is the incorporation of antioxidants such as butylated hydroxytoluene (BHT) or the peroxide reducing agent triphenylphosphine (TPP) in the sample collection and preparation solutions, as well as purging solutions and vessels with argon or nitrogen to minimize autoxidation during sample handling. Moreover, to achieve optimal results sample preparation should be performed under argon (Griffiths et al. 2016).

The high background of cholesterol interfering with the conventional analytical methods and the isomeric variation of oxysterols have favored the use of different mass spectrometry (MS)-based approaches for oxysterol analyses (Griffiths et al. 2013). The most traditional of these is gas chromatography-mass spectrometry (GC-MS) (Dzeletovic et al. 1995). To achieve maximal sensitivity, the MS is usually operated in the selected ion monitoring (SIM) mode, which avoids scanning over uninteresting, redundant *m/z* regions. This methodology is moderately expensive and labor intensive. It requires the cleavage of conjugates such as fatty acyl esters (saponification, solvolysis), solid-phase extraction (separation of oxysterols from cholesterol), and derivatization (typically to trimethylsilyl ethers) prior to the analysis (Griffiths et al. 2016). In liquid chromatography-mass spectrometry (LC-MS) and LC tandem mass spectrometry (LC-MS/MS) derivatization is not necessarily required and sterol glucuronides and sulfates can be analyzed without hydrolysis and solvolysis. Separation of oxysterol isomers can here be achieved through reversed-phase LC. Electrospray ionization (ESI) and multiple reaction monitoring (MRM) are often employed for oxysterol analyses (Griffiths et al. 2016). A drawback of LC-MS/MS is its limited sensitivity in the analysis of neutral molecules such as oxysterols. The sensitivity can be improved by the incorporation of charged groups into the analytes, designated charge-tagging, for which a number of different methods and reagents have been devised, such as derivatization to picolinyl esters, *N,N*-dimethylglycine esters, oximes, or Girard hydrazones, as well as enzyme-assisted derivatization for sterol analysis (EADSA). This, however, further complicates the analysis protocols. Of note, McDonald et al. (2012) introduced a semi-high-throughput method (50 samples/day) based on a combination of

high-performance liquid chromatography (HPLC)-MS and GC-MS, for the analysis of plasma sterols (including oxysterols) and secosteroids.

The complexity of the GC-MS or LC-MS/MS-based analytical approaches makes their use in routine high-throughput laboratory analyses cumbersome and expensive. Moreover, the analytical methods require thorough standardization, since there are marked inconsistencies between measurements carried out in different laboratories (Lutjohann et al. 2018). If some of the oxysterols identified as putative disease biomarkers should be brought to clinical practice, simple and fast, well-standardized targeted analytical approaches need to be introduced, besides introduction of the autoxidation inhibiting protocols into the pipelines for the collection and handling of patients' blood or tissue samples. At the moment there are no such rapid high-throughput analytical methods suited for targeted detection of oxysterol species, but the methodology thus far relies on the above MS-based approaches.

In the following paragraphs, we will briefly comment on the prospects of potentially employing oxysterols as novel biomarkers for atherosclerotic cardiovascular diseases (ASCVD), neurological diseases, and certain cancers.

18.2 Prospects in the Use of Oxysterols as Cardiovascular Biomarkers

Atherosclerosis represents a chronic combination of cholesterol accumulation and inflammation of the arterial wall, which may lead to intimal destruction, thrombosis, and end-organ ischemia. Atherosclerosis is as a rule associated with elevated circulating concentrations of low-density-lipoprotein cholesterol (LDLc) and remnant particles of triglyceride-rich lipoproteins (TRL) containing a wealth of cholesterol. Atherosclerotic plaques contain elevated concentrations of oxysterols, mainly autoxidation products [primarily 7-ketocholesterol (7-KC), 7-α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 5,6-α-epoxycholesterol (α-EPOXC), 5,6-β-epoxycholesterol (β-EPOXC), and cholestane-3β, 5α,6β-triol (triolC)] as well as the enzymatically derived 27-HC (Carpenter et al. 1995; Garcia-Cruset et al. 1999, 2001; Maor et al. 2000; Vaya et al. 2001). Oxysterol concentrations in the plaques exceed their plasma concentrations at least 100-fold (Brown and Jessup 1999), and the plaque oxysterols are suggested to induce apoptosis and necrosis in cells of the vascular wall, thus promoting the advancement of the lesion and eventually its destabilization (Gargiulo et al. 2017; Larsson et al. 2006). A number of studies have also associated circulating oxysterol concentrations (7β-HC, 7-KC, β-EPOXC, 25-HC, 27-HC, or total free oxysterols) with coronary or carotid artery plaque development and cardiovascular risk (Rimner et al. 2005; Salonen et al. 1997; Song et al. 2017; Yasunobu et al. 2001; Zieden et al. 1999). In fact, serum 7β-HC concentration was found to be one of the strongest predictors of the progression of carotid atherosclerosis (Salonen et al. 1997) and a potentially useful biomarker for coronary artery disease (Khatib and Vaya 2014). Of note, the oxysterols commonly found in plasma from hypercholesterolemic patients are also present in atherosclerotic plaques.

There are currently a multitude of diagnostic tools for assessing cardiovascular risk or confirming the presence of atherosclerotic lesions (Aboyans et al. 2018). The progression of atherosclerosis is mainly monitored by evaluation of the arterial lumen diameter or determination of blood flow. Importantly, vulnerable plaques do not necessarily obstruct the arterial lumen, and many of the above methods do not perform well in detecting subclinical atherosclerosis. Therefore, new biomarkers are needed for the detection of atherosclerotic CVD at the earliest possible stage, to allow therapeutic interventions at the early stages of the disease, when they are most effective. Here, oxysterols, especially the species generated through cholesterol autoxidation (such as 7β-HC, 7-KC, triolC) and found elevated in the plasma of subjects with ASCVD, might provide useful biomarkers. It is unclear how these

oxysterols end up in the circulation: Do they leak into the plasma from atherosclerotic lesions, thus reflecting the lesion burden in a person's arteries, or do they rather reflect a general oxidative stress status? Of note, a number of oxysterols, including the above species, are also found elevated in other diseases associated with oxidative stress, such as chronic kidney disease (Miyazaki-Anzai et al. 2014) and neurological diseases such as Parkinson's disease (Lee et al. 2009) and Niemann-Pick C (Tint et al. 1998; Zhang et al. 2008). Thus, the oxysterols produced by autoxidation are not completely specific for ASCVD, but could nevertheless show value in assessing a person's plaque burden at an early stage of the disease, thus improving the diagnosis of subclinical atherosclerosis. However, this would require (1) improved high-throughput oxysterol analytical methodology and (2) extensive longitudinal clinical investigations to validate the biomarker value of circulating oxysterols in predicting the development of atherosclerotic CVD.

18.3 Do Oxysterols Hold Promise for the Diagnostics of Neurological Diseases?

The brain is an enormously cholesterol-rich organ, containing 25% of the total body cholesterol despite its weight accounts for only 2% of our body weight. Epidemiological studies suggest firm connections between cholesterol metabolism and neurodegenerative diseases. One of the most abundant oxysterols in human circulation, 24S-HC (also called cerebrosterol), originates from the central nervous system, where it plays important roles as both a means of removing sterol from the brain into the circulation and as an intercellular signaling compound (Sodero 2021). 24S-HC is generated from cholesterol by the neuronal cytochrome p450 enzyme cholesterol 24-hydroxylase, CYP46A1. In addition to 24S-HC, the brain and cerebrospinal fluid (CSF) also contain 27-HC, which mainly originates from the circulation and is generated in many different cell types by the enzyme CYP27A1. The circulating levels of 27-HC correlate positively with cholesterol, and the elevated 27-HC, part of which enters the CNS through a leaky blood–brain barrier, is suggested to provide an explanation to the observed link between serum cholesterol and neurodegenerative diseases (Leoni et al. 2003).

Altered plasma or CSF levels of specific oxysterol species are found associated with a number of neurological and neurodegenerative diseases, including Alzheimer's disease (AD), Niemann-Pick C disease (NPC), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MuS), amyotrophic lateral sclerosis (ALS), spastic paraplegia type 5 (SPG5), and Smith-Lemli-Opitz syndrome (SLOS). These associations, which have previously been reviewed by Zmyslowski and Szterk (2019), and their relevance for future diagnostics of the diseases are briefly reviewed in the following paragraphs.

18.3.1 Alzheimer's Disease (AD)

One of the major challenges in treating AD is early detection of the disease, before the cognitive symptoms become evident. Patients with neurodegenerative disorders typically present with elevated 24S-HC concentrations in the CSF and reduced concentrations of this oxysterol in the circulation (Bjorkhem et al. 2006; Heverin et al. 2004; Wang et al. 2016). Circulating 24S-HC apparently reflects the number or mass of metabolically active neurons in the gray matter, and therefore drops during progression of the disease, while the 24S-HC released from dying neurons apparently accumulates in the CFS. While 24S-HC is suggested to suppress amyloid formation by promoting α-secretase activity, 27-HC failed to do this and might counteract the beneficial effect of 24S-HC (Famer et al. 2007). Increased brain and CSF 27-HC in patients with AD indicates a defect in the blood–brain barrier, dysfunction of which is suggested to play an important role in AD development. A number of studies have suggested 24S- and 27-HC as putative biomarkers for AD, besides the conventional decreased amyloid $A\beta_{42}$ or reduced $A\beta_{42}$:$A\beta_{40}$

ratio combined with elevated CSF tau or phospho-tau. The 27-HC/24S-HC ratio seems to be a possible parameter to be measured as AD biomarker, since its elevation is associated with the accumulation of Aβ and neurofibrillary tangles implicated in the neurodegeneration (Bjorkhem et al. 2009, 2006; Glockner et al. 2011).

However, in terms of early biomarkers is it not useful to measure oxysterol concentrations in AD patients who already have cognitive symptoms and are diagnosed by the conventional approaches. Leoni et al. identified 24S-HC as the most sensitive biomarker of mild cognitive impairment (Leoni et al. 2006). To what extent might measurement of circulating 24S-HC and 27-HC promote the early diagnosis of AD? Importantly, the alterations in 24S-HC levels depend on the disease stage. In early stages of AD, the circulating 24S-HC levels are reported to increase rather than decrease, possibly due to destruction of cholesterol-rich neuronal membranes upon cell death, so in fact a higher 24S-HC/27HC ratio was reported to predict cognitive impairment during 8-year follow-up (Hughes et al. 2012). Thus, the 24S-HC/27-HC ratio might provide a valuable tool for early diagnostics of AD, promoting opportunities for early intervention, symptomatic treatment, and improved patient function.

18.3.2 Multiple Sclerosis (MuS), Parkinson's Disease (PD), and Huntington's Disease (HD)

Reminiscent of AD, a major oxysterol whose circulating levels are affected in MS, PD, and HD, is 24S-HC. In younger or early-stage MuS patients, plasma 24S-HC levels were found normal or even elevated, while they were reduced in more severely affected relapse remitting (RR)-MuS or primary progressive (PP)-MuS subjects with a high degree of neurodegeneration (Leoni et al. 2002; Teunissen et al. 2003; Vejux et al. 2021). Reduced concentrations of 27-HC and 7α-HC were found in patients with MuS as compared to healthy controls. Moreover, elevated 7-KC in PP-MS as compared to RR-MuS was reported. These observations suggest a disturbance of an oxysterol metabolic network in MuS, enhanced oxidative stress in subjects with PP-MuS, and are consistent with the neurodegeneration in PP-MuS (Leoni et al. 2002; Mukhopadhyay et al. 2017; Teunissen et al. 2003).

PD patients were shown to exhibit elevated plasma 7-KC, 7β-HC, and 27-HC concentrations but reduced 24S-HC as compared to healthy controls (Lee et al. 2009). Similar to AD, CSF 24S-HC concentration was increased in the patients and correlated with disease duration, suggesting that this oxysterol could be used as a biomarker for PD progression (Bjorkhem et al. 2013).

Similar to the above neurodegenerative disorders, circulating 24S-HC levels were found significantly reduced in HD patients and decreased progressively with progression of the disease (Leoni et al. 2008, 2011, 2013). The reduction of plasma 24S-HC was parallel to brain atrophy and thus most likely the neuronal loss in the gray matter.

The data for MuS, PD, and HD suggest that the circulating oxysterol concentrations could be employed to monitor the progression of these diseases. This would be particularly important in the case of PD, since specific diagnostic tests for PD progression are lacking.

18.3.3 Niemann-Pick C Disease (NPC) and Spastic Paraplegia Type 5 (SPG5)

NPC is a rare autosomal recessive neurovisceral disorder characterized by hepatosplenomegaly and progressive neurodegeneration (Chang et al. 2005). Lysosomal cholesterol accumulation is a hallmark of the disease, resulting in non-enzymatic cholesterol oxidation to yield 7-KC and triolC, which are found elevated in the patients' plasma (Tint et al. 1998; Zhang et al. 2008). These oxysterols can be considered as relatively specific biomarkers of NPC disease, and triolC, in particular, seems to show good

discrimination power in NPC patients and healthy controls (Boenzi et al. 2014; Jiang et al. 2011; Pajares et al. 2015; Porter et al. 2010; Reunert et al. 2016). For this rare inherited disorder, laboratories in the USA are already employing measurement of the above oxysterols in plasma for NPC diagnostics. The disease is so rare that automatized high-throughput analytic platforms are apparently not necessary for this purpose.

SPG5 is another rare autosomal recessive neurodegenerative disorder. It is characterized by progressive degeneration of corticospinal tract motor neurons caused by mutations in *CYP7B1*. This gene encodes oxysterol 7α-hydroxylase, an enzyme hydroxylating carbon 7 of side chain-hydroxylated oxysterols, crucial for their routing for bile acid synthesis (Schols et al. 2017). In patients with SPG5, the circulating levels of 25-HC and 27-HC are significantly elevated. 27-HC was found elevated both in the plasma (6–9-fold) and in the CSF (30–50-fold), while plasma 25-HC was found elevated 100-fold (Schule et al. 2010). Another study found in the patients' plasma 19- and 11-fold increases of 25- and 27-HC, respectively (Marelli et al. 2018). These observations suggest that measurement of circulating 25- and 27-HC levels might provide a useful addition to the diagnostic toolbox for SPG5, provided that their diagnostic value in first confirmed in larger clinical investigations.

18.3.4 Amyotrophic Lateral Sclerosis (ALS)

ALS (aliases motor neuron disease or Lou Gehrig's disease) is a fatal neurodegenerative disease deteriorating motor neurons. Of this disease affecting on average 1 out of 100,000 individuals both familial and sporadic forms are known, 90% of the cases being sporadic. In addition to the degeneration of spinal and bulbar motor neurons, the disease often leads to dementia due to neuron loss (Vejux et al. 2018). A handful of studies have assessed the concentrations of oxysterols in the CSF or plasma of patients with ALS. The CSF levels of 24S-HC, 25-HC, and 27-HC, as well as plasma 25-HC were found elevated in ALS patients not treated with the drug riluzole, as compared to a drug-treated group or healthy controls (Kim et al. 2017). However, also contradicting observations have been made: Wuolikainen et al. (2014) found no significant difference in 24S- and 25-HC levels in the plasma of patients with ALS, while La Marca et al. (2016) reported reduced levels of 24S-HC in both the plasma and the CSF of subjects with ALS. It could be that, similar to the oxysterol levels in AD, the circulating and CSF concentrations of patients with ALS may depend on the disease stage, or differ between different etiologies of ALS. A lot of further research will be needed before judgement on the usefulness of oxysterols as biomarkers for ALS can be made.

18.3.5 Smith-Lemli-Opitz Syndrome (SLOS)

SLOS is a rare autosomal recessive malformation disorder caused by defects in 7-dehydrocholesterol reductase (DHCR7), the last enzyme in the Kandutsch-Russell pathway of cholesterol biosynthesis. The enzyme defect results in the accumulation of 7-dehydrocholesterol (7-DC) and its isomer 8-dehydrocholesterol (8-DC) (Porter 2008; Waterham and Wanders 2000). 7-DC accumulates most in the brain of SLOS patients, but also in other tissues such as the liver and in body fluids. 7-DC and 8-DC are converted to a number of enzymatic and non-enzymatic oxysterol derivatives, none of which are present in the plasma of healthy subjects (Korade et al. 2013; Xu et al. 2011, 2012). Of note, also the plasma concentrations of 7-KC and 7β-HC were found elevated in the SLOS patients, and 7-KC correlated positively with the severity score, indicating that 7-KC might, in addition to 7- and 8-DC or their oxysterol derivatives, be used as a diagnostic tool to monitor the disease progression (Liu et al. 2013). However, more research and method development are clearly required before

oxysterols can be considered as biomarkers for the disease.

18.4 Oxysterols as Biomarkers for Cancer: Future Perspectives

It is well established that sterol metabolism is connected with cancers. Hypercholesterolemia is associated with an increased risk of breast, prostate, and colon cancer, while it is connected with a reduced risk of stomach and liver cancers (Kitahara et al. 2011). Moreover, high dietary cholesterol intake increases the risk of breast cancer in post-menopausal women and the risk of a number of other cancers (Hu et al. 2012). As the circulating cholesterol and concentrations of a number of oxysterols correlate positively (Bjorkhem et al. 1998; Leoni et al. 2003), it has been postulated that oxysterols may play an important role in mediating the cholesterol–cancer association. Due to the multiple physiologic functions of oxysterols in processes such as cellular lipid homeostasis, cell migration, proliferation, immune/inflammatory reactions, and apoptosis, oxysterols have been actively studied in the context of cancers. The status of the knowledge of the roles of these compounds in malignancies has been extensively reviewed (Kloudova et al. 2017; Kloudova-Spalenkova et al. 2021; Kovac et al. 2019).

In this section, we focus on the value of oxysterols as biomarkers for cancer or its progression and mention examples of studies bringing up this possibility, having the emphasis on future perspectives.

18.4.1 Breast Cancer (BC)

27-HC is identified as a selective estrogen receptor modulator (SERM) (DuSell et al. 2008; Umetani et al. 2007). Hypercholesterolemia is an established risk factor for estrogen receptor (ER)-positive BC and associates with an impaired response to endocrine therapies. 27-HC increases ER-dependent tumor growth and liver X receptor-dependent metastasis in mouse models of BC. In human tumors, the tumor grade was shown to correlate with the expression of CYP27A1, the enzyme generating 27-HC (Nelson et al. 2013). The effects of cholesterol on tumor pathology were shown to depend on its conversion to 27-HC and were attenuated by inhibitors of CYP27A1. Therefore, it is not surprising that 27-HC is the major oxysterol studied in the context of BC, and of several other cancers. Despite promising results from mouse model studies and the observed association between tumor grade and CYP27A1 expression (Nelson et al. 2013), work by (Wu et al. 2013) revealed that serum 27-HC levels did not differ between BC patients and controls, although the range of 27-HC variation in the patients was higher than in the controls. A more recent large prospective study by Lu et al. (2019) on a 25,000-subject cohort (EPIC-Heidelberg cohort) similarly failed to detect an association of serum 27-HC with BC risk. However, stratification into pre- and post-menopausal subjects revealed in the post-menopausal subjects an inverse association of 27-HC with BC risk. This is surprising since 27-HC promoted growth and metastasis in experimental models of estrogen receptor-positive mammary cancer (DuSell et al. 2008; He and Nelson 2017). Moreover, albeit (Wu et al. 2013) failed to detect differences in serum 27-HC levels between BC patients and controls, they did find significantly elevated levels of oxysterol in the tumor tissue as compared to tumor-adjacent issue or to normal tissue from healthy control subjects, consistent with the data of Nelson et al. (2013).

In addition to 27-HC, also 25-HC (Dalenc et al. 2017), triolC (Kloudova-Spalenkova et al. 2020), and 7-KC (Dalenc et al. 2017; Soucek et al. 2018) were found elevated in subjects with BC, the association of 25-HC being detected in patients with metastatic BC (Dalenc et al. 2017). Interestingly, triolC may possess diagnostic/prognostic value: Its level in plasma is significantly associated with the disease-free survival of luminal BC patients (Kloudova-Spalenkova et al. 2020). Another interesting aspect in the oxysterol analyses of BC patients is monitoring changes in their levels after treatment. Dalenc et al. (2017) showed that tamoxifen treatment increased the

serum levels of 4β-HC and decreased the levels of 24S-HC, 7α-KC, and 25-HC, while aromatase inhibitor treatment increased 27-HC and β-EPOXC. Likewise, the level of 7-KC was increased 12–24 months after surgery and initiating the treatment (Soucek et al. 2018). These observations bring up the possibility of employing circulating oxysterol concentrations to monitor the success of BC therapies—however, this idea still requires extensive investigation with large patient cohorts.

Further potential biomarkers in BC tissue are the oxysterol metabolite dendrogenin A (DDA), which arises through stereoselective enzymatic conjugation of 5,6α-epoxy-cholesterol with histamine, and 6-oxo-cholestan-3β,5α-diol (OCDO). While elevated levels of OCDO were found in BC tumor biopsies (Voisin et al. 2017), reduced levels of DDA were found in such specimens (de Medina et al. 2021), consistent with the proposed pro- and anti-tumor functional effects of OCDO and DDA (Poirot and Silvente-Poirot 2018). However, an obvious problem in measuring oxysterols in tumor specimens is the large intratumor variability in oxysterol concentrations, which is likely caused by tumor heterogeneity (Solheim et al. 2019).

18.4.2 Other Cancers

In addition to BC, serum/plasma or tumor tissue oxysterol concentrations have been studied in lung, pancreatic, gastric, bladder, and colorectal cancers as well as in glioblastoma, mostly by employing small numbers of patients and control subjects (Kloudova-Spalenkova et al. 2021). In a small study using plasma samples from the EPIC-Heidelberg cohort, 7β-HC was significantly associated with lung carcinoma risk (Linseisen et al. 2002). In a metabolomics study on pancreatic carcinoma, α-EPOXC was identified as one of the metabolites with the capacity to discriminate the patients from the controls (Di Gangi et al. 2016).

An interesting development in gastric cancers is the analysis of oxysterols in gastric juice. The levels of several oxysterols (24,25-EPOXC, 4β-HC, 7α-HC, 7β-HC, 25-HC, and 25-HC-3-sulfate) were found significantly elevated in the juice of gastric cancer patients relative to healthy controls (Guo et al. 2018). The use of gastric juice in such biomarker analysis definitely deserves further study on larger patient cohorts. In this same study, the levels of 27-HC and 24,25-EPOXC were increased in the gastric carcinoma tissues as compared to adjacent non-tumor tissue.

Tumor tissue analysis of 27-HC revealed an elevation of this oxysterol in colorectal cancer TNM stage III specimens as compared to tumor-adjacent tissue (Rossin et al. 2019), similar to the above findings in BC and gastric carcinoma. Furthermore, the levels of 25-HC were found elevated in bladder carcinoma tissue as compared to adjacent non-tumor tissue, and a high 25-HC concentration was associated with poor survival of the patients (Wang et al. 2020). Interestingly, elevated 27-HC concentration in glioblastoma tissue specimens connected with a poor outcome of the patients, and was thus suggested to have prognostic value (Liu et al. 2019).

18.5 Conclusions on Oxysterols as Biomarkers

An increasing number of reports suggest circulating or tissue oxysterols as putative biomarkers for diseases such as cardiovascular and neurodegenerative diseases or cancers (Fig. 18.1). So far most of the studies have been carried out on small study populations. To achieve routine biomarker use, large prospective cohort studies are absolutely required. This, again, would necessitate thorough standardization of the oxysterol analytical methodology across the different laboratories, which now employ different technologies resulting in inconsistencies in the measured oxysterol levels. Routine use of oxysterol biomarkers would also necessitate the development of a new targeted analytical methodology suitable for high-throughput platforms. Employing circulating oxysterols as new non-invasive disease biomarkers is certainly a promising approach in cardiovascular medicine, where specific oxysterols could function as

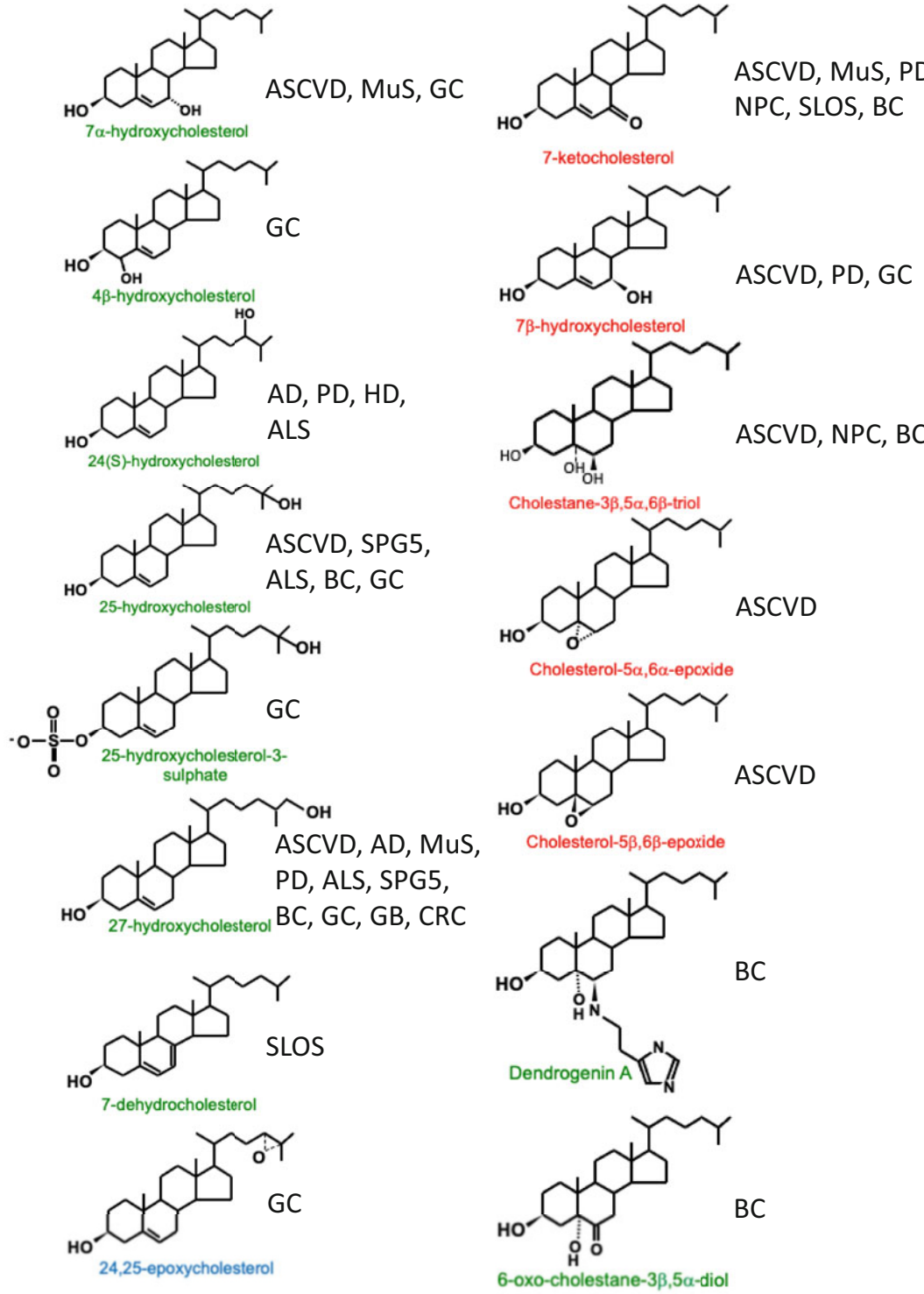

Fig. 18.1 Chemical structures of select oxysterols and related compounds, and diseases for which they are suggested to function as biomarkers. Names of enzymatically generated sterols are printed in green and cholesterol autoxidation products in red. 24,25-Epoxycholesterol (blue) originates from a shunt of the cholesterol biosynthetic pathway. Abbreviations: ASCVD atherosclerotic cardiovascular disease, MuS multiple sclerosis, GC gastric cancer, AD Alzheimer's disease, PD Parkinson's disease, HD Huntington's disease, ALS amyotrophic lateral sclerosis, SPG5 spastic paraplegia 5, BC breast cancer, GB glioblastoma, CRC colorectal cancer, SLOS Smith-Lemli-Opitz syndrome, NPC Niemann-Pick C disease

markers for atherosclerotic plaque burden or possibly even vascular wall inflammation. In neurodegenerative diseases specific oxysterols species are already used for diagnostics (of NPC), and there is promising data for other diseases of this group. However, their use is in certain cases (such as AD and MuS) complicated by disease stage-specific differences in the circulating oxysterol concentrations. There are a large number of published studies that have measured circulating or tissue oxysterol concentrations in subjects with different types of cancer. Time will show what their role in cancer diagnostics and prognosis will be, in relation to other non-invasive biomarkers that are constantly being sought. Moreover, since circulating oxysterol concentrations are affected by a number of interfering factors such as oxidative stress, one must always pay attention to the specificity of the oxysterols employed as biomarkers—could an altered concentration actually reflect an interfering oxidative stress factor? Despite the challenges in the biomarker use of oxysterols, they do hold a lot of promise; Further vigorous research efforts on large patient cohorts are needed to make a reality of these promises.

18.6 Phytosterols

18.6.1 Phytosterols and Phytostanols and Their Presence in Food

The collective name phytosterols include phytosterols and their 5-α-saturated derivatives phytostanols. Phytosterols are structurally similar to cholesterol except for slight differences in the side chain (Fig. 18.2). Phytosterols and phytostanols are present in plants and in plant-based foods in different forms such as in free, fatty acid ester, glucosides, and esterified glucoside forms.

Especially vegetable oils, such as corn oil, rapeseed (Canola) oil, soybean oil, and sunflower oil, and seeds, nuts, breads, and cereals are rich in

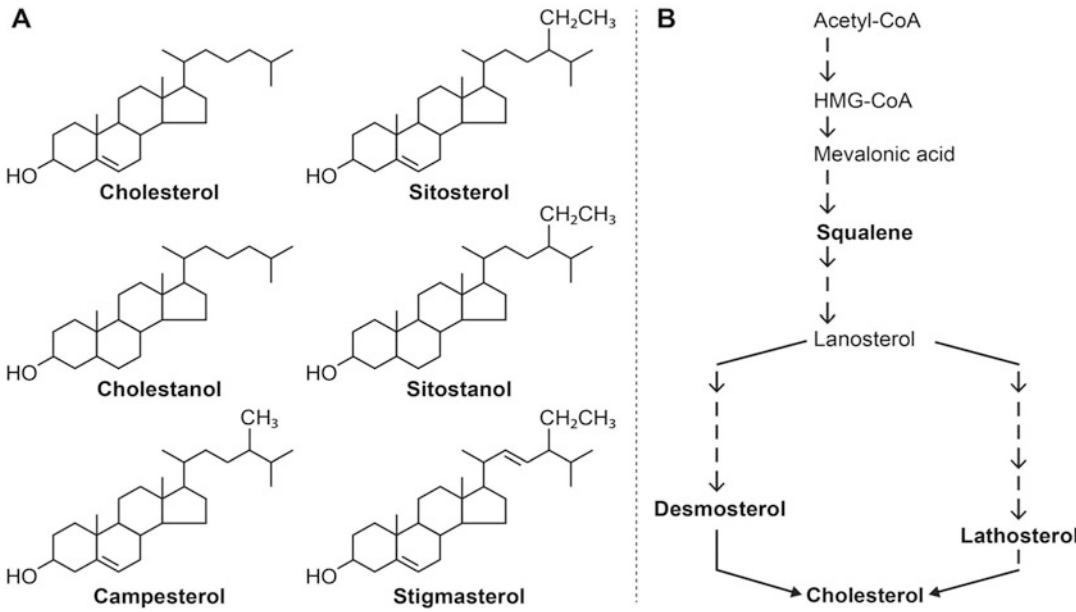

Fig. 18.2 (Panel **A**) The structures of cholesterol and its saturated derivative cholestanol, the phytosterols sitosterol, campesterol, stigmasterol, and sitostanol, the saturated derivative of sitosterol. (Panel **B**) A modification of the cholesterol synthesis pathway emphasizing those cholesterol precursors dealt with in this study and indicated in bold font. Broken arrows denote omitted precursors

phytosterols (Piironen and Lampi 2004). Cereals contain also moderate phytostanols.

Normal Western diets contain phytosterols of approximately 300–360 mg/day (Escurriol et al. 2010; Jaceldo-Siegl et al. 2017; Klingberg et al. 2008), which is approximately the same amount as the dietary intake of cholesterol. In contrast, the amount of phytostanols in diets is much less, about 20 mg/day in a typical Finnish diet (Valsta et al. 2004).

The most abundant individual phytosterols in food items are sitosterol, campesterol, and stigmasterol, and the main phytostanols are sitostanol and campestanol. Their relative quantities in food have been evaluated, e.g., in the large EPIC-Norfolk cohort study including about 25,000 individuals (Klingberg et al. 2008). Of the total dietary intake of phytosterols and phytostanols, the proportion of sitosterol was 66%, and those of campesterol 22%, stigmasterol 8%, sitostanol 3%, and campestanol 1%, respectively.

18.6.2 Phytosterol and Phytostanol Metabolisms in Humans

As a consequence of the structural differences between cholesterol, phytosterols, and phytostanols, their intestinal absorption, circulating and tissue concentrations, metabolism, and elimination differ from each other. Phytosterols and phytostanols are not synthesized in the human body, so that their variability in serum is dependent also on their supply in the diet. Together with cholesterol, they are taken up from the micellar phase in the upper small intestine to the enterocytes by the membrane transporter Niemann-Pick C1 Like 1 (NPC1L1) (Davis Jr. et al. 2004). However, about half of cholesterol and most of the phytosterols and phytostanols taken into the enterocyte are actively excreted back to the intestinal lumen by the adenosine triphosphate (ATP)-binding cassette (ABC) G5/8 transporters ABCG5 and ABCG8 (ABCG5/8) (Berge et al. 2000). NPC1L1 and ABCG5/8 transporters are also located in the membranes of hepatocytes functioning similarly as in the enterocytes but regarding biliary excretion.

Genetic regulation of the NPC1L1 and ABCG5/8 transporters is coordinated mainly by the cellular transcription factors liver X receptors (LXRs) (Duval et al. 2006; Repa et al. 2002). LXRs are activated by high intracellular concentrations of cholesterol and oxysterols, and they upregulate the expression of genes diminishing cholesterol absorption and increasing its removal from the body. Interestingly, it has been demonstrated that stigmasterol, but not sitosterol or campesterol, is able to disrupt cellular cholesterol homeostasis in a cell-based assay by activating the LXR system (Yang et al. 2004). In addition, stigmasterol also reduces cellular cholesterol synthesis by inhibiting the processing of the sterol regulatory element-binding transcription factor 2 (SREBP-2) (Yang et al. 2004).

The sterols and stanols are taken up into the enterocytes in selective order, which in mice is cholesterol > campesterol > sitosterol > campestanol > sitostanol (Igel et al. 2003). Also in mice, more than 97% of sitostanol is recovered in feces compared with 88% of sitosterol (Ikeda and Sugano 1978). Similarly, the sterols are excreted also from the hepatocytes to bile in selective order, which in mice is stigmasterol > sitosterol > campesterol > cholesterol (Yu et al. 2004).

In healthy individuals during phytosterol and phytostanol controlled test diets, the absorption efficiency of phytosterols was <2% and that of phytostanols <0.2% (Ostlund Jr. et al. 2002). More specifically, sitosterol was absorbed by 0.51%, campesterol by 1.89%, sitostanol by 0.04%, and campestanol by 0.15%.

Because of the low absorption and effective biliary elimination, the serum concentrations of phytosterols and especially those of phytostanols are very low, ≤24 μmol/L (≤1 mg/dL) for phytosterols and ≤0.3 μmol/L (≤0.012 mg/dL) for phytostanols (Gylling et al. 2014). In circulation, approximately 60% of phytosterols and phytostanols are carried mainly as fatty acid esters in low-density lipoproteins (LDLs) from liver to tissues, and 29% are carried in high-density lipoproteins (HDLs) from tissues back to liver (Miettinen and Gylling 2003b). The rest is carried in very-low (VLDLs) and intermediate-

density lipoproteins (IDLs) from liver to circulation.

Thus, phytosterols and phytostanols are transported in serum and distributed in lipoproteins similar to cholesterol. This suggests that the higher the LDL level and LDL cholesterol concentration, the higher is also the phytosterol concentration in subjects without gene variants impacting their absorption efficiency. To obtain their relevant serum concentrations, they should be standardized to serum cholesterol concentration and expressed as ratios to cholesterol. The standardization also enables to compare phytosterol and phytostanol levels between individuals and populations with varying serum cholesterol concentrations. Phytosterols and phytostanols are distributed from serum into tissues in similar proportions as they are present in serum without any observed tissue accumulation (Genser et al. 2012; Simonen et al. 2015; Windler et al. 2023).

Phytosterolemia (sitosterolemia) is a rare Mendelian disease, which is caused by homozygous or compound heterozygous loss-of-function (LoF) mutations in *ABCG5/8* genes (Windler et al. 2023). It was first described in 1974 in two overall healthy sisters aged 20 and 22 years with normal plasma cholesterol levels, but they had tendon xanthomas and extremely high serum total phytosterol levels, 37 mg/dL and 26 mg/dL (the reference value being ≤1 mg/dL) (Bhattacharyya and Connor 1974). The absorption rate of sitosterol in these two sisters was markedly increased being 24% and 28% (the reference value being <0.51%). In three other phytosterolemia patients, sitosterol absorption was about 20%, cholesterol absorption efficiency was 61–63%, and endogenous cholesterol elimination via bile to feces was very low, one-fifth from the controls (Lutjohann et al. 1996; Miettinen 1980). The prevalence of phytosterolemia is estimated to be approximately 1 in 200,000 individuals, but since the serum phytosterols cannot be detected with routine lipid measurements, the prevalence may be underestimated (Brinton et al. 2018). The risk of the development of atherosclerotic cardiovascular diseases (ASCVDs) in phytosterolemia varies, but, if anything, it is assumed to be linked to the LDL cholesterol concentrations, which vary greatly between the phytosterolemia families and especially in childhood (Nomura et al. 2020; Tada et al. 2022).

18.6.3 Cholesterol Homeostasis and High Cholesterol Absorption

The absorption of cholesterol from intestine to liver and its elimination from the body via bile are active and selective processes combined tightly with whole-body cholesterol synthesis. Basically, a balance between the absorption, excretion, and synthesis of cholesterol are considered the main components of cholesterol homeostasis, i.e., a steady-state of cholesterol metabolism (Grundy et al. 1969). As a consequence, when cholesterol absorption is low, cholesterol synthesis and biliary cholesterol elimination to feces are elevated, and when cholesterol absorption is high, cholesterol synthesis and biliary cholesterol elimination to feces are low (Gylling and Miettinen 2002; Lutjohann et al. 1995).

Distribution of the percentage absorption of dietary cholesterol, called cholesterol absorption efficiency herein, was studied in a population-based overall healthy study population aged 17–80 years (Bosner et al. 1999). Cholesterol absorption efficiency ranged from 29% to 80% with a mean value of 56%. Thus, in about 40% of the participants, cholesterol absorption efficiency was 50–60%, which can be considered the "normal" reference values (Lutjohann et al. 1993; Miettinen et al. 1990). In about one-third of the population, it was higher than 60%, which is considered high cholesterol absorption.

The concept of high cholesterol absorption is important because it renders the cholesterol metabolism more atherogenic, and will for that reason be briefly discussed. In high cholesterol absorbers, because of low biliary cholesterol elimination, excess cholesterol accumulates in tissues including arterial walls and increases their atherosclerotic burden (Lin et al. 2017; Sehayek and Hazen 2008). In addition, the

aggregability of LDL particles, which is associated with the risk of ASCVD death in coronary patients (Ruuth et al. 2018), is increased in high cholesterol absorbers. Moreover, the risk of recurrent coronary events was increased twofold in high compared with low cholesterol absorbers during a 5-year follow-up (Miettinen et al. 1998). Statin treatment alone did not inhibit recurrent coronary events (Miettinen et al. 1998; Yamaguchi et al. 2018) or in-stent restenosis in high cholesterol absorbers (Otto et al. 2022). In these subjects, a combination therapy with statin-ezetimibe decreasing cholesterol synthesis and absorption was needed to reduce the atherosclerotic event risk (Otto et al. 2022; Yamaguchi et al. 2018).

18.7 Phytosterols as Serum Biomarkers of Cholesterol Absorption

18.7.1 The Definition of a Biomarker

National Institutes of Health (NIH) have published a consensus statement regarding the definition of a biomarker as follows: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). Their use in cardiovascular medicine has been critically discussed, and if properly validated, confounding factors identified, and thoughtfully and critically used, they are considered valuable tools from basic, translational, and clinical research to patient care (Libby and King 2015). For example, LDL, HDL, and triglycerides are considered lipid biomarkers of the pathogenic pathways in atherosclerotic cardiovascular diseases (ASCVDs), and cardiac troponins as the biomarkers of myocardial injury.

18.7.2 Serum Phytosterols and Cholesterol Absorption

Since cholesterol absorption and cholesterol synthesis are tightly interconnected, three cholesterol precursors depicted in Fig. 18.2 and cholesterol synthesis are discussed to some extent also in the following.

The assessment of absolute cholesterol absorption (mg/day) is a complicated and time-consuming process. The first methods were based on intestinal perfusion techniques, but the current methods are mainly non-invasive, based on consuming stable isotopes and non-isotopic tracers, keeping dietary recalls, and collecting fecal samples, all lasting from 3 to 7 days in general (Crouse and Grundy 1978; Lutjohann et al. 1993; Matthan and Lichtenstein 2004). The prerequisite for the metabolic studies is that the study participants have a steady-state cholesterol metabolism and follow stable conditions regarding living habits and dietary cholesterol intake. The analyses require sophisticated chromatography-mass spectrometry techniques and tracer countings. From these assays, the absolute values of dietary, biliary, and total cholesterol absorption and cholesterol absorption efficiency are obtained. Cholesterol absorption efficiency is generally considered to reflect not only the dietary but also the total (dietary and biliary) relative cholesterol absorption (Miettinen et al. 2011).

These complicated and time-consuming methods limit the feasibility of the absolute measurements especially in large population studies. The finding that serum cholesterol precursors quantified with gas-liquid chromatography correlated with absolute cholesterol synthesis cleared the way for the discovery that some of the serum cholesterol precursors and phytosterols could be used as markers of cholesterol synthesis and absorption, respectively (Miettinen 1970; Tilvis and Miettinen 1986).

As ratios to cholesterol, serum phytosterols sitosterol and campesterol, and serum cholestanol, a metabolite of cholesterol, correlate with cholesterol absorption efficiency and absolute absorption of dietary cholesterol (Table 18.1)

Table 18.1 Correlations between variables of cholesterol metabolism and their serum biomarkers in a randomly selected male population aged 50 years

Variables	Serum sterols, μmol/mmol of cholesterol			
	Lathosterol $n = 63$	Sitosterol $n = 63$	Campesterol $n = 63$	Cholestanol ($n = 61$)
Cholesterol absorption efficiency, %	−0.529***	0.301*	0.423***	0.317**
Dietary cholesterol absorbed, mg/kg/day	−0.478***	0.383**	0.362**	−0.090
Cholesterol synthesis, mg/kg/day	0.411**	−0.300*	−0.337**	−0.422***
Fecal endogenous sterols, mg/kg/day	0.284*	−0.431***	−0.331***	−0.357**
Serum sitosterol[a]	−0.235	1.000	0.855***	0.366**
Serum campesterol[a]	−0.222	0.855***	1.000	0.238
Serum cholestanol[a]	−0.379*	0.365*	0.238	1.000

Modified from Miettinen et al. (1989, 1990)
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$
[a]μmol/mmol of cholesterol

(Miettinen et al. 1989, 1990; Tilvis and Miettinen 1986). Moreover, they correlate negatively with cholesterol synthesis and fecal endogenous sterols. Fecal endogenous sterols denote biliary cholesterol elimination and cholesterol eliminated via the so-called trans-intestinal cholesterol elimination pathway (TICE) to feces, and they mainly contain endogenous cholesterol from the hepatic cholesterol pool and its bacterial conversion products coprostanol and coprostanone. Serum phytosterols and cholestanol correlate also with each other, and negatively with cholesterol synthesis markers.

The demethylated cholesterol precursors desmosterol and lathosterol, and squalene in type 2 diabetes, correlate as ratios to cholesterol with absolute cholesterol synthesis and negatively with cholesterol absorption efficiency, as shown for lathosterol in Table 18.1 (Miettinen et al. 1990; Simonen et al. 2008).

Thus, these findings suggest that sitosterol, campesterol, and cholestanol as ratios to cholesterol are valid biomarkers of cholesterol absorption efficiency and dietary cholesterol absorption. In addition, serum desmosterol and lathosterol ratios to cholesterol are valid biomarkers of cholesterol synthesis, but serum squalene is valid only in specific populations.

The validity of the biomarkers is not only based on the studies discussed above. The ratios to cholesterol of the absorption and synthesis biomarkers have been validated against the absolute methods in different populations regarding age, gender, body mass index (BMI), ethnicity, and main chronic diseases related to cholesterol metabolism and/or atherosclerosis. This has been studied under specific dietary challenges, during inhibition of cholesterol absorption with ezetimibe or with phytosterol/phytostanol ester-enriched dietary products and supplements, with the activity of the enzyme hydroxy-methyl-glutaryl CoA reductase (HMG-CoAR), or in individuals with LoF genetic variations in the NPC1L1 or ABCG5/8 transporters (Bjorkhem et al. 1987; Cohen et al. 2006; Grundy 2013; Lutjohann et al. 1995; Miettinen et al. 2011; Stellaard and Lutjohann 2017; Stellaard et al. 2017).

The homeostasis of cholesterol metabolism can be assessed using the biomarkers as demonstrated in Table 18.1 and confirmed with the respective absolute methods.

The negative correlation between serum sitosterol, campesterol, and cholestanol ratios to cholesterol and fecal endogenous sterols indicates that the absorption biomarkers also reflect the activity of biliary endogenous cholesterol elimination in inverse proportion. This finding was confirmed later in a study population of over 80 individuals (Lin et al. 2017). The atheroprotective effect of both low cholesterol absorption and effective elimination of endogenous cholesterol has been demonstrated in humans (Lin et al. 2017) and in laboratory animals (Greenberg et al. 2009).

18.7.3 Do the Serum Sitosterol, Campesterol, and Cholestanol Ratios to Cholesterol Fulfill the NIH Definitions for a Biomarker?

Regarding the first NIH requirement for a biomarker that it should be objectively measured (Biomarkers Definitions Working Group 2001), the two serum phytosterols, cholestanol, and the three cholesterol precursors fulfill the criteria when quantitated with gas- or liquid chromatography with flame ionization or mass-selective detection (Lutjohann et al. 2019). The methodology is demanding throughout the whole process from sample preparation to interpretation of the sterol chromatograms. The first international survey of the quality of the chromatographic measurements of serum cholesterol, sitosterol, campesterol, lathosterol, and cholestanol was performed in 22 sterol research laboratories located in Brazil, Canada, Europe, and USA. The laboratories received a calibration solution from the standard stock, but used their own internal standards. Even though excellent results were observed between some of the laboratories, there were also high interlaboratory variations in the analyses of cholesterol and noncholesterol sterols, so it was encouraged to harmonize the analytical methods between the laboratories and to adopt regular quality control programs.

Regarding the second NIH requirement for a biomarker to be evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention, the biomarkers of cholesterol absorption and synthesis fulfill all these three prerequirements as discussed above (Bjorkhem et al. 1987; Cohen et al. 2006; Grundy 2013; Lutjohann et al. 1995; Miettinen et al. 2011).

18.7.4 The Caveats Related to the Use of Phytosterols as Serum Biomarkers of Cholesterol Absorption

The biomarkers of cholesterol absorption (and cholesterol synthesis) are generally accepted and widely employed globally in the field of cholesterol research. Prerequisites to their use imply that, like all metabolic studies, they should be performed under stable conditions. This can be confirmed by analysis of both the absorption and synthesis biomarkers. Still, they are relative indicators of cholesterol metabolism, and their validity is not self-evident in different populations. Their validity should therefore be confirmed in "new" populations or unexplored pathogenic processes. In obesity, high cholesterol synthesis leads to increased output of biliary cholesterol, which may dilute the intestinal cholesterol absorption markers and also result in reduced serum phytosterol and cholestanol levels, and thus flaw the results of cholesterol absorption. In addition, during consumption of phytosterol- or phytostanol-enriched food products or supplements, serum phytosterols cannot be used as biomarkers of cholesterol absorption. Since each of the serum biomarkers has their individual metabolism, it is worthwhile to use several instead of only one to ensure a valid result.

18.8 Future Perspectives of Phytosterols as Serum Biomarkers of Cholesterol Absorption

The use of biomarkers of cholesterol absorption (and synthesis) enables to investigate cholesterol absorption and synthesis from a serum sample and with less laborious measures and a less demanding methodology than is necessary for the respective absolute measurements. The biomarker analysis is feasible also in large population-based studies. The drawbacks are the relatively complicated technique related to the capillary gas-liquid chromatography-flame

ionization detection (GC-FID), GC-mass selective detection (MS), and liquid chromatography (LC)-MS, the requirement of a steady-state cholesterol metabolism of the study population, and the validity checking of "new" populations with absolute measurements, especially if the serum biomarkers of cholesterol absorption and synthesis fail to correlate with each other.

At the same time, the use of serum biomarkers enables to explore of cholesterol metabolism in individuals, in families, and in large populations to recognize (1) individuals and families with high cholesterol absorption, (2) individuals and families with phytosterolemia, and (3) individuals with high cholesterol absorption that can be induced an increased risk of ASCVD. These conditions are genetically determined, and their diagnosis requires either genetic analyses or quantitation of the serum cholesterol absorption biomarkers. These conditions cannot be diagnosed by routine blood lipid analyses. If also LDL cholesterol concentration is within the reference values in high cholesterol absorbers or in phytosterolemia, these individuals will be missed out on proper treatment.

High cholesterol absorption modulates cholesterol metabolism more atherogenic. To optimally reduce the risk of ASCVD events, the patients need a combination therapy with statin and ezetimibe or alternatively statin-phytostanol/phytosterol products. In phytosterolemia, the risk of ASCVD varies, but a low phytosterol/phytostanol diet and ezetimibe or statin-ezetimibe combination therapy will control the LDL cholesterol and phytosterol levels. Since in both conditions, if untreated and even in the presence of reasonable LDL cholesterol levels, ASCVD can develop unnoticed from young adulthood for decades as subclinical atherosclerosis (Fernandez-Friera et al. 2015).

The future perspectives on phytosterols, how to utilize the information on cholesterol metabolism and the ASCVD risk, include, first, that the analysis of biomarkers of serum cholesterol metabolism is available in routine lipid laboratories. Second, it is important to generally acknowledge that the profile of cholesterol metabolism can reveal the risk of the development of ASCVD. Third, the screening of the profile of cholesterol metabolism will be included in the ASCVD risk surveys, e.g., in families with a history of early onset or frequent ASCVDs and in young adults aged 18–20 years to exclude the presence of high cholesterol absorption. Individuals in high cholesterol absorption families need preventive measures from young adulthood to decrease cholesterol absorption in order to inhibit the development and progression of atherosclerosis. In primary prevention, the dietary intake of phytosterol/phytostanol-enriched food items or supplements is recommended, because a daily intake of 2–3 g of phytostanols as esters reduces LDL cholesterol by 0.33–0.42 mmol/L (9–12%) (Musa-Veloso et al. 2011) and decreases cholesterol absorption by −17% (Miettinen and Gylling 2003a). The reduction in cholesterol absorption is reflected in increased cholesterol elimination from the body, and these changes render cholesterol metabolism less atherogenic.

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Part V

Pharmacological Applications

19 Therapeutic Applications of Oxysterols and Derivatives in Age-Related Diseases, Infectious and Inflammatory Diseases, and Cancers

Mohamed Ksila, Imen Ghzaiel, Khouloud Sassi, Amira Zarrouk, Valerio Leoni, Giuseppe Poli, Leila Rezig, Vivien Pires, Smail Meziane, Atanas G. Atanasov, Sonia Hammami, Mohamed Hammami, Olfa Masmoudi-Kouki, Oumaima Hamdi, Pierre Jouanny, Mohammad Samadi, Anne Vejux, Taoufik Ghrairi, and Gérard Lizard

M. Ksila
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Neurophysiology, Cellular Physiopathology and Valorisation of Biomolecules (LR18ES03), Department of Biology, Faculty of Sciences, University Tunis El Manar, Tunis, Tunisia

I. Ghzaiel
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

K. Sassi · V. Pires · A. Vejux · G. Lizard (✉)
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France
e-mail: gerard.lizard@u-bourgogne.fr

A. Zarrouk
Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

Faculty of Medicine, University of Sousse, Laboratory of Biochemistry, Sousse, Tunisia

V. Leoni
Department of Laboratory Medicine, University of Milano-Bicocca, Azienda Socio Sanitaria Territoriale Brianza ASST-Brianza, Desio Hospital, Desio, Italy

G. Poli
Department of Clinical and Biological Sciences, University of Turin, San Luigi Hospital, Turin, Italy

L. Rezig
University of Carthage, National Institute of Applied Sciences and Technology, LR11ES26, LIP-MB 'Laboratory of Protein Engineering and Bioactive Molecules', Tunis, Tunisia

University of Carthage, High Institute of Food Industries, El Khadra City, Tunis, Tunisia

S. Meziane
Institut Européen des Antioxydants (IEA), Neuves-Maisons, France

A. G. Atanasov
Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Magdalenka, Poland

S. Hammami
Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

University Hospital Fattouma Bourguiba, Monastir, Tunisia

M. Hammami
Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

O. Masmoudi-Kouki · T. Ghrairi
Laboratory of Neurophysiology, Cellular Physiopathology and Valorisation of Biomolecules (LR18ES03), Department of Biology, Faculty of Sciences, University Tunis El Manar, Tunis, Tunisia

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Abstract

Oxysterols, resulting from the oxidation of cholesterol, are formed either by autoxidation, enzymatically, or by both processes. These molecules, which are provided in more or less important quantities depending on the type of diet, are also formed in the body and their presence is associated with a normal physiological activity. Their increase and decrease at the cellular level and in biological fluids can have significant consequences on health due or not to the interaction of some of these molecules with different types of receptors but also because oxysterols are involved in the regulation of RedOx balance, cytokinic and non-cytokinic inflammation, lipid metabolism, and induction of cell death. Currently, various pathologies such as age-related diseases, inflammatory and infectious diseases, and several cancers are associated with abnormal levels of oxysterols. Due to the important biological activities of oxysterols, their interaction with several receptors and their very likely implications in several diseases, this review focuses on these molecules and on oxysterol derivatives, which are often more efficient, in a therapeutic context. Currently, several oxysterol derivatives are developed and are attracting a lot of interest.

Keywords

Oxysterols · Therapeutic applications · Age-related diseases · Alzheimer's disease · Cardiovascular diseases · Eye diseases · Osteoporosis · Infectious diseases · Inflammatory diseases · Cancer

O. Hamdi
University Hospital Fattouma Bourguiba, Monastir, Tunisia

Pôle Personnes Agées, CHU de Dijon, Centre de Champmaillot, Dijon Cedex, France

P. Jouanny
Pôle Personnes Agées, CHU de Dijon, Centre de Champmaillot, Dijon Cedex, France

M. Samadi
Laboratory of Chemistry and Physics Multi-Scale Approach to Complex Environments, Department of Chemistry, University Lorraine, Metz, France

Abbreviations

20S-OHC	20(S)-Hydroxycholesterol
22R-OHC	22(R)-Hydroxycholesterol
22S-OHC	22(S)-Hydroxycholesterol
24S-OHC	24(S)-Hydroxycholesterol
25-OHC	25-Hydroxycholesterol or 5-Cholestene-3β,25-diol
27-OHC	27-Hydroxycholesterol
4β-OHC	4β-Hydroxycholesterol
5α,6α-EC	5α,6α-Epoxycholesterol
5β,6β-EC	5β,6β-Epoxycholesterol
7KC	7-Ketocholesterol
7α,25 DHC	7α,25-Dihydroxycholesterol
7α-OHC	7α-Hydroxycholesterol
7β-OHC	7β-Hydroxycholesterol
AMD	Age-related macular degeneration
CH25H	Cholesterol 25-hydroxylase
ChEH	Cholesterol epoxide hydrolase
CT	Cholestane-3beta-5alpha-6beta-triol
LXRs	Liver X receptors α or β

19.1 Oxysterols: From Key Players in Various Diseases to Therapeutic Applications

Oxysterols are derived from the oxidation of cholesterol (also named: cholesterin, cholesteryl alcohol, and cholest-5-en-3beta-ol; PubChem CID 5997) (Fig. 19.1). Oxysterols are formed by autoxidation and/or enzymatically are identified at variable levels in all cells of the body as well as in biological fluids, and are associated with normal physiological activity (Mutemberezi et al. 2016). From one cell type to another, depending on the tissue and the organ considered, the

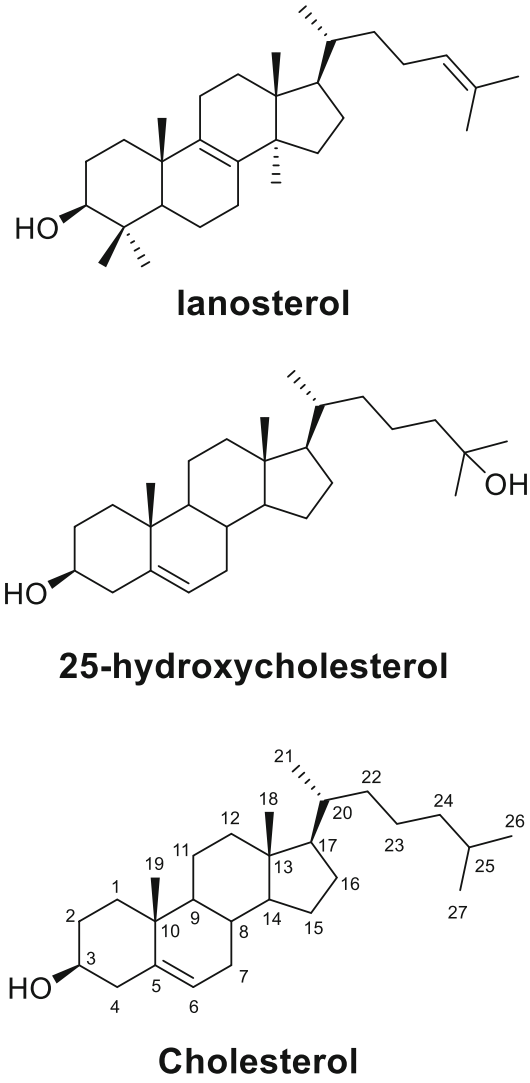


Fig. 19.1 Prevention of cataract with sterols. Lanosterol and 25-hydroxycholesterol (25-OHC, also named 5-Cholestene-3β,25-diol) clarify the lens. 25-OHC is known as VP1-001 for its use in cataract as eye drops to clarify the crystallin (https://newdrugapprovals.org/2022/06/20/vp1-001/, accessed on February 20, 2023). Cholesterol, which is the precursor of all oxysterols (formed either by autoxidation, enzymatically, or both), is shown as a reference (Mutemberezi et al. 2016)

oxysterol profile can be different, and it can vary under the influence of intrinsic factors (genetic factors, metabolism) and extrinsic factors (lifestyle, physical and emotional stress, diet) (Dias et al. 2019). Oxysterol levels, depending on the diseases concerned and their stages, can be either decreased or increased, highly or slightly. In addition, oxysterols are involved in the regulation of RedOx balance, cytokinic and non-cytokinic inflammation, lipid metabolism, and the activation of cell death which are hallmarks of aging and age-related diseases (De Freitas et al. 2021; De Medina et al. 2022). Some of them, especially those oxidized on the side chain, also interact with specific receptors such as: (a) nuclear receptors (liver X receptors (LXRs) α or β (Viennois et al. 2011), retinoic acid receptor-related orphan receptor α and γ (RORα [NR1F1], and RORγ [NR1F3]) (Wang et al. 2010); (b) cytoplasmic receptors such as SREBP (sterol regulatory element binding transcription protein) (Sato 2010), NPC1 (NPC intracellular cholesterol transporter 1/Nieman-Pick type C1) (Yu et al. 2014), FXR (NR1H4, farnesoid X receptor alpha) (Kovač et al. 2019), oxysterols binding proteins (OSBPs), OSBPs-related proteins (ORPs) (Olkkonen and Ikonen 2022; Arora et al. 2022), and cholesterol epoxide hydrolase (ChEH) [also named anti-estrogen binding site (AEBS) which is an hetero-oligomeric complex comprising 3β-hydroxysterol-δ(8)-δ(7)-isomerase (D8D7I) and 3β-hydroxysterol-δ (7)-reductase (DHCR7)] (Silvente-Poirot and Poirot 2012); and (c) the Epstein–Barr virus-induced gene 2 receptor (EBI2, also known as GPR183) (Hannedouche et al. 2011; Liu et al. 2011; Emgård et al. 2018). Some of these receptors are involved in cholesterol trafficking, cell proliferation, and cell death. There are also several lines of evidence that RORs, FXR, LXRs, and EBI2 are involved in inflammation (Spann and Glass 2013; Pascual-García and Valledor 2012; Fessler 2018; Cariello et al. 2021). 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-OHC), either minimally or do not interact with these receptors. However, 7KC could interact with tyrosine kinase receptors (Kim and Lee 2010). Due to the important biological activities of oxysterols, their interaction with several receptors and their very likely implications in several diseases especially age-related diseases (cardiovascular and eye diseases, neurodegenerative diseases, osteoporosis) (Zarrouk et al. 2014), infectious and inflammatory diseases, such as COVID-19 (Marcello

et al. 2020) and inflammatory bowel disease (Offei et al. 2019), as well as some cancers, especially breast cancer (Decker et al. 2023) and prostate cancer (Celhay et al. 2019), this has led to study these molecules in a pharmacological context and to develop oxysterol derivatives for therapeutic applications (Brown et al. 2021). In addition, oxysterol levels may be employed as biomarkers for the diagnosis of specific diseases or in predicting the incidence rate of diseases such as age-related diseases, infectious and inflammatory diseases, and cancers (Samadi et al. 2021).

19.2 Pharmacological Interest of Oxysterols and Derivatives in Age-Related Diseases

19.2.1 Eye Diseases

Cataract and age-related macular degeneration (AMD) are the most frequent age-related eye diseases. In these diseases, the involvement of oxysterols is highly likely (Vejux et al. 2011; Zhang et al. 2021).

19.2.1.1 Cataract

Cataracts can begin to develop as early as the age of 50 years and symptoms can occur from 60 to 70 years of age. In cataracts, characterized by a progressive opacity of the crystallin, the contribution of oxysterols, especially those formed by autoxidation, such 7KC, is widely suspected (Girão et al. 1998; Vejux et al. 2011; Reyes et al. 2023). At the moment, an efficient surgical treatment is available. However, due to its cost, this treatment is not necessarily accessible to all patients. Consequently, the identification of drugs allowing a non-surgical treatment, which would be less expensive, is an important challenge (Lian and Afshari 2020; Lu et al. 2022). In addition, an alternative to surgical correction may be not only applied to adult patients without access to surgical care but also to infantile cataracts which cannot be cured by surgical treatment (Skinner and Miraldi Utz 2017; Xu et al. 2020). At the moment, lanosterol and 25-hydroxycholesterol (25-OHC/5-cholesten-3b,25-diol also named VP1-001) have been reported to combat the aggregation of proteins in the crystallin both in vivo and ex vivo (Zhao et al. 2015; Chen et al. 2018; Wang et al. 2022b; Lee and Afshari 2023) (Fig. 19.1). The ability of 25-OHC/VP1-001 to bind αβ-crystallin, a chaperone maintaining protein stability and preserving lens transparency (Horwitz 1992; Rajagopal et al. 2015), seems important to reverse lens opacity in mouse models of cataract (Molnar et al. 2019). The efficiency of lanosterol and 25-OHC/VP1-001 is, however, controversed: both sterols would fail to reach an acceptable threshold binding scores for good predictive binding to the αB-crystallin (Daszynski et al. 2019).

19.2.1.2 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) concerns people over the age of 50 years. In AMD which affects the retina and results in a loss of central vision, two forms exist: the dry form (the most common), and the exudative form, which is rare and results from the evolution of the dry form to an advanced stage (https://www.nei.nih.gov/learn-about-eye-health/eye-conditions-and-diseases/age-related-macular-degeneration, consulted on September 28, 2023).

AMD is characterized by the presence of high 7KC levels in lipid deposits called drusens which are localized between the Bruch membrane and the basement membrane of retinal pigment epithelial cells (Malvitte et al. 2006; Rodríguez and Larrayoz 2010; Rodriguez et al. 2014). Due to the pro-oxidant, pro-inflammatory, and pro-angiogenic activities of 7KC and to its capacity to trigger cell death (Vejux and Lizard 2009; Amaral et al. 2013), this oxysterol could contribute to favor retinal damages including retinal neovessels formation. Analysis of post-mortem eyes of patients with AMD showed that retinal pigment epithelial cells die by apoptosis (Dunaief et al. 2002) and investigations conducted with 7KC and 7β-OHC on human retinal cell lines (R28, ARPE-19) (Ong et al. 2003; Rodriguez et al. 2004) and on primary cell cultures (neuroretina cells, primary porcine retinal pigment epithelial cells) (Chang and Liu 1998; Joffre

et al. 2007) described cytotoxic and pro-inflammatory effects with 7KC and 7β-OHC. The cytotoxic effect of 7β-OHC was also studied on human pigment retinal epithelial cells (ARPE-19) (Malvitte et al. 2008). It was shown that 7KC can be converted in 7β-OHC by the enzyme hydroxysteroid dehydrogenase (11β-HSD1) expressed in the retinal pigment epithelium and choroid (Zola et al. 2022; Ghzaiel et al. 2022). On ARPE19 cells, 7β-OH induces a caspase-3-independent mode of cell death associated with lysosomal destabilization (Malvitte et al. 2008). At the moment, the cytotoxic effects of 7KC have been extensively studied to clarify its role in the pathophysiology of AMD in order to identify pharmacological targets and new treatment strategies (Pariente et al. 2019). In the context of AMD, but also of cataract, new therapeutic approaches based on the degradation of 7KC or its inactivation are promising avenues (Ghosh and Khare 2017; Ghzaiel et al. 2022).

It is important to emphasize that oxysterols oxidized on the lateral chain are also present at the retinal level. These oxysterols formed enzymatically from cholesterol via the enzyme CYP-46A1 to give 24(S)-hydroxycholesterol (24S-OHC) and via the enzyme CYP-27A1 to give 27-hydroxycholesterol (27-OHC) are present in cone and rod cells as well as in nerve cells such as Müller cells (Léger-Charnay et al. 2019; Zhang et al. 2021). These oxysterols depending on their concentrations may also influence the activity of retinal cells. So, in AMD, decrease and/or increase expression and/or activity of CYP46-A1 and CYP27-A1 may be involved. In agreement with this hypothesis, mice deficient in CYP46-A1 and/or CYP27-A1 in the retina have disturbed electroretinograms indicating a decrease in the vision (Petrov et al. 2019).

19.2.2 Alzheimer's Disease

In France, Alzheimer's disease affects 3% of the age group over 65 years and more than 20% of the population over 80 years. Currently, in Alzheimer's disease the amyloid theory is the best known and the most studied (Paroni et al. 2019; Ma et al. 2022). The latter has more or less put other fields of research in second place such as the involvement of lipid metabolism (fatty acids, cholesterol) as well as the involvement of oxysterols in this frequent and highly disabling neurodegenerative disease with significant social and economic consequences (Björkhem et al. 2006; Kenigsberg et al. 2009). These two approaches, amyloid and lipid theory of Alzheimer's disease, are not as distinct: indeed, amyloids can have consequences on lipid metabolism, and the latter can interact with amyloidogenesis (Sjögren et al. 2006; Zarrouk et al. 2018). For several years, it is important to underline that the involvement of cholesterol and its precursors as well as of some of its oxidized derivatives (oxysterols) has been widely suspected (Vaya and Schipper 2007).

In Alzheimer's disease, a prominent role is mainly attributed to 24S-OH, initially named cerebrosterol (Di Frisco et al. 1953). Indeed, when 24S-OHC is quantified in cerebrospinal fluid and plasma, its quantity makes it possible to assess brain status (brain mass, neuronal metabolism) (Leoni and Caccia 2011). At the beginning of the disease, the destruction of neurons can lead to an increase in 24S-OHC, while in advanced stages, when brain mass is decreased, the amount of 24S-OHC is decreased. In humans and mouse models, CYP46A1 activity is altered. Currently, in addition to 24S-OHC, 27-OHC formed from cholesterol by the enzyme CYP-27A1 also appears to be involved in the pathophysiology of Alzheimer's disease (Wang et al. 2016). Indeed, 27-OHC can cross the blood–brain barrier which is altered in Alzheimer's disease (Björkhem 2006), and there is evidence indicating its influence on the progression of the disease (Loera-Valencia et al. 2019). Interestingly, synthetic oxysterols inducing the production of the LXR agonists 24S-OHC by upregulating *CYP46A1*, encoding the enzyme converting cholesterol into 24S-OHC, were also described (Zhan et al. 2023).

Whereas 24S-OHC and 27-OHC are potentially most closely involved in the pathogenesis of Alzheimer's disease, the possible involvement

of oxysterols resulting from cholesterol autoxidation, including 7KC and 7β-OHC, is now emerging (Testa et al. 2016; Zarrouk et al. 2020). In a systematic analysis of oxysterols in post-mortem human Alzheimer's disease brains, classified by the Braak staging system of neurofibrillary pathology, several oxysterols deriving from cholesterol autoxidation were identified; 7KC, 7β-OHC, 7α-hydroxycholesterol (7α-OHC), 4β-hydroxycholesterol (4β-OHC), 5α,6α-epoxycholesterol (5α,6α-EC), and 5β,6-β-epoxycholesterol (5β,6β-EC) (Testa et al. 2016).

By initiating cholesterol efflux and activating the cholesterol synthesis pathway, CYP46A1 is the key enzyme that ensures brain cholesterol turnover (Pikuleva and Cartier 2021) and constitutes a new therapeutic target in Alzheimer's disease (Djelti et al. 2015).

19.2.3 Osteoporosis

The effects of oxysterols on osteogenesis have been reported since 2004 by Kha et al. in the murine multipotent bone marrow stromal M2-B104 cells which differentiate in osteoblasts and adipocytes (Kha et al. 2004).

On M2-B104 cells, pro-osteogenic effects have been observed with 22(R)-hydroxycholesterol (22R-OHC), 22(S)-hydroxycholesterol (22S-OHC), and 20(S)-hydroxycholesterol (20S-OHC), while anti-osteogenic effects have been found with 7KC (Kha et al. 2004) as well as with cholestane-3beta-5alpha-6beta-triol (CT) on rat bone marrow stromal cells (Liu et al. 2005) (Fig. 19.2). 22R-OHC and 22S-OHC, which are LXR agonist and antagonist, respectively (Janowski et al. 1996), are osteogenic inducers, whereas they have opposing effects on ROS production (Shouhed et al. 2005). On M2-10B4 and C3H10T1/2, osteogenesis induced by 20S-OHC and 22S-OHC involves protein kinase A (PKA)- and protein kinase C (PKC)-associated signaling pathways (Richardson et al. 2007) as well as the Hedgehog pathway (Dwyer et al. 2007; Kim et al. 2007; Amantea et al. 2008). The combination of 22S-OHC and 20S-OHC also promotes periodontal regeneration using periodontal ligament stem cells and alveolar bone healing models (Lee et al. 2017; Che et al. 2022). It has also been shown that 27-OHC, which can be found at high level in the plasma of post-menopausal women and interacts with the estrogen receptor (Nelson et al. 2014; He and Nelson 2017), could also promote osteoporosis (Chang et al. 2019). 25-OHC formed from cholesterol by the enzyme CH25H also inhibits differentiation into osteoblasts (Moseti et al. 2020). This oxysterol triggers oxiapoptophagy (Nury et al. 2021) in human osteoblast-like MG-63 cells and in the femoral bone of a mouse model of osteoporosis obtained by ovariectomy (Seo et al. 2023). Consequently, there are several proofs in favor of an involvement of oxysterols formed either by autoxidation (7KC) and enzymatically (22R-OHC, 22S-OHC, 20S-OHC, 27-OHC) or both (25-OHC) in osteoporosis.

At the moment, several oxysterol derivatives of 20S-OHC have been synthesized to identify molecules more suitable for clinical applications. Two structural derivatives of 20S-OHC, Oxy34, and Oxy49 (Fig. 19.3), were identified as inducing osteogenic differentiation and inhibiting the adipogenic differentiation of murine bone marrow stromal cells (M2-10B4) through activation of Hedgehog signaling (Johnson et al. 2011). Oxy49 also induces osteogenic differentiation in rabbit bone marrow stromal cells (Hokugo et al. 2013). Despite the great promise of Oxy34 and Oxy49, it was important to obtain molecules as efficient with a significantly lower production cost. Oxy133 (Fig. 19.3) which induces significant expression of osteogenic markers Runx2, osterix, alkaline phosphatase, bone sialoprotein, and osteocalcin in C3H10T1/2 mouse embryonic fibroblasts and in M2-10B4 cells, was obtained (Montgomery et al. 2014).

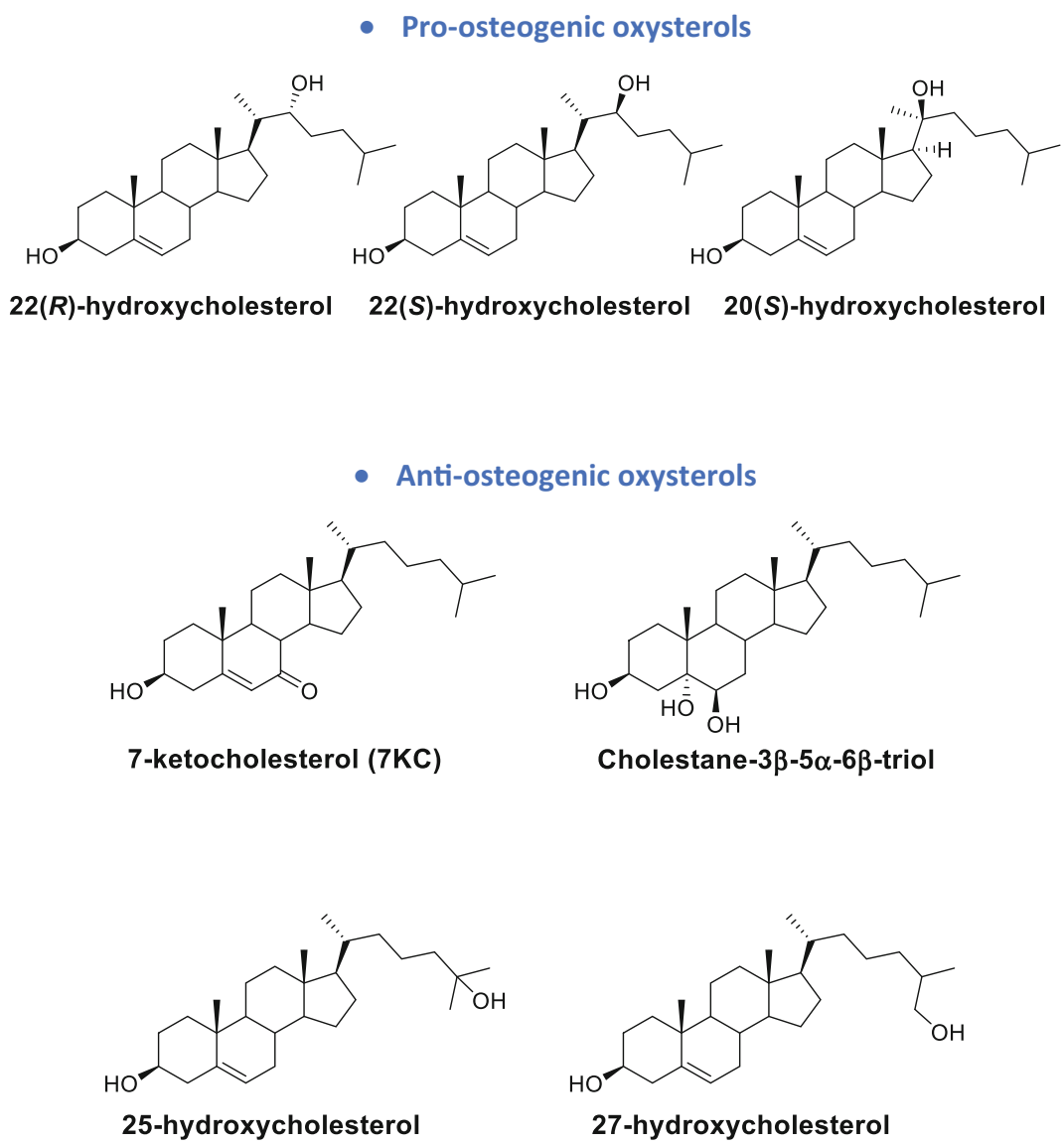
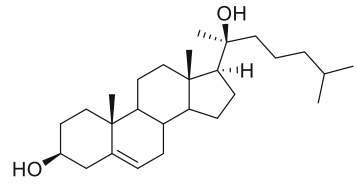
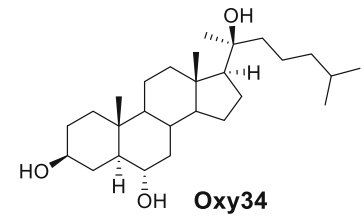


Fig. 19.2 Osteoporosis and oxysterols. Pro-osteogenis oxysterols: 22R-OHC 22(R)-hydroxycholesterol, 22S-OHC 22(S)hydroxycholesterol, 20S-OHC 20(S)-hydroxycholesterol; Anti-osteogenic oxysterols: 7KC 7-ketocholesterol, CT cholestane-3beta-5alpha-6beta-triol, 25-OHC 5-Cholestene-3β,25-diol or 25-hydroxycholesterol, 27-OHC 27-hydroxycholesterol

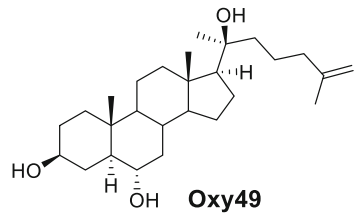
- **Oxysterol derivatives obtained from 20(S)-hydroxycholesterol with pro-osteogenic activities**



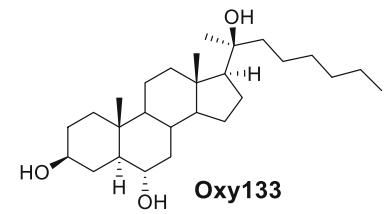
20(*S*)-hydroxycholesterol



Oxy34

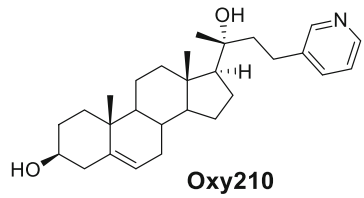


Oxy49

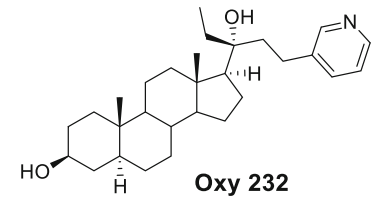


Oxy133

- **Oxysterol derivatives inhibiting viral replication of SARS-CoV2**

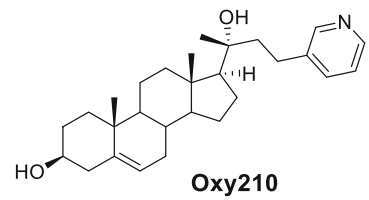


Oxy210



Oxy 232

- **Anti-inflammatory oxysterol derivative**



Oxy210

Fig. 19.3 Oxysterol derivatives. Oxy34, Oxy49, and Oxy133 are oxysterol derivatives of 20(S)-hydroxycholesterol (20S-OHC) and induce osteogenic differentiation; Oxy210 and Oxy232 reduce viral replication of SARS-CoV2 as well as 7KC, 22R-OHC, 24S-OHC, and 27-OHC; Oxy210 has marked anti-inflammatory activities

19.3 Oxysterols and Derivatives in the Pathophysiology and Treatment of Infectious Diseases

Some oxysterol such as 25-OHC and its metabolite 7α,25-dihydroxycholesterol (7α,25 DHC) synthesized from 25-hydroxycholesterol by cytochrome P450 family 7 subfamily B member 1 (CYP7B1) enzyme as well as 27-OHC have emerged as important lipid mediators in the response to both bacterial and viral infections mostly through the modulation of the immune response (Bah et al. 2017; Foo et al. 2022); however, these oxysterols can also reduce viral entry in the cells, reduce viral replication, assembly, and release (Foo et al. 2022); CH25H may inhibit SARS-CoV-2 and other coronaviruses by depleting membrane cholesterol and blocking membrane fusion (Wang et al. 2020; Zang et al. 2020). 25-OHC is also described as a potent SARS-CoV-2 inhibitor (Zu et al. 2020). In addition, there are lot of evidences that 25-OHC and 7α,25-DHC have wide-ranging influences on innate and adaptive immunity (Cyster et al. 2014). Oxysterols bearing an additional ketone or hydroxyl group in the sterol ring, namely in the B ring, such as 7KC, 7α-OHC, and 7β-OHC, also have some antiviral activities (Lembo et al. 2016). In patients with severe forms of COVID-19, physiological serum levels of 27-OHC were significantly decreased compared to the matched control group whereas 7KC and 7β-OHC levels were significantly increased (Marcello et al. 2020) leading to suggest that 7KC might be involved in the pathophysiology of COVID-19 by contributing to the acute respiratory distress syndrome via its pro-oxidant and pro-inflammatory activities (Ghzaiel et al. 2021). In addition, 7KC, 22R-OHC, 24S-OHC, and 27-OHC, substantially inhibited SARS-CoV-2 propagation in cultured cells, and the oxysterol derivatives Oxy210 and Oxy232 (Fig. 19.3) reduced viral replication by more than 90% at 10 μM and 99% at 15 μM, respectively (Ohashi et al. 2021).

19.4 Oxysterols and Derivatives in Inflammatory Diseases and Immunotherapy

There are lot of evidence supporting the involvement of oxysterols in frequent inflammatory human diseases such as atherosclerosis, inflammatory Bowel disease, and arthritis (Testa et al. 2018). 7KC and 7β-OHC which are found at increased levels in the plasma and atherosclerotic plaques of patients with cardiovascular diseases are strongly pro-inflammatory oxysterols (Vejux and Lizard 2009). Several adverse activities (including stimulation of oxidative stress and induction of inflammatory processes) of 7KC, 7β-OHC, 5α,6α-EC, and 5β,6β-EC often present at elevated concentrations in processed and/or stored cholesterol-rich foods are suspected in inflammatory Bowel disease (Biasi et al. 2009). Slight but significant increases of 7KC and CT were also observed in silicosis (Aksu et al. 2020). In addition, critical roles of 25-OHC [formed from cholesterol by the enzyme cholesterol 25-hydroxylase (CH25H)] and its metabolite 7α,25 DHC, which interacts with EBI2, also known as GPR183, are suspected in inflammatory Bowel disease. Thus, 25-OHC limits intestinal IgA plasma cell differentiation (Trindade et al. 2021), and 7α,25 DHC promotes colonic inflammation in mice (Emgård et al. 2018). CH25H may contribute to intestinal fibrosis which is a common complication of Crohn's disease (Raselli et al. 2019). 25-OHC could also be involved in osteoarthritis; this oxysterol induces apoptosis on chondrocytes and caspase-dependent apoptosis as well as proteoglycan loss in the articular cartilage of cultured rat knee joints (Seo et al. 2020). Noteworthy, Oxy210 (Fig. 19.3) has marked anti-inflammatory activities in human and mouse macrophages, THP-1 and RAW264.7, respectively, via inhibition of Toll-Like Receptor (TLR) 4 and TLR2 signaling (Wang et al. 2022a).

19.5 Oxysterols and Derivatives in Cancer: Focus on New Therapeutic Tools and Concept of Metabolic Therapy

Lipid metabolism is an important criterion to take into account in carcinogenesis because it affects cell proliferation and differentiation, oxidative stress, inflammation, and multidrug resistance which are all parameters involved in the control of cell death (Huang and Freter 2015). It is currently well established that some oxysterols interacting or not with receptors are involved in carcinogenesis because of their mutagenic and carcinogenic properties, their effects on cell differentiation, and their pro-oxidant and inflammatory properties (Jusakul et al. 2011; De Freitas et al. 2021). However, the contribution of oxysterols in carcinogenesis seems to be more subtle since some of their derivatives, such as dendrogenin-A (DDA), may have anti-tumor properties (Poirot and Silvente-Poirot 2018) while others such as 6-oxo-cholestan-3β,5α-diol (OCDO) can be pro-tumoral (Poirot et al. 2018). The involvement of oxysterols and metabolites in common cancers, therefore, represents a promising field of study to better understand certain cancers and to identify new treatments, in particular, in the context of metabolic therapy (targeting, for example, cholesterol metabolism) (Sassi et al. 2021; Griffiths and Wang 2022), which can be considered as a promising therapy with few side effects. It should be noted that even if aging does not cause cancer, it is more common in people over 65 years of age (Serraino 2007).

19.5.1 Breast Cancer

Breast cancer is the most common cancer in women in the world and is also the most common form of cancer (Fitzmaurice et al. 2018). In breast cancer, cholesterol metabolism as well as certain oxysterols (7KC, 5α,6α-EC, 5β,6β-EC, CT, and 27-OHC) may play an important role in tumor development and progression (Sawada et al. 2020) (Fig. 19.4). In 2010, de Medina et al. identified cholesterol-5,6-epoxide hydrolase (ChEH) as a target for tamoxifen and anti-estrogen binding site (AEBS) ligands; they show (1) that AEBS is a hetero-oligomeric complex composed of 3beta-hydroxysterol-Delta8-Delta7-isomerase (D8D7I) and 3beta-hydroxysterol-Delta7-reductase (DHCR7) which binds different structural classes of ligands, including ring B oxysterols, (2) that the substrates of ChEH, 5,6α-EC and 5,6β-EC, and its product (CT) are competitive ligands of tamoxifen binding to the AEBS, and (3) that each AEBS ligand is an inhibitor of ChEH activity (De Medina et al. 2010). The 5,6-ECs are metabolized in breast cancers to the tumor promoter OCDO whereas, in normal breast tissue, they are metabolized to the tumor suppressor metabolite, DDA (De Medina et al. 2021). These data pave the way for new therapeutic strategies in breast cancer. There is evidence that 7KC and 27-OHC could contribute to drug resistance. Thus, in human breast tumor MCF-7 cells, which have a high estrogen receptor (ER)α/ERβ ratio, 7KC and 27-OHC decreased the toxicity of doxorubicin, and 7-KC stimulated the efflux function of P-glycoprotein (P-gp) and increases the mRNA and protein levels of P-gp (Wang et al. 2017). There are also more and more evidences that 27-OHC, which can be enhanced in the plasma of post-menopausal women, and which interacts with the estrogen receptors, could contribute to breast cancer development (Nelson et al. 2014; Biasi et al. 2022).

19.5.2 Glioblastoma

Despite the progress made in recent years in the field of oncology, the status of glioblastoma treatment remains unsatisfactory. Glioblastoma is the most common malignant primary brain tumor and is invariably fatal to affected patients. Major challenges are the limitation of irreversible brain damage and the infiltrative part of the tumor tissue which is the ultimate cause of recurrence. At the time of recurrence, treatment options are very limited with modest activity. Oxysterols belong to a class of bioactive lipids that are implicated in

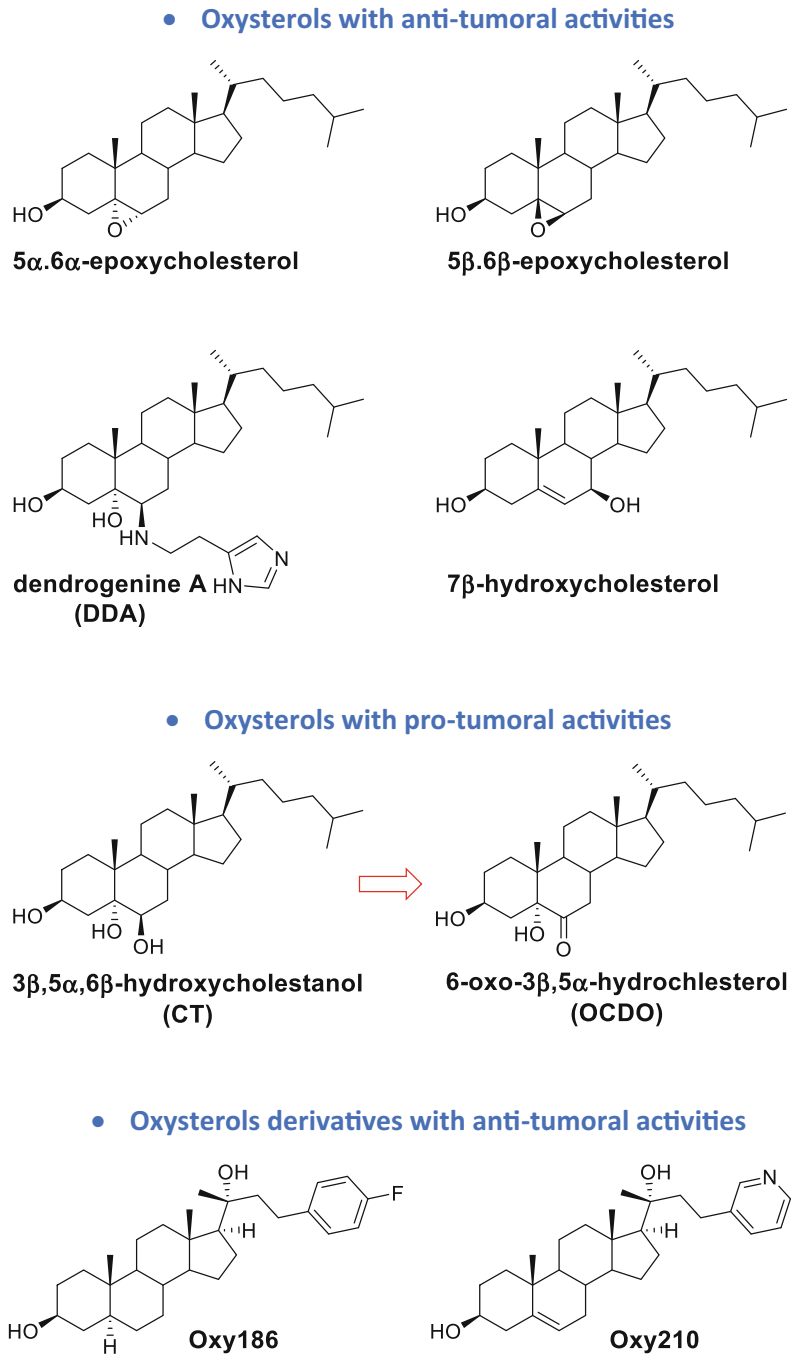


Fig. 19.4 Cancer, oxysterols, and oxysterol derivatives. Oxysterols with anti-tumoral activities: 5α,6α-EC 5α,6α-epoxycholesterol, 5β,6β-EC 5β,6β-epoxycholesterol, DDA dendrogenine-A, 7β-OHC 7β-hydroxycholesterol; oxysterols with pro-tumoral activities: CT cholestane-3beta-5alpha-6beta-triol, OCDO 6-oxo-cholestan-3β,5α-diol [27-hydroxycholesterol (27-OHC); and 7-ketocholesterol are shown in Fig. 19.2]; oxysterol derivatives with anti-tumoral activities: Oxy 186 and Oxy210. CT is metabolized into OCDO by the OCDO synthase identified as 11β-hydroxysteroid dehydrogenase of type 2 (11β-HSD2) (Poirot et al. 2018)

neurological diseases and are associated with various tumor types (De Weille et al. 2013a; Kloudova-Spalenkova et al. 2021). Oxysterols are potent inhibitors of the endogenous cholesterol biosynthesis in the brain and show in vivo a correlation between cholesterogenesis (Wang et al. 2008) and reactive astrocyte proliferation (Velázquez et al. 2006). In addition, oxysterols can also inhibit astrogliosis and intracranial glioblastoma growth (Bochelen et al. 1995).

Glioblastoma is remarkably dependent on cholesterol for survival, rendering these tumors sensitive to LXR agonist-dependent cell death (Villa et al. 2016). Some oxysterols, especially those oxidized on the lateral chain, are major activators of LXR (Janowski et al. 1996; Janowski et al. 1999). It has been shown that LXR-mediated pathways interfere with cholesterol metabolism (Janowski et al. 1996). LXRs and SREBPs constitute a negative feedback loop to regulate the homeostasis of cellular cholesterol metabolism, and numerous studies have found that a variety of drugs alter the cholesterol levels in glioblastoma cells by acting on these two pathways (Guo et al. 2022). Oxysterols such as 24-OHC/25-OHC can inhibit cholesterol synthesis by inhibiting SREBP (Goldstein et al. 2006; Han et al. 2020). Thus, it is not surprising that some oxysterols are able to inhibit cancer cell proliferation including glioblastoma (Guo et al. 2011). Furthermore, gene expression and lipidomic analyses revealed that LXR-β regulates the expression of immune response gene sets and lipids known to be involved in immune modulation. Thus, therapeutic targeting of LXR-β in glioblastoma might be effective through diverse mechanisms (Patel et al. 2019). Moreover, dysregulation of lipids in phospholipidosis inhibits glioblastoma cell proliferation and may play key roles in the induction of apoptosis by oxysterols. Anticancer activity of these compounds may be related to the immobilization of cancer cells as a result of the stiffening effect caused by oxysterols (Wnętrzak et al. 2021). In addition to lowering cellular cholesterol, oxysterols inhibit glioblastoma growth through multiple mechanisms. The 7-β-hydroxycholesterol (7β-OHC) induces ROS overproduction in rat C6 glioma cells, resulting in a non-apoptotic mode of cell death (Sassi et al. 2019). The effect of 7β-OHC on the growth and mitogen-activated protein kinase (MAP)-kinase activity in C6 cell line was investigated. The MAP kinase activity decrease is correlated with the toxic effect of 7β-OHC and occurs at first stages of 7β-OHC action (Adamczyk et al. 1998). It has also been demonstrated that 7β-OHC provokes transient activation of AMP-activated protein kinase (AMPK) in C6 cells (Clarion et al. 2012). De Weille et al. have shown that 7β-OHC provokes both metabolic stress, as witnessed by AMPK activation, increases cell cycle time, changes the affinity of pyruvate kinase to its substrate, phosphoenol pyruvate, and changes in lipid raft composition in C6 cells (De Weille et al. 2013b). The 7-β-hydroxycholesteryl-3beta-oleate possesses anti-tumor properties against the C6 cells injected in the frontal cortex of 6-day-old Wistar rats (Werthle et al. 1994). The effect of an analog of this molecule, 7β-hydroxycholesterol-3beta-O-oleyl, was investigated (Rakotoarivelo et al. 2006). The 7β-ether and 7β-ester forms displayed similar anti-tumor activities whereas the 7β-ether form was more active on well-developed glioblastoma. The 7β-ether form had a cytostatic rather than a cytotoxic effect. It is suggested that the absence of "etherases" enhances the anti-tumor activity of this type of compound. Thus, an original therapeutic approach for glioblastoma treatment may be envisaged with such compounds (Rakotoarivelo et al. 2006). Local administration of 7β-hydroxycholesteryl-3-oleate inhibits the growth of C6 cells and might be useful for local glioblastoma chemotherapy (Werthle et al. 1994). Furthermore, 7β-OHC and 7β-hydroxycholesteryl-3-esters have anti-proliferative and anti-inflammatory properties (Bochelen et al. 1992). The 7β-OHC cytotoxicity has been shown in vivo and in vitro to be dependent on the accumulation of its esters (Clarion et al. 2012). Clarion et al. demonstrated that 7β-OHC exerts cytotoxicity in glioblastoma cells via the accumulation of 7β-OHC esters in lipid rafts, which triggers energy stress, activates a variety of signaling pathways, such as extracellular signal-regulated kinases (ERK), AMPK and phosphoinositide 3-kinase (PI3K)/Akt, and finally activates the P38 signaling pathway, leading to cell death (Clarion et al. 2012). The 24S-OHC exerts different effects on U-87 MG human glioblastoma cells depending on its level. At lower concentrations, it stimulates cellular processes critical to maintain redox homeostasis, while at higher doses it induces a dose-dependent toxicity associated with increased reactive oxygen species production (Cigliano et al. 2019). The

25-OHC, can suppress IL-1β production, thus reducing inflammation but it is ineffective to restore autophagy flux and to decrease apoptosis levels in human glioblastoma cell line (U87-MG) (Tricarico et al. 2017). Eibinger et al. found that 25-OHC acts as a chemokine to promote the recruitment of tumor-related macrophages (Eibinger et al. 2013), suggesting that 25-OHC may be related to tumorigenesis and tumor progression. Many studies have elucidated the effects of 27-OHC in glioblastoma. It is not surprising that its effects are conflicting, because oxysterol can have different effects in various cancers. The 27-OHC increased the proliferation, colony formation, emigration, and invading of U251 and U118 MG cells which are human glioblastoma cell lines. In U251 and U118 MG cell lines, 27-OHC administration was likewise related to an increase in the production of glioblastoma-initiating cells. The 27-OHC has been shown to increase the expression of phosphorylated MAK (pMAPK), phosphorylated mammalian target of rapamycin (pmTOR), phosphorylated AKT (pAKT), p70S6K, and YKL40 in glioblastoma cells (Liu et al. 2019). Furthermore, elevated concentrations of 27-OHC in glioblastoma tissues were linked to a poor prognosis in sufferers. Finally, 27-OHC could have a crucial function in developing glioblastoma (Abdalkareem Jasim et al. 2022). Nevertheless, another study found that 27-OHC inhibits cell viability and induces apoptosis by reducing cholesterol in C6 glioma cells (An et al. 2017). The comparative study of natural and synthetic oxysterols effects on rat C6 glioma cells underlines that they can potentially constitute a new group of molecules to treat glioblastoma (Sassi et al. 2021). Therefore, we believe that some oxysterols are good candidates as new therapeutic molecules, in the context of metabolic therapy of glioblastoma, and could constitute an alternative to the currently aggressive treatments of this brain cancer (radiotherapy, chemotherapy with memantine), and/or that they could be of interest to potentialize the activity of current treatments.

19.5.3 Myeloma

Multiple myeloma is an hematological cancer with frequent patient relapse due to drug resistance. On JJN3 and U266 human myeloma cells and on ex vivo in myeloma patients' sorted CD138+ malignant cells, two cholesterol derivatives (5α,6α-EC and 5β,6β-EC isomers) induce a mode of cell death by oxiapoptophagy (Nury et al. 2021) through concomitant oxidative stress, caspase-3-mediated apoptosis and autophagy (Jaouadi et al. 2021). These data highlight anti-tumor activity of 5α,6α-EC and 5β,6β-EC against human myeloma cells, paving the way for future therapeutic strategies in multiple myeloma.

19.5.4 Non-Small Cell Lung Cancer

Non-small cell lung cancer (NSCLC) is a leading cause of cancer often associated with metastasis and drug resistance. In patients with NSCLC, the 27-OHC content was significantly higher in the tumor region than in the non-tumor region (Takada et al. 2022). Oxy186 and Oxy210 are oxysterol derivatives (Fig. 19.4). In the human NSCLC cell line A549, Oxy186 and Oxy210 inhibit WNT/β-catenin signaling and suppress the proliferation of A549 cells; Oxy186 (50 mg/kg; oral gavage; daily) also inhibits A549 tumor growth in mice (Tang et al. 2022). Oxy210 also inhibits proliferation as well as tumor growth factor (TGF)-β and Hedgehog signaling in A549 NSCLC cells (Stappenbeck et al. 2019). In mice, Oxy210 has drug-like properties, including chemical scalability, metabolic stability, and oral bioavailability in mice.

19.5.5 Prostate Cancer

Prostate cancer is the most frequent cancer in older men. More than 60% of prostate cancers appear from the age of 65 years, and this cancer is rare before the age of 50 years. Its evolution, generally slow, spreads over several years and

can lead to metastases. Different treatments (surgery, radiotherapy, homonotherapy, chemotherapy) are often associated with side effects and have limited effectiveness. Developing new physical or chemical treatments is therefore a major challenge. In prostate cancer, some oxysterols and their metabolites as well as LXR receptors may contribute to the evolution of this tumor. The oxysterols concerned are 27-OHC and CT (Kloudova et al. 2017). Treatment with the synthetic LXR agonist T0901317 downregulates the protein kinase B/AKT survival pathway and triggers apoptosis in human prostate LNCaP PCa cancer cells in both xenografted nude mice and cell culture (Pommier et al. 2010). LXR receptors appear to be good candidates for treating prostate cancer (De Boussac et al. 2013; Bousset et al. 2018).

19.5.6 Other Types of Cancers

Oxysterols can be involved in several types of cancers, and in any case their beneficial or detrimental effects must always be considered (Kloudova et al. 2017). 7KC could favor drug resistance by inducing P-glycoprotein expression (Wang et al. 2013). 7KC and 7β-OHC induce cell death in different tumoral cells such as colon carcinoma cells (Roussi et al. 2006; Roussi et al. 2007), human monocytic leukemia cells (U937) and human hepatoma cells (HepG2) (O'Callaghan et al. 2002). 22R-hydroxydesmosterol also induces cell death in hepatoma cells (Hietter et al. 1984). Noteworthy, selective cytotoxic effects of 7β-OHC have been reported on murine lymphomas, lymphoblasts, and lymphocytes (Hietter et al. 1986). As for 27-OHC, it has beneficial or detrimental activities in breast cancer, prostate cancer, colon cancer, gastric cancer, ovarian cancer, endometrial cancer, lung cancer, melanoma, glioblastoma, thyroid cancer, adrenocortical cancer, and hepatocellular carcinoma (Abdalkareem Jasim et al. 2022).

19.6 Conclusion and Perspectives

The involvement of oxysterols obtained by cholesterol autoxidation (7KC, 7β-OHC, 7α-OHC, 4β-OHC, 5α,6α-EC, 5β,6β-EC, CT) or enzymatically (20S-OHC, 22R-OHC, 22S-OHC, 24S-OHC, 27-OHC, 25-OHC, 7α,25 DHC) or both (7α-OHC, 25-OHC) in several diseases (age-related diseases, infectious and inflammatory diseases, cancer) is now well established and some oxysterol derivatives (Oxy34, 49, 133, 186, 210, 232) could be new therapeutic tools (Johnson et al. 2011; Montgomery et al. 2014; Ohashi et al. 2021; Stappenbeck et al. 2019; Tang et al. 2022) (Fig. 19.5). The receptors with which they may interact, the signaling pathways on which they act and the effects they induce are increasingly well known. As a result, the pharmacological interest in these poorly studied molecules is growing and oxysterol derivatives are being synthesized for therapeutic purposes. It can be expected that better knowledge of the pharmacology of oxysterols will lead to new drugs for diseases with a limited therapeutic arsenal.

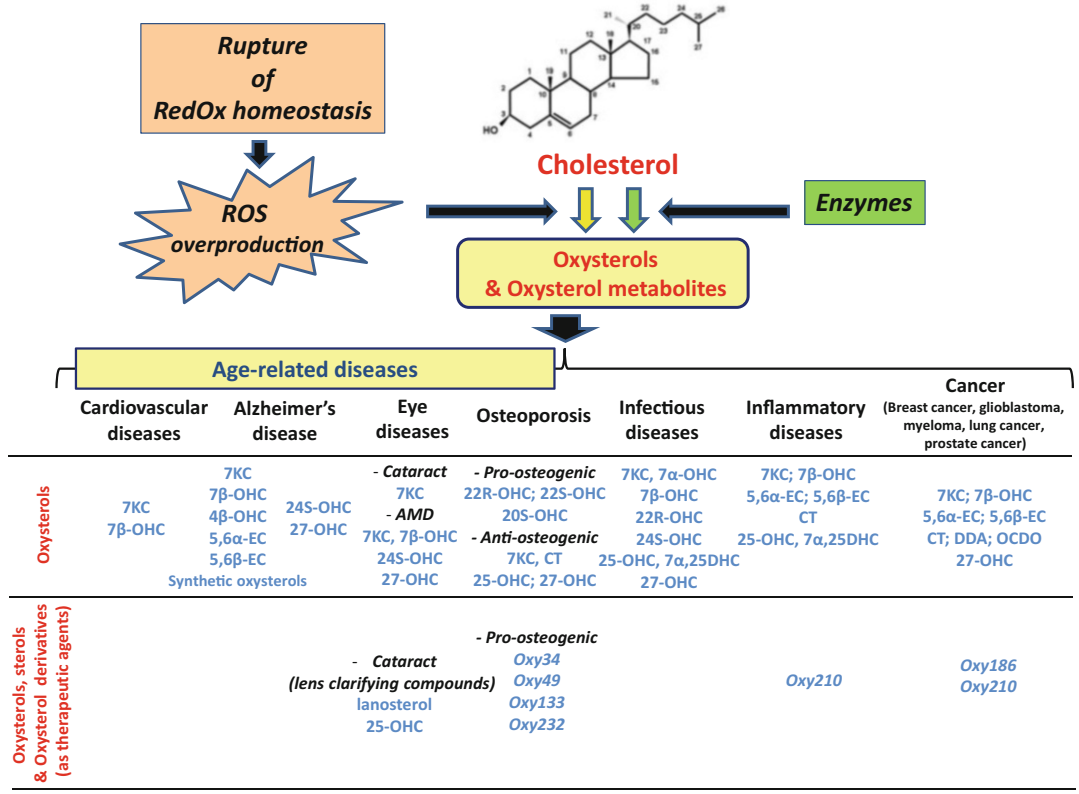


Fig. 19.5 Contribution of oxysterols in age-related diseases, infectious and inflammatory diseases, and cancers. Oxysterols can be either beneficial or detrimental in several diseases. Several oxysterol derivatives (Oxy) have anti-inflammatory (Oxy210) and anti-tumoral (Oxy186, Oxy210) activities whereas some others favor osteogenesis (Oxy34, Oxy49, Oxy133, Oxy232) (Johnson et al. 2011; Montgomery et al. 2014; Ohashi et al. 2021; Stappenbeck et al. 2019; Tang et al. 2022). Synthetic oxysterols inducing the production of 24S-OHC have been described by Zhan et al. (2023)

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Conflicts of Interest The authors declare no conflict of interest.

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Part VI

Industrial Aspects

Current and New Insights on Delivery Systems for Plant Sterols in Food

20

V. Blanco-Morales, D. Mercatante, M. T. Rodriguez-Estrada, and G. Garcia-Llatas

Abstract

Plant sterols are minor bioactive components of food lipids, which are often used for the formulation of functional foods due to their cholesterol-lowering properties. However, they have low solubility and tend to crystallize, which may affect their biological effects, the sensory profile of the sterol-enriched food, and its consumer acceptability. Moreover, due to the unsaturated structure of sterols, they are susceptible to oxidation, so different encapsulation systems have been developed to improve their dispersibility/solubility, stability, delivery, and bioaccessibility. This chapter provides an overview of the main encapsulation systems currently used for plant sterols and their application in model and food systems, with a particular focus on their efficiency and impact on sterol bioaccessibility.

Keywords

Plant sterols · Phytosterols · Encapsulation · Physicochemical characteristics · Delivery systems · Efficiency · Bioaccessibility · Safety

20.1 Introduction

Encapsulation is a technique in which one or more compounds (core or internal phase) are surrounded by one or more materials (carrier or wall material) to protect them from adverse environmental factors (light, oxygen, heat, and moisture) (Feng et al. 2022). Encapsulation techniques using different wall materials and technologies have also demonstrated great potential for improving the handling, stability, and bioaccessibility of bioactive compounds (McClements and Öztürk 2021).

Several techniques have been applied for the encapsulation of plant sterols (PS) to enhance their application in the food industry; this is of great interest due to their beneficial physiological effects, but it represents a major challenge due to their physicochemical properties (Pavani et al. 2022).

This chapter aims to present an overview of the main limitations of PS inclusion in food, the encapsulation techniques and technologies

V. Blanco-Morales · G. Garcia-Llatas
Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Valencia, Spain
e-mail: virginia.blanco@uv.es; guadalupe.garcia@uv.es

D. Mercatante
Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, Bologna, Italy
e-mail: dario.mercatante2@unibo.it

M. T. Rodriguez-Estrada (✉)
Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, Bologna, Italy

CIRI-Agrifood (Interdepartmental Centre of Industrial Agrifood Research), Alma Mater Studiorum—University of Bologna, Cesena, Italy
e-mail: maria.rodriguez@unibo.it

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developed to overcome these shortcomings and the main results obtained to improve their stability, bioaccessibility, and food application.

20.2 PS Characteristics

20.2.1 Biological Effects

PS have two health claims authorized by the European Union (EU). The first states that a minimum daily intake of 0.8 g of PS contributes to the maintenance of normal blood cholesterol levels in a healthy adult population (Commission Regulation (EU) 432/2012). Regarding the disease risk reduction, the second authorized claim reports that a daily intake of 1.5–3 g of PS has a beneficial effect on the reduction of blood cholesterol in subjects with moderate hypercholesterolaemia (Commission Regulations (EU) 983/2009 and 384/2010). However, the intake of PS from food sources in the European population is estimated to range from 211 to 325 mg/day, reaching up to 600 mg/day or 896–1047 mg/day in vegetarian or vegan diets, respectively (Feng et al. 2020a; Jie et al. 2022). For this reason, given that their intake from food is not enough to attain a hypocholesterolemic effect, food enrichment with PS is an increasingly common practice in the food industry. Several clinical studies have demonstrated the cholesterol-lowering effect of PS-enriched food, showing a dose-dependent relationship between intakes of 0.6–3.3 g PS/day and reductions of 6–12% in LDL-cholesterol levels (Zhang et al. 2022). In addition, to improve the blood lipid profile, PS consumption has been associated with other beneficial health effects, such as anticancer, antiobesity, antidiabetic, antimicrobial, anti-inflammatory, and immunomodulatory effects (Nattagh-Eshtivani et al. 2022; Khan et al. 2023).

20.2.2 Main Industrial Sources

Tall oil and oil refining distillates (mainly from rapeseed and soybean oils) are the major sources of PS used for the development of functional foods (Zhang et al. 2022). Tall oil is a by-product obtained from the wood pulp industry. During the production of sulfate or kraft paper, wood chips are heated with an alkaline mixture (sodium sulfide and sodium hydroxide). The pulp solution obtained is concentrated until the soaps (as sodium salts) and the unsaponifiable fraction are separated. After being skimmed off, the tall oil soap is acidulated, and an aqueous phase and an oil phase are obtained. The oil phase is also known as crude tall oil and contains approximately 10–20% neutral matter whose main component is β-sitosterol. Diverse methodologies such as solvent extraction and molecular distillation can be used to separate PS from vegetable oils, but recoveries are low. During chemical or physical refining of crude vegetable oils, the deodorization step is carried out using steam-distillation at high vacuum and high temperature, which allows the removal of residual volatile compounds and the generation of a PS-rich deodorized distillate as refining by-product. PS extraction from the deodorized distillate involves different steps: (1) conversion into free PS by hydrolysis or transesterification; (2) free fatty acid esterification; and (3) PS recovery or concentration. This last step can be performed by physical (crystallization), chemical (solvent or mixture of solvents), or physicochemical procedures (from esterified and transesterified mixtures by adduct formation). The PS content of deodorized distillates is approximately $<5\%$ esterified PS (E-PS) and 2–15% free sterols (up to 18% in soybean oil distillates) (García-Llatas and Rodríguez-Estrada 2011; Maniet et al. 2019; Dikshit et al. 2020; Zhang et al. 2022).

Although these are the main approaches to obtain PS, there are other possible sources and isolation methodologies that have been developed in recent years. Fermentation is a relatively new method based on the ability of microorganisms to synthesize sterols during their growth and metabolism. In addition, solvent extraction assisted by auxiliary technologies, such as microwaves or ultrasounds, has been used to extract sterols using a smaller amount of solvent in a shorter time. Moreover, supercritical carbon dioxide ($SC\text{-}CO_2$) extraction can also be used as a more

selective method than traditional PS solvent extraction (Zhang et al. 2022).

20.2.3 Physical and Chemical Properties

Certain physicochemical properties of PS limit their application in food products. One of the most important aspects is that PS are poorly soluble in water and slightly soluble in lipid medium, which results in a gritty texture that may affect sensory attributes and consumer acceptability when incorporated into food (Moreno-Calvo et al. 2014; Corrêa et al. 2017). In general, their level of hydrophobicity is related to the length and unsaturation degree of the PS side chain. Thus, PS with longer side chains and double bonds are more hydrophobic and have lower micellar solubilities (Feng et al. 2022; Zhang et al. 2022).

The low water solubility of PS and their susceptibility to precipitate in aqueous gastrointestinal fluids lead to a low PS absorption rate, which results in most of the ingested PS being excreted rather than digested and absorbed. Specifically, campesterol has the highest intestinal absorption rate (9.4–14.8%), followed by β-sitosterol (3.1–4.5%), stigmasterol (∽4%), and campestanol and sitostanol (<2%) (García-Llatas and Rodríguez-Estrada 2011). This leads to low PS blood concentrations, ranging from 7–24μmol/L (0.3–1.0 mg/dL) for phytosterols and 0.05–0.3μmol/L (0.002–0.012 mg/dL) for phytostanols (Jie et al. 2022; Zhang et al. 2022).

PS are also prone to oxidation, generating phytosterol oxidation products (POPs). In food products, autoxidation is the main pathway of formation. 7α/β-hydroperoxy derivatives are produced in the first reactions of autoxidation, and during storage or heating, they can be decomposed into 7α/7β-hydroxysterols or 7-ketosterols. Other secondary products of sterol oxidation include the epimers 5,6α/β-epoxysterols and 5α,6β-triol derivatives (García-Llatas and Rodríguez-Estrada 2011; Lin et al. 2016; Jie et al. 2022). The POPs content in food depends on their composition, industrial processing, storage conditions, and the culinary procedures used, as there are certain conditions related to these processes that are key promoting factors of sterol oxidation: high temperature, presence of oxygen, exposure to light, surrounding lipid matrix, and the presence of antioxidants and water in food (Barriuso et al. 2017).

20.2.4 Industrial Chemical Modifications for Food Applications

The hydrophobic and lipophobic properties of PS can be modulated by several chemical modifications. Regarding the solubility of PS in oil, direct esterification with fatty acids (saturated, unsaturated or blends) has been shown to increase their hydrophobicity, and to decrease melting temperature and crystallization temperature compared to parent PS. Chemical synthesis of E-PS is the most widely used method, in which several catalysts (such as mineral acid, organic sulfonic acid, acid salt, acid ion exchange resin, basic mineral salts, sodium alkoxide, or hydroxide) can be used. Enzyme-catalyzed synthesis (by direct reaction with fatty acids or transesterification with triacylglycerols or fatty acid methyl esters) and ionic liquid-catalyzed synthesis (a medium composed of specific anions and organic cations) are also used, with esterification yields similar to those of chemical synthesis (>90%). The enhancement of PS water solubility can be approached by direct addition or spacer-mediated coupling of a water-soluble moiety at the hydroxyl group located at position 3 (Corrêa et al. 2017; Feng et al. 2022).

Although these modifications can improve PS solubility, their addition to food is still limited by the adverse effects on sensory properties discussed above. For this reason, research on PS encapsulation methodologies has undergone major development in recent decades as a suitable alternative to overcome these drawbacks.

20.3 Theoretical Aspects of Delivery Systems

Encapsulation enables the incorporation of PS into food products by their entrapment in coating materials that can be composed of different natural biopolymers (lipids, polysaccharides, and proteins). The composition and structure of the delivery system is critical to determine their functionalities. The following content focuses on the most common techniques and processing innovations used for PS encapsulation.

20.3.1 Emulsions

Emulsions are colloidal dispersions composed of an immiscible oil and an aqueous phase, which constitute a stable and homogenous system in the presence of a suitable emulsifier and/or co-emulsifiers (Mohammadi et al. 2020). Different emulsion systems are represented in Fig. 20.1a, b. The oil phase may consist entirely of the bioactive lipid or a carrier oil in which the compound of interest is dissolved. The physicochemical characteristics of the lipids constituting the oil phase have a direct impact on the stability of the system. In this sense, oil phases with lower viscosity and interfacial tension (e.g., essential or flavored oils) result in a smaller droplet size than emulsions composed of long-chain triacylglycerols (Salvia-Trujillo et al. 2017). The aqueous phase composition is also of interest because it can impact the formation, stability, or functional properties of emulsion systems due to changes in pH and/or ionic strength. Emulsifier addition is required to stabilize the emulsion systems and must be properly selected according to the oil phase composition, the type of emulsion [oil-in-water (O/W) or water-in-oil (W/O)] and the emulsifier hydrophilic-lipophilic balance (HLB). By decreasing the interfacial tension, emulsifiers arrange at the interface, facilitating the formation of small droplets; the strong electrostatic and/or steric repulsive forces generated between emulsifier-coated oil droplets protect against emulsion destabilizing phenomena (such as coalescence, flocculation, etc.). The most frequently used emulsifiers for food-grade emulsions are macromolecules (e.g., proteins) and small-molecule surfactants (e.g., Tweens). Surfactants can be classified as cationic, anionic, non-ionic, or zwitterionic based on their electrical characteristics, which can greatly influence the emulsion system (Salvia-Trujillo et al. 2017; McClements and Öztürk 2021). Other parameters that define emulsion properties and stability are the emulsification method (e.g., spontaneous emulsification, phase inversion temperature methods, high-pressure homogenization) and the oil:emulsifier ratio.

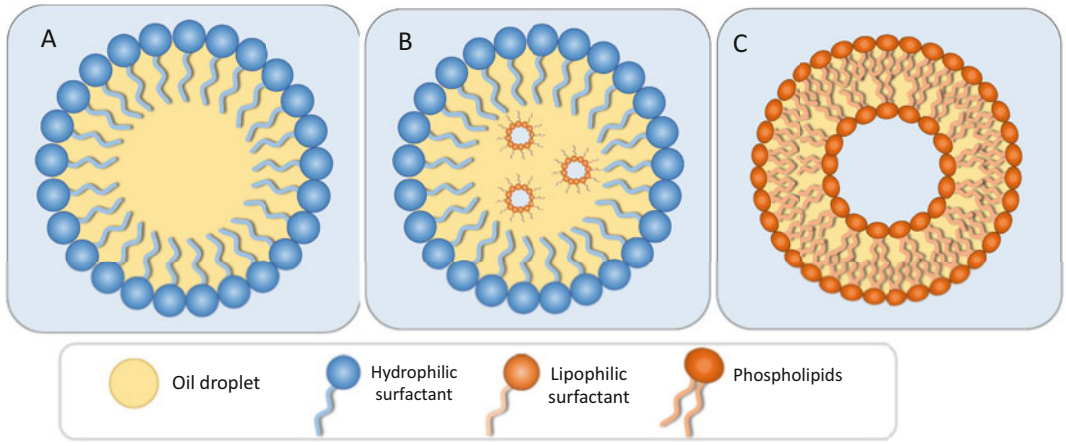

Fig. 20.1 Oil-in-water emulsion (O/W) (**a**), double emulsion (W/O/W) (**b**) and liposome (**c**) representation

Microemulsions and nanoemulsions are the most common types of colloidal dispersions. The main difference between both systems is based on their thermodynamic stability: microemulsions refer to stable systems, whereas nanoemulsions are unstable ones. Although microemulsions and nanoemulsions have similar particle sizes (radius < 100 nm), their particle size distribution (PSD) are different; in fact, microemulsions only show one narrow peak, while nanoemulsions may have one or more peaks that could be narrow or broad (McClements 2012). Moreover, microemulsions can have a spherical or non-spherical shape, whereas nanoemulsions tend to be spherical because the interfacial tension is higher (McClements 2012; McClements and Öztürk 2021). Microemulsions can form spontaneously, even though stirring or heating is often necessary to facilitate the formation process; in contrast, nanoemulsions require an external energy input to form the colloidal dispersion system by means of either high- or low-energy methods. High-energy methods involve the use of high disruptive forces (high-pressure homogenizers or sonicators), while low-energy methods focus on changing the solution or environmental conditions (spontaneous emulsification or phase-inversion tempering methods) (Salvia-Trujillo et al. 2017). The higher energy inputs used in the formation of nanoemulsions generate a smaller droplet with reduced interface area and higher stability against gravitational separation. Although microemulsions show better stability than conventional emulsions, they may require a large amount of surfactant for stabilization, which could have adverse health effects (Mohammadi et al. 2020).

20.3.2 Liposomes

Liposomes are composed of an aqueous core and one or more concentric phospholipid bilayers (see Fig. 20.1c). Phospholipid molecules are self-assembled under shearing conditions (homogenization or sonication). Since lipid components can be entrapped within the hydrophobic domain formed by nonpolar phospholipid tails, liposomes can be used as encapsulation, delivery, and controlled release systems (Mohammadi et al. 2020; McClements and Öztürk 2021). Liposomes are a nontoxic, biocompatible, and biodegradable technique and can also be used for co-encapsulation of compounds with different solubilities. However, they have certain disadvantages, such as low encapsulation efficiency (EE) or low stability during storage (coalescence or aggregation) and in the gastrointestinal environment (Pavani et al. 2022). Liposome size can range from 0.025μm to 2.5μm. The size and number of bilayer membranes of the vesicles determine liposome classification, which includes multilamellar vesicles (MLVs) (with an onion-like structure) and unilamellar vesicles (with a single bilayer). The latter, in turn, comprise two categories: large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) (Akbarzadeh et al. 2013). Lipid film hydration, reverse-phase evaporation, and ethanol injection are the most commonly used methods for the formation of MLVs and LUVs. The application of additional mechanical processes to MLVs and LUVs, such as homogenization, ultrasonication, microfluidization, and membrane extrusion, leads to the formation of SUVs.

20.3.3 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid lipid nanoparticles (SLNs) are formed by a lipid phase organized into highly regular crystalline structures surrounded by an emulsifier coating (Fig. 20.2a). Bioactive compounds are entrapped in the lipid crystal structure, so their mobility is very low. These features provide some advantages over other encapsulation methods, such as high loading capacity (LAC), reduced bioactive leakage, and improved chemical stability (Mohammadi et al. 2020). Crystallization of the lipid phase may lead to the ejection of bioactive molecules toward the droplet surface (which

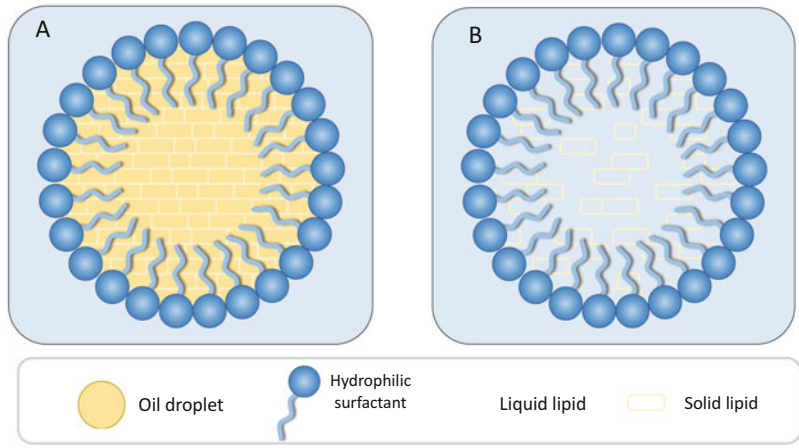

Fig. 20.2 Solid lipid nanoparticles (SLNs) (**a**) and nanostructured lipid carriers (NLCs) (**b**) representation

may reduce their chemical stability), as well as to the modification of the nanoparticle shape into a non-spherical shape (thus increasing the surface area and the susceptibility to aggregation) (Mohammadi et al. 2020; McClements and Öztürk 2021). To overcome these drawbacks, a modified version of SLNs has been proposed. Nanostructured lipid carriers (NLCs) have the same composition as SLNs, but the difference is that the lipid phase is composed of a mixture of solid and liquid lipids (Fig. 20.2b). The incorporation of a liquid oil reduces the crystalline structure, which results in enhanced LAC and EE and fewer changes in particle size with respect to SLNs (Mohammadi et al. 2020; McClements and Öztürk 2021). As in emulsion systems, the selection of the lipid blends and type of emulsifier determines SLNs and NLCs properties. Moreover, it is necessary to consider the solubility of the bioactive compounds in the oil phase and the miscibility of solid and liquid lipids at the concentrations studied to obtain a suitable lipid mixture to be used as a delivery system. SLNs and NLCs are commonly prepared by making a nanoemulsion or emulsion at high temperature or with a high-energy method, and subsequently, they are cooled below the melting point to crystallize the lipid phase. Both low- and high-energy methods can be used for its elaboration (Mohammadi et al. 2020; McClements and Öztürk 2021).

20.3.4 Gels

Gels are composed of biopolymer molecules (e.g., polysaccharides and proteins) that form a three-dimensional network capable of trapping a considerable amount of water, oil, or air (hydrogel, oleogel, and aerogel, respectively) (see Fig. 20.3) (Abdullah et al. 2022). Lipophilic bioactive compounds can be encapsulated directly in the biopolymer network or by preloading them into lipophilic carriers. Particle gelation takes place by modifying the solution or environmental conditions (e.g., thermal, ion, osmotic, or covalent gelation). Gel properties (stability, retention, and release) are determined by many factors, such as pore size and particle dimension and morphology, which can be controlled by altering the biopolymer nature, concentration, and cross-linking level (Zhang et al. 2015; McClements 2017).

20.3.5 Complex Coacervation

This encapsulation method is based on the electrostatic interaction generated between two or more polymers with opposite charges (protein/polysaccharides) dispersed in water solution. This interaction results in two immiscible liquid phases: the polymer-rich dense phase, known as coacervate, and the polymer-poor continuous phase. For the encapsulation of hydrophobic

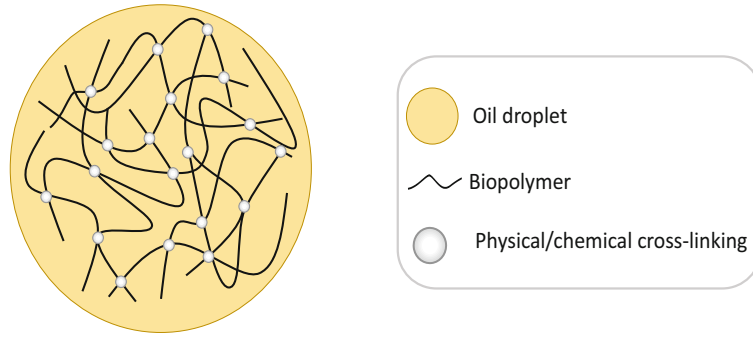

Fig. 20.3 Oleogel schematic representation

compounds, a previous emulsification step is needed. Then, the complex coacervation point is achieved by adjusting the temperature and pH (below the isoelectric point) to obtain the interaction between positively charged proteins and negatively charged polysaccharides. In this way, coacervate microdroplets are formed and separated from the continuous phase (polymer-poor) to form a continuous shell on the nucleus surface. Finally, the coacervate layer obtained on the surface of the oil droplets is further hardened by adding some cross-linking agent, such as transglutaminase or glutaraldehyde. The summarized process is shown in Fig. 20.4. A suitable combination of biopolymer charges is mandatory, so the evaluation of their zeta potential (ζ-potential; see definition in Sect. 20.3.8.2) is a priority point in this approach; the combination of gelatin and arabic gum is widely used. Different conditions can influence the complex coacervation process: concentration, ratio, and molecular weight of biopolymers used and process parameters (pH, ionic strength, temperature, and homogenization degree). The advantages of this encapsulation method include the biocompatibility, nontoxicity, and biodegradability of the biopolymeric complex coacervate. However, this encapsulation method also presents some limitations; for instance, a post-processing step (e.g., spray-drying or freeze-drying) is usually needed to stabilize the system (Tavares et al. 2021; Muhoza et al. 2022; Pavani et al. 2022).

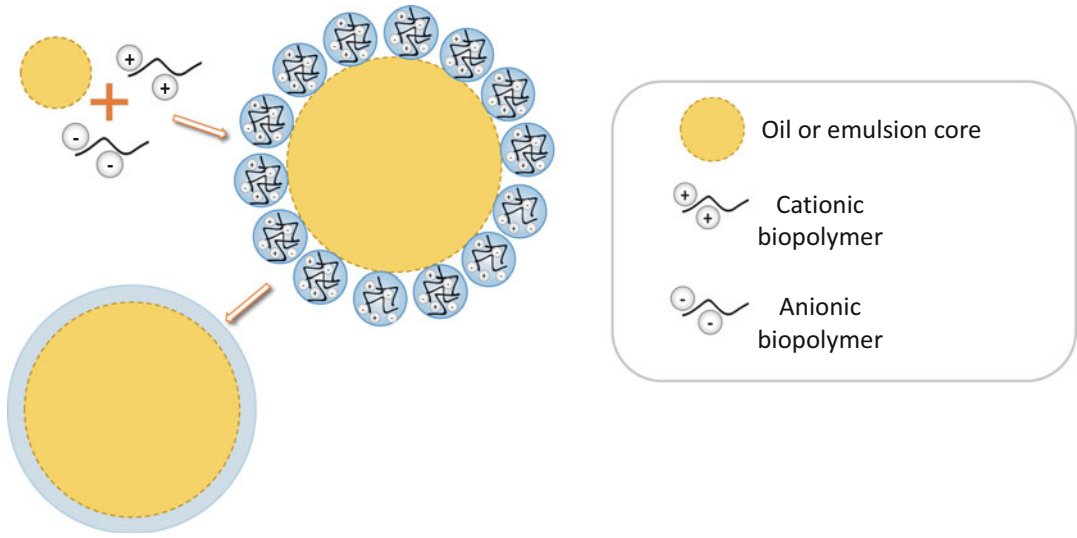

Fig. 20.4 Representation of complex coacervation process

20.3.6 Inclusion Complexation: Cyclodextrins

Cyclodextrin (CD) molecules consist of a diverse number of glucopyranose units (six, seven and eight for α-, β-, and γ-CD, respectively) linked by α-1,4 bonds. Their structures lead to particular three-dimensional shapes as truncated cones, with a hydrophilic outer surface and a hydrophobic inner cavity (see Fig. 20.5). Therefore, CDs can encapsulate guest molecules through the formation of inclusion complexes. Hydrophobic compounds can be partially or fully included in the inner cavity of CDs, replacing water molecules and stabilizing the complex by means of hydrophobic, dipole–dipole and van der Waals interactions. The process, known as complexation, is thermodynamically favored. Inclusion complexes are not considered microcapsules, but they also allow the controlled release of the compound of interest, as well as its protection against environmental conditions. Moreover, CDs are "Generally Recognized as Safe" (GRAS) by the Food and Drug Administration (FDA, USA) (Comunian and Favaro-Trindade 2016; Cid-Samamed et al. 2022; Paiva-Santos et al. 2022).

20.3.7 Other Techniques for Encapsulation: Powdered Forms

The production of PS-loaded particles in powdered forms is a suitable strategy to facilitate their use and incorporation during the manufacturing process of food products. Spray-drying, freeze-drying, electrospinning and electrospraying are the most commonly used technologies to obtain powder forms.

In the spray-drying method, a homogenous solution, suspension or emulsion of the wall material and active ingredient is atomized in a drying chamber at high temperature (120–150 °C) for a short contact time (few seconds) and generates microparticles or microspheres (Comunian and Favaro-Trindade 2016; Pavani et al. 2022). The microparticles or capsules formed are collected by means of a cyclone separator. In the case of PS encapsulation, the high temperature used during spray-drying may affect PS stability; wall materials and processing conditions must be selected to minimize adverse effects. On the other hand, freeze-drying is carried out under vacuum conditions, and the frozen solution undergoes a sublimation process until

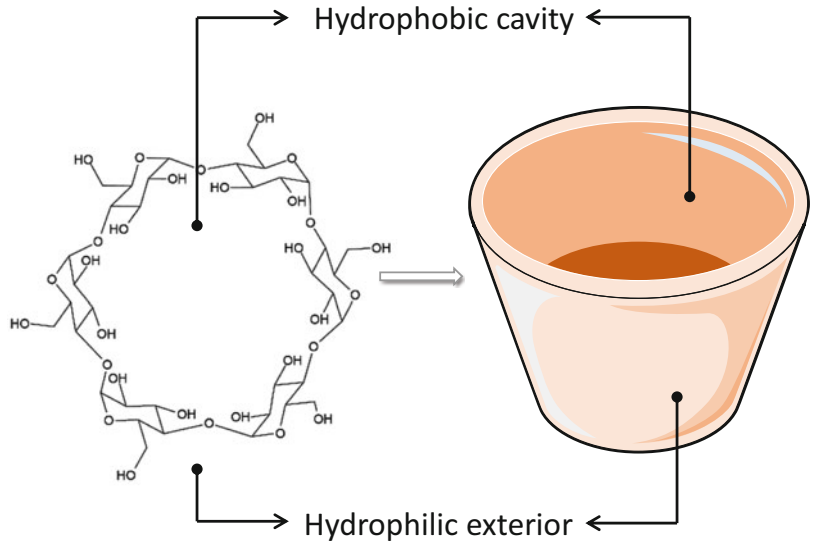

Fig. 20.5 Cyclodextrin as encapsulation technique

complete drying. Freeze-drying must be employed as a complementary technique because it does not generate nano- or microparticles. In contrast, electrospinning and electrospraying are nonthermal electrohydrodynamic techniques used to produce fibers or particles when a polymeric solution is spun or sprayed, respectively, using a high potential electric field (1–30 kV). The properties of the polymer solution (e.g., viscosity, concentration) determine the formation of fibers or particles. Briefly, the solution is pumped from a syringe at a given rate and expelled through the needle while an electrical charge is applied. Electrostatic forces between the needle (positively charged) and the collector plate (negatively charged or used as grounding point), together with the Coulombic force of the external electric field, lead to an elongation of the droplet at the tip of the needle, creating a conical shape known as a Taylor cone. In the electrospinning process, when the solution concentration is high, the polymer jet stabilizes and is ejected from the Taylor cone, resulting in jet elongation by a whipping instability mechanism. On the other hand, the electrospraying process takes place when the concentration of the solution is low. In this case, the jet is not stabilized, and fine droplets are formed. Due to droplet self-dispersion in space, agglomeration and coagulation are avoided. In both processes, the voltage causes evaporation of the solvent, and the solidified solution is deposited on the collector plate (Bhushani and Anandharamakrishnan 2014).

Table 20.1 summarizes the main factors that can influence the drying process, as well as the principal advantages and disadvantages of the described methodologies.

Table 20.1 Principal characteristics of the technologies used to obtain powdered forms

	Factors to control	Advantages	Disadvantages
Spray-drying	• Feed solution: wall material: bioactive core ratio, total solid content, viscosity, surface tension. • Equipment: inlet air temperature, feed flow rate. • Type of atomizer, spray-drying predisposition.	• Rapid, simple, and easy operating system. • Very cost-effective. • High encapsulation efficacy. • Possibility to control particle size, morphology, and porosity by changing operation conditions.	• Not suitable for heat-sensitive materials (100–200 °C). • High capital and maintenance cost.
Freeze-drying	• Previous encapsulation technique.	• Minimum damages to the product. • Suitable for heat-sensitive products.	• Complementary technique (it does not generate nano- or microparticles). • Long drying time (24–36 h). • Difficult to change process conditions for improving encapsulation efficacy. • High capital and maintenance cost.
Electrospinning and electrospraying	• Feed solution: polymeric concentration and solvent (pH, conductivity, viscosity, surface tension). • Equipment: voltage, flow rate, and distance between needle and plate collector. • Environmental conditions: temperature, humidity, air speed.	• Nonthermal technique. • High encapsulation efficiency.	• Process optimization. • Industrial scale-up. • Applicable only to volatile solvents.

20.3.8 Characterization of Delivery Systems

The materials and fabrication methods used for the encapsulation process lead to particles with different compositions, morphologies, or interfacial properties that determine their functional characteristics.

20.3.8.1 Size

Particle size is one of the most important factors in the development of delivery systems, as it can interfere with the final texture and flavor of food products and affect the bioaccessibility and stability of the encapsulated bioactive compound as well. In this sense, the smaller the particle size is, the higher the stability to creaming, sedimentation and aggregation due to the reduced impact of the gravitational and attractive forces. In addition, the reduction of the particle size leads to an increase in surface area, so the digestion process can be faster due to the increased rate of interfacial chemical reactions, and thus, bioaccessibility can be improved. However, PS are prone to oxidation, so the reduction in particle size also means increased exposure to the aqueous phase with the consequent possibility of increased oxidative degradation (Sani et al. 2022).

Particle size distribution, polydispersity index (PDI) or central tendency and width of the distribution are parameters that can be used to refer to the encapsulation size. Other parameters that are often used [such as surface mean diameter ($D_{(3,2)}$) and volume mean diameter ($D_{(4,3)}$)] are associated with the presence of large and small particles, respectively (Jafari et al. 2017; Recharla et al. 2017). Particle shape can also impact solubility, with nonsymmetrical particles being more soluble than symmetrical ones.

20.3.8.2 Electrical Characteristics

The electrical properties of delivery systems are related to the ionic material employed for their formulation (free fatty acids, phospholipids, proteins, and ionic polysaccharides) and can modulate several aspects, such as the stability, release, and physicochemical properties of the particles (Jafari et al. 2017). Typically, the electrical properties are characterized by measuring the ζ-potential as a function of pH. The ζ-potential determines the electrostatic interactions between nanoparticles and among nanoparticles and other charged substances. The higher the electrostatic repulsion between the nanoparticles, the stronger the ζ-potential (positive or negative), and the lower the tendency to aggregate. Usually, particles with absolute values of ζ-potential greater than 30 mV are considered strongly cationic or anionic, and the generated repulsive force is enough to inhibit aggregation (Sani et al. 2022). Other factors that can influence the ζ-potential are the interfacial composition and the pH and ionic strength of the surrounding aqueous phase.

20.3.8.3 Physical State

The determination of the physical properties of the encapsulation systems can provide useful information about their stability and functional features. In this sense, X-ray diffraction is used to identify the degree of crystallinity of a material or food ingredient and is a useful tool to confirm the loading of the bioactive compound into the delivery system. In the case of PS, this information is of great significance since amorphous forms have greater aqueous solubility than crystalline ones. Differential scanning calorimetry (DSC) provides information related to enthalpy changes of the encapsulation systems (glass transition temperature, heat capacity, melting point, or recrystallization times), which are particularly interesting for SLNs and NLCs. Fourier transform infrared spectroscopy generates a spectrum that allows a fast identification and quantification of the sample's components, thus providing the main information for the chemical characterization of the encapsulation system material (Jafari et al. 2017).

20.4 Current Delivery Systems for PS Encapsulation

In this section, the most relevant applications of emulsions, nanoemulsions, nanocapsules, nanodispersions, and NLCs for PS encapsulation

will be discussed, focusing on their impact on EE, stability, and PS bioaccessibility.

20.4.1 Encapsulation Efficiency and Stability

Table 20.2 shows the applications of emulsions and nanoemulsions as PS encapsulation systems. These two systems are considered the simplest and fastest to encapsulate PS, and there are several studies about their utilization in both model systems and food. In the case of model systems (Table 20.2), Engel and Schubert (2005) used different types of oily phases [medium chain triacylglycerols (MCT) oil, tricaprylin, triolein or tristearin], emulsifiers [various Tween groups or biodegradable pH-sensitive surfactant (BPS)] and crystallization inhibitors [distilled monoacylglycerols (MAG) or lecithin] to formulate O/W emulsions with β-sitosterol (0–30% in the oil phase). They observed a decrease in the crystallization temperature of β-sitosterol when using distilled MAG or lecithin, while the best performances were observed in emulsions formulated with MCT, Tween 20, 30% lecithin, and 30% PS. On the other hand, Khalid et al. (2017) observed that a more stable emulsion was obtained when using the microchannel emulsification technique and lower concentrations of β-sitosterol (0.5–4.0%) and γ-oryzanol (0.5–4.0%) for an O/W emulsion prepared with MCT and decaglycerol monolaurate (ML-750) or Tween 20 (1%) (see Table 20.2 for further details). In the emulsion formulated with 0.5% β-sitosterol and 1.0% γ-oryzanol, no crystallization was observed after 24 h, and an EE $>$ 80% was achieved at 4 °C and 25 °C during a 30-day storage. When the emulsion was formulated with ML-750 as emulsifier, EE decreased during storage due to the interaction between ML-750 and γ-oryzanol. Partitioning of γ-oryzanol molecules from the oil phase to the water phase could take place due to the thinning of the interfacial layer around their droplets with storage time. Exchange phenomena could also occur if γ-oryzanol degraded in the water phase and repartitioned into the oil phase, especially at 25 °C.

In another study, Chaijan and Panpipat (2020) formulated an O/W emulsion using β-sitosterol or β-sitosterol oleate as the core material (5% in the oily phase), tripalmitin as the oily phase and Brij35 as the emulsifier (see Table 20.2 for further details); the emulsions were subjected to accelerated storage (65 °C/35 days), showing an increase in different primary and secondary lipid oxidation parameters [peroxide value (PV), thiobarbituric acid reactive substances (TBARS), and *p*-anisidine value (AnV)]. Moreover, in emulsions prepared with β-sitosterol oleate as the core material, there was an increase in its oxidation products derived from the monomolecular oxidation pathway, in particular 7-ketositosterol, thus confirming that β-sitosterol was more stable than β-sitosterol oleate in these model system conditions.

Cheng and Cui (2022), instead, used stigmasterol (0.05% in the oil phase) as the core material for an O/W emulsion formulated with MCT as the oily phase and sodium dodecyl sulfate (SDS) and SDS micelles as surfactants (see Table 20.2 for further details). An initial coarse emulsion was formulated with MCT containing stigmasterol and SDS and then passed through a high-pressure homogenizer 3 times at 12,000 psi. To study the effect of additional surfactant micelles, the emulsions were then added with a further 0–2% SDS to formulate final O/W emulsions containing 500 ppm stigmasterol and various concentrations of additional SDS micelles; the emulsions were thereafter subjected to accelerated storage (60 °C/35 days) to assess their physicochemical stability. The results showed that stigmasterol and stigmasterol hydroperoxides rapidly solubilized in the aqueous phase, while the addition of SDS micelles in the coarse emulsion increased the presence of stigmasterol and stigmasterol hydroperoxides in the aqueous phase by 8% and 64%, respectively. The latter behavior could be due to the fact that SDS micelles might have made the stigmasterol hydroperoxides and their downstream alkoxyl radicals less effective at attacking the unoxidized stigmasterol and speeding up the sterol oxidation process.

Table 20.2 Review of the most relevant results of emulsions and nanoemulsions used for PS encapsulation

Core material	Oil phase	Aqueous phase	Encapsulation methodology	Main results	Ref.
Model systems					
β-sitosterol (0–30% in the oil phase)	MCT **or** tricaprylin **or** triolein **or** tristearin (10%)	Tween (20, 40, 60, 65 or 80) **or** BPS (5 or 30) (1%)	– Pre-emulsion (magnetic stirring) – High-pressure homogenization (1000 bar, 90 °C)	– Distilled MAG **or** lecithin ↓ β-sitosterol's crystallization temperature. – Highest PS concentration without crystallization: 30% of PS with MCT, Tween 20, and 30% of lecithin.	Engel and Schubert (2005)
γ-oryzanol (0.5–4.0%) **and** β-sitosterol (0.5–4.0%)	MCT	ML-750 **or** Tween 20 (1%)	Microchannel emulsification: – Ultrasonication of microchannel array chip + oil phase (100 kHz, 20 min) – Injection of aqueous phase (flow rate 1–14 mL/h)	– Optimized concentration without crystallization after 24 h: 0.5–1.0% of γ-oryzanol and β-sitosterol. – EE > 80% at 4 °C and 25 °C during 30-day storage. – Storage: ↓EE (ML-750 > Tween 20).	Khalid et al. (2017)
PS (20%)	–	Sunflower lecithin emulsion (10%)	– PS suspension in water **or** water-ethanol solution: homogenization (7200 rpm) and ultrasonication (15 s to 3 min) – Injection of PS suspension into lecithin emulsion – Homogenization (1200 rpm, 1 min), stirring (240 rpm, 30 min), centrifugation (14,000 rpm, 10 min) – Drying with vacuum oven (50 °C)	– Dispersity degree of PS suspension: polar solvent > water. – Optimized ultrasonic treatment at 50 s: 80% particles have <0.35μm. – PS in emulsion: EE of 88%.	Bugaets et al. (2020)
β-sitosteryl oleate **or** β-sitosterol (5% in the oil phase)	Tripalmitin (20%)	Brij35 (1.5%)	– Homogenization (2000 rpm, 5 min) – Accelerated storage (65 °C, 35 days)	– Storage: ↑ PV, ↑ TBARS, ↑ AnV and ↑ 7-ketositosterol (β-sitosteryl oleate > β-sitosterol). – Stability: β-sitosterol > β-sitosteryl oleate.	Chaijan and Panpipat (2020)
Stigmasterol (0.05% in the oil phase)	MCT (5%)	SDS (0.25%) **and** additional SDS micelles (0–2%)	– Pre-emulsion (superfine homogenization) – High-pressure homogenization (12,000 psi, 3 times) – Addition of SDS micelles – Accelerated storage (60 °C, 35 days)	– Stigmasterol and stigmasterol hydroperoxides solubilize rapidly in the aqueous phase. – Additional SDS micelles: ↑ stigmasterol and stigmasterol hydroperoxides in the aqueous phase (8% and 64%, respectively).	Cheng and Cui (2022)

(continued)

Table 20.2 (continued)

Core material	Oil phase	Aqueous phase	Encapsulation methodology	Main results	Ref.
PS (1%)	MCT (4%)	LPC **or** PC (10%) Glycerol (30%)	– Pre-emulsion (20,000 rpm, 3 min) – Ultrasonication (30% amplitude, 12 min) – Storage (4 °C **or** 37 °C, 30 days)	– Mean diameter: 111 nm for PC and 68 nm for LPC. – Stability: LPC < PC. – Storage T influences stability. – ↑ Particle size: 4 °C > 37 °C. – ↑ PV: LPC > PC, 37 °C > 4 °C.	Acevedo-Estupiñan et al. (2019)
Food					
E-PS (1.6%)	*Echium* oil (5%)	Tween 20 (0.6%)	High-pressure homogenization (500 bar, ice bath) (a) Without antioxidants, with antioxidants or prooxidants (4.42–5.35 mg/g oil) Heat treatment (84 °C, 90 min) Storage (room T, 30 days) (b) With addition of phenolic compounds (200 ppm) Heat treatment (90 °C, 40 min) Storage (room T, 14 days) (c) With the addition of selected phenolic compounds (quercetin, rutin, and sinapic acid) (500 **or** 1000 ppm) Heat treatment (90 °C, 40 min) Storage (room T, 14 days)	– Higher antioxidant capacity (↓PV and ↓TBARS values): quercetin at 500 ppm, and rutin and sinapic acid at 200 ppm. – Identification of 16 POPs: 7-keto derivatives were the most abundant and ↑during heat treatment.	Espinosa et al. (2015)
β-Sitosterol (2%)	Corn oil	Internal: polyglycerol polyricinoleate (0.5%) External: Tween 20 (3%)	– Primary emulsion: homogenization (500 rpm, 15 min) – Final multiple emulsion: shear (0–6000 rpm, 0–5 min) and high-pressure homogenization (100–300 bar, 1–3 cycles)	– Optimized conditions (300 bar, 3 cycles without high shear homogenization): average droplet size of 0.13 nm, PDI of 0.08, ζ-potential of 29.3 mV, stability of 100%, solubility of 99%, EE of 53%, and β-sitosterol release of 8.5%.	Momeny et al. (2017)
PS (3% **or** 6%)	Anhydrous milk fat (10%)	WPI (1%) alone **or** with SL (3%) **or** MAG (3%)	– Stirring (300 rpm, 2 min) – Pre-emulsion (3200 rpm, 60 °C, 1 min) – Emulsion (high-	– PS concentration increased crystallization. – SL and MAG ↓PS and milk crystallization. – PS crystallization:	Zychowski et al. (2018)

(continued)

Table 20.2 (continued)

Core material	Oil phase	Aqueous phase	Encapsulation methodology	Main results	Ref.
			pressure homogenization): 1. 300 bar, 1 pass (D = ~1.0μm) 2. 1000 bar, 3–5 passes (D = ~0.2μm)	Smaller droplets > larger droplets. – Stability: Smaller droplets > larger droplets. – Particle size: WPI alone < WPI + SL or MAG.	
PS (0.3% **or** 0.6%)	Anhydrous milk fat (10%)	WPI (1%)	– High-pressure homogenization (300 bar, 1 pass, 60 °C)	– Particle size: 0.1–16.4μm. – ↑PS concentration: ↓$D_{(4,3)}$ – WPI and PS: ↓interfacial tension through a synergistic effect.	Zychowski et al. (2019)
PS (4%)	Flavored oils (orange, lemon, **or** peppermint) (10–50%)	TS (0.5–3.0%), **or** QS, SM, SL, SC, OS (3%), **or** Tween 80 (3%)	– Sonication (20 kHz, 200–360 W at a pulsed pattern, alternating 5 s sonication + 5 s without sonication) – Spray-drying (inlet T: 150 °C, outlet T: ~70 °C) – Storage (room T, 1 month)	– Particle size: lemon > orange and peppermint oil. – Particle size at 3% of emulsifier: SC > TS > OS > QS. – Optimized preparation/formulation (ultrasonication time of 5 min, 280 W, 3% TS, 20% oil, 4% PS): higher stability, EE (~90%), good fluidity, and redispersion behavior.	Chen et al. (2021)

AnV *p*-anisidine value, *BPS* biodegradable pH-sensitive surfactant, *D* average particle diameter, $D_{(4,3)}$ volume mean particle diameter, *E-PS* esterified phytosterols, *EE* encapsulation efficiency, *LPC* lysophosphatidylcholine, *MAG* monoacylglycerols, *MCT* medium chain triacylglycerols, *ML* decaglycerol monolaurate, *OS* octenylsuccinate starch, *PC* phosphatidylcholine, *PDI* polydispersity index, *POPs* phytosterols oxidation products, *PS* phytosterols, *PV* peroxide value, *QS* *Quillaja* saponin, *SC* sodium caseinate, *SDS* sodium dodecyl sulfate, *SL* soybean lecithin, *SM* sorbitan monooleate, *TBARS* thiobarbituric acid reactive substances, *TS* tea saponin, *WPI* whey protein isolate, ζ-potential zeta-potential

Other studies investigated the use of a mixture of unesterified PS at different concentrations as core material for emulsions, modifying the emulsion formulation. Acevedo-Estupiñan et al. (2019) used a mixture of PS (1%), MCT, glycerol, lysophosphatidylcholine (LPC), or phosphatidylcholine (PC) to formulate an O/W emulsion (see Table 20.2 for further formulation details) that was stored at 4 °C or 37 °C for 30 days. During storage, it was observed that preservation affected the stability of the emulsion; in particular, PV increased in samples stored at 37 °C when using LPC as an emulsifier. Moreover, it was observed that the particle size was affected by the temperature; in fact, a larger particle size was found in the samples stored at 4 °C than in those kept at 37 °C.

Bugaets et al. (2020) also used a mixture of unesterified PS (20% of the total emulsion) as the core material and sunflower lecithin as the emulsifier to formulate an O/W emulsion that had water or a mixture of water:ethanol (55:35) as the aqueous phase (see Table 20.2 for further details). The results showed that the dispersity degree of PS was higher in the ethanol-containing emulsion than in the water emulsion; ultrasonic treatment for 50 s led to a size <0.35μm for 80% of the particles. Finally, the emulsions completely resolubilized and remained stable for at least 24 h when the encapsulated PS (EE 88%) had been

dispersed in water at a ratio of 1:10 to 1:50 at a temperature of 23 ± 2 °C.

Several studies have been carried out on the formulation of emulsions using food grade oils or other types of products. As shown in Table 20.2, several researchers have used *Echium* oil or corn oil as the oil phase. *Echium* oil was utilized to formulate an O/W emulsion containing esterified PS as the core material and Tween 20 as the emulsifier. The study was performed with three types of emulsions: (1) a base emulsion; (2) emulsions containing tert-butylhydroquinone (TBHQ) and a mixture of ascorbic acid and $FeSO_4$ as negative and positive controls of oxidation, respectively; and (3) emulsions with different types of antioxidants, including a mixture or single phenolic compounds (quercetin, rutin, and sinapic acid) (see Table 20.2 for further details on heat treatment and storage conditions). The results showed that samples containing quercetin at 500 ppm and rutin and sinapic acid at 200 ppm had the highest antioxidant capacity and the lowest PV and TBARS levels. In addition, 16 POPs were identified in all samples, among which 7-keto derivatives were the most abundant and tended to increase during heating (Espinosa et al. 2015).

Momeny et al. (2017), instead, used β-sitosterol as a core material to formulate a double emulsion with corn oil as the oil phase and polyglycerol polyricinoleate (PGPR) and Tween 20 as emulsifiers. To prepare the double emulsion, a first W/O emulsion with corn oil, PGRP and β-sitosterol (2%) was formulated (see Table 20.2 for further details). The final β-sitosterol multiple emulsion was prepared under different experimental conditions of pre-homogenization and high-pressure homogenization. The results showed that the optimal conditions to obtain this type of emulsion were 300 bar and 3 cycles without high shear homogenization; in fact, the emulsion had an average droplet size of 0.13μm, a PDI of 0.08 and a ζ-potential of 29.3 mV. This type of emulsion showed very high stability and solubility (100% and 99%, respectively), with an EE of 53% and β-sitosterol release of 8.5%. These optimized formulation and preparation conditions provided similar results to those obtained with the predictive statistical model (Momeny et al. 2017); however, it must be noted that the EE values were lower than those found in the literature (53% vs. ≥70%) (Engel and Schubert 2005; Bugaets et al. 2020).

In another study, Zychowski et al. (2018) formulated an emulsion using milk fat products as the oil phase. First, anhydrous milk fat was combined with soybean lecithin (SL) or MAG and a mixture of unesterified PS (3% or 6%) to prepare bulk oily mixtures. The O/W emulsions were then formulated with 10% of the aforementioned bulk oily blends and an aqueous protein phase consisting of whey protein isolate (WPI) (1% protein + 89% water). The two phases were combined into a pre-emulsion at 3200 rpm for 1 min and then homogenized at 60 °C using two different processes (1 pass at 300 bar pressure or 3–5 passes at 1000 bars) to obtain either larger (∼1.0μm in average diameter) or smaller emulsion droplets (∼0.2μm in average diameter), respectively; the emulsions were finally kept at 4 °C. It was found that the crystallization of the emulsion increased with increasing PS concentration; however, the addition of the two surfactants (SL or MAG) helped reduce PS crystallization by increasing the PS solubility or by forming a dense crystalline network in which PS was entrapped. In addition, the diffraction patterns showed that the reduction in PS crystallization was mainly due to the decrease in droplet size rather than to the addition of SL or MAG. However, within the smaller PS emulsions with a $D_{(4,3)}$ of 0.2μm, samples with added MAG were found to be the most unstable, while the 0.6% PS emulsion with SL was the most stable over time. In a subsequent study, Zychowski et al. (2019) formulated O/W emulsions containing unesterified PS (0.3% or 0.6%), anhydrous milk fat (10%) and WPI (1%). In this case, they noted that as the concentration of PS increased, there was a decrease in $D_{(4,3)}$; moreover, the combination of PS and WPI was able to lower the interfacial tension to a greater extent than either component alone, thus demonstrating synergism between the two classes of compounds.

In a more recent study by Chen et al. (2021), instead, oleogel-in-water flavored emulsions with flavored oil (orange, lemon, or peppermint) and PS were prepared and stabilized with various types of emulsifiers [tea saponin (TS), octenylsuccinate starch (OS), *Quillaja* saponin (QS), sodium caseinate (SC), SL, sorbitan monooleate (SM), or Tween 80] using ultrasonication (see Table 20.2 for further details). The oleogel-in-water flavored emulsions were subjected to spray-drying to obtain a powdered form. The emulsions formulated with TS exhibited excellent stability and a higher EE of PS compared to conventional emulsifiers (90% vs. 60%). In the emulsions prepared with SL and Tween 80, large PS aggregates precipitated on the surface; this trend was still observed after 1 month of storage at room temperature. In the case of the spray-dried powders, those stabilized with natural saponins showed excellent flowability, redispersibility, and low PS crystallinity.

Table 20.3 reports information about several studies dealing with nanodispersions and nanocapsules to encapsulate PS. Regarding nanodispersions, Leong et al. (2011a) used a mixture of unesterified PS as the core material and tested different types of organic solvents as the aqueous phase (hexane, isopropyl alcohol, ethanol, or acetone) in which PS were dissolved, while Tween 20 was used as the surfactant. The best results were obtained with high-pressure homogenization at 80 MPa (1 cycle), giving a mean particle size of 50 nm. However, it was observed that as the pressure increased during nanodispersion operations, the PS loss also increased (3–28%). The authors highlighted that attention must also be paid to the organic solvent evaporation step, as it could also cause PS loss (0.5–9%).

In another study, the same authors (Leong et al. 2011b) formulated a nanodispersion with unesterified PS dispersed in hexane as the core material and Tween 20 as the surfactant; in this case, they added a step of high-pressure homogenization (0.1–80 MPa, 1 cycle) to conventional homogenization (1000–9000 rpm, 1–20 min). The results showed that a higher pressure led to an increase in particle size (52 nm) and a decrease in PS retention (345.40 mg/L) due to the high shear force and cavitation generated during the application of high-pressure homogenization; this brief, intense heating was, in fact, able to trigger PS degradation in the nanodispersion. The optimized conditions for the preparation of the nanodispersion were 15 min of homogenization at 7000 rpm and a subsequent cycle of high-pressure homogenization at 42 MPa. In a third study, Leong et al. (2011c) focused on the effects of the surfactant [sucrose stearate (SS), sucrose palmitate (SP), sucrose laureate (SLA), or sucrose oleate (SOL) (0.1–5.0%)] and high-pressure homogenization (80 MPa, 1 cycle) on the stability of unesterified PS nanodispersions prepared with hexane as the solvent phase. Most surfactants resulted in a particle size <100 nm, except for SOL. In terms of PS retention, values varied between 230.4 and 504.6 mg PS/L, while residual hexane was $<1.5\mu L/L$ of nanodispersion.

In recent studies, Feng et al. (2020b, 2021) tested free stigmasterol as a core material and a mixture of pectin/zein (ratio 0:10–5:10) and pectin/soy protein isolate (SPI) (ratio 0:10–5:10) as surfactants. Two organic solvents (ethanol and hexane) were tested for dissolving stigmasterol. In the ethanol-formulated nanodispersions, the pectin/zein ratio $<$ 2:10 resulted in a smaller mean particle size (374.05–584.40 nm) and ζ-potential (38.52–38.57 mV) and a higher EE (70.10–90.11%) and LAC (9–95 g/100 g). The results obtained with scanning electron microscopy and DSC showed the successful encapsulation of stigmasterol with pectin and zein, which formed an elastic network gel over the nanodispersions. In the presence of pectin, in fact, the stability of nanodispersions under different environmental conditions and simulated digestion conditions was improved (Feng et al. 2020b). On the other hand, when hexane was used as solvent (Feng et al. 2021), a pectin/SPI ratio of 1:10 resulted in a nanodispersion with an almost spherical shape, reduced stigmasterol crystallinity, long-term stability (PDI = 0.17), an average particle size of 477 nm, an EE of 89%, and a LAC of 18%; this behavior was also associated with an increase in the stability of the

Table 20.3 Summary of the main results of nanodispersions and nanocapsules used for PS encapsulation

Organic phase	Aqueous phase	Emulsifier	Encapsulation methodology	Results	Ref.
Nanodispersion					
PS	Organic/aqueous phase ratio (1:9–5:5)	Tween 20 (0.2%)	**(1)** In hexane, isopropyl alcohol, ethanol, **or** acetone (45 °C, 5 min) **(3)** Conventional homogenization (500–10,000 rpm, 5–40 min) **or** emulsification-evaporation method (20–80 MPa, 1–3 cycles) **(4)** Evaporation (5 °C, 0.17 bar)	– Optimized conditions (high-pressure homogenization at 80 MPa, 1 cycle): mean particle size of 50 nm with monomodal distribution. – ↑Pressure: ↑PS loss (3–28%). – Evaporation of organic phase: 0.5–9% PS loss.	Leong et al. (2011a)
PS	Organic/aqueous phase ratio (1:9)	Tween 20 (0.2%)	**(1)** In hexane (45 °C, 5 min) **(3)** Conventional homogenization (1000–9000 rpm, 1–20 min) + high-pressure homogenization (0.1–80 MPa, 1 cycle) **(4)** Evaporation (5 °C, 0.17 bar)	– ↑Pressure: ↑particle size, ↑PDI, ↓PS retention. – ↑Mixing speed: ↓particle size. – ↑Mixing time: ↑PDI. – Optimized conditions (15 min of mixing, 7000 rpm, 42 MPa): particle size of 52 nm, PDI of 0.34 and PS content of 336 mg/L.	Leong et al. (2011b)
		SS, SP, SLA **or** SOL (0.1–5.0%)	**(1)** In hexane (45 °C, 5 min) **(3)** Conventional homogenization (5000 rpm, 5 min) + high-pressure homogenization (80 MPa, 1 cycle) **(4)** Evaporation (5 °C, 0.25 bar)	– Particle size: 2.8–259.9 nm. – PS retention: 230.4–504.6 mg/L. – SS, SP and SL: particle size <100 nm with monomodal distribution. – Hexane residue <1.5μL/L nanodispersion.	Leong et al. (2011c)
Stigmasterol	PS/zein ratio (1:1–1:15)	–	**(1)** In ethanol, hexane, **or** methanol **(2)** In ethanol (70 **or** 80%) **(3)** High-speed homogenization (14,500 rpm) **or** ultrasonication (585 J/mL, 200–800 W, continuous **or** 1 s, 4 s, 8 s pulse mode) **(4)** Evaporation (45 °C) – Addition of deionized water – Lyophilization	– Best solvents for PS and zein: ethanol and 80% ethanol. – Optimized formulation (ratio stigmasterol/zein of 1:5) and conditions (ultrasonication at 200 W, pulse mode of 1 s): D of 337 nm, PDI of 0.1127, ζ-potential of 57 mV, EE of 97% and LAC of 19 g/100 g. – Ultrasonication: ↓D and ↑ζ-potential, ↑EE and ↑LAC.	Feng et al. (2019)
	Pectin/zein ratio (0:10–5:10)		**(1)** In ethanol **(3)** Ultrasonication (585 J/mL, 200 W, 1 s pulse mode, 3 min) **(4)** Evaporation (45 °C) – Addition of deionized water	– Pectin/zein ratio < 2:10: ↓average particle size, ↓ζ-potential, and ↑higher EE and ↑LAC. – Nanoparticles without pectin have a near-spherical shape. – Encapsulation: ↓stigmasterol crystallinity. – Pectin improved stability in different environmental conditions and simulated digestion conditions.	Feng et al. (2020b)

(continued)

Table 20.3 (continued)

Organic phase	Aqueous phase	Emulsifier	Encapsulation methodology	Results	Ref.
	Pectin/SPI ratio (0:10–5:10)		**(1)** In hexane **(3)** High-speed homogenization (10,000 rpm) + ultrasonication (585 J/mL, 200 W, pulse mode, 3 min) **(4)** Evaporation (45 °C)	– Pectin/SPI (ratio 1:10): near-spherical shape, ↓stigmasterol crystallinity, long-term stability (PDI = 0.17), average particle size of 477 nm, EE of 89%, LAC of 18%. – ↑Stability with different pH and ionic strength conditions. – Stigmasterol in vitro release: limited under gastric conditions, promoted at intestinal environment.	Feng et al. (2021)
Nanocapsules					
PS (φ: 0.1–0.4)	WPC **or** SPI **or** SC (0.5–3%)	–	**(1)** In hexane (45 °C) **(2)** In deionized water. Stored overnight (4 °C) **(3)** High-speed homogenization (5000 rpm) + stirring (5000 rpm, 2 min) + microfluidization treatment (40 MPa, 1 pass) **(4)** Rotary evaporator at 45 °C – Addition of deionized water – Centrifugation (8000 g, 10 min) – Lyophilization – Storage stability (4 °C, 25 °C, and 60 °C, 28 days)	– Selection of SC: lower particle size, similar EE and LAC, higher ζ-potential. – Optimized formulation (φ: 0.3, 1% SC): D_z of 143 nm, PDI of 0.12, EE of 90%, LAC of 19 g/100 g protein, ζ-potential of −38 mV, good redispersion behavior and low crystallinity of PS. – PS bioaccessibility: unencapsulated < encapsulated (18% vs. 29%). – Storage: ↓LAC (PS loss of 8% at 4 °C and 28% at 60 °C).	Cao et al. (2016)
PS (1%)	SC **or** SC/dextran (1:1) (organic/aqueous phase ratio, 1:5)	–	**(1)** In ethanol **(2)** In Milli-Q water and continuous stirring (2 h) **(3)** High-speed homogenization (11,000 g, 2 min) + ultrasonication (250 W, 25 min) **(4)** Evaporation (45 °C) – Lyophilization	– SC/dextran vs. SC formulation: EE (79% vs. 74%), gastric acid resistance (9% vs. 5%) and PS released in intestinal fluid (52% vs. 33%). – Encapsulation: ↓PS crystallinity.	Li et al. (2021a, b)
PS	SC **or** SC/pectin (1:1) (organic/aqueous phase ratio, 3:1–1:3)	–	**(1)** In ethanol (45 °C, 30 min) **(2)** In deionized water. Stored overnight (4 °C) **(3)** Homogenization (8000 rpm, 3 min) **(4)** Evaporation (45 °C) – Addition of deionized water – pH adjustment (3–7) and addition of gluconolactone for composite agglomeration (1 h) – Thermal stability (70–100 °C, 30 min) – Storage stability (4 °C and 25 °C, 15 days)	– Optimized formulation (SC/pectin ratio of 2:1, organic/aqueous phase ratio of 1:3): particle size of 240 nm, PDI < 0.3, EE of 91%, LAC of 21%. – pH and thermal stability: SC/pectin > SC formulation. – PS bioaccessibility: SC/pectin > SC formulation. – PS retention: Storage at 4 °C > 25 °C.	Gan et al. (2022)

PS (1%)	SPI (0.25–1.25%)	SL (PS/SL ratio, 1:1–1:5)	**(1)** In ethanol (8–24 mL) **(2)** In deionized water. Stored overnight (4 °C) **(3)** High-speed homogenization (10,000 rpm) + high-pressure homogenization (90 MPa, 6 passes) **(4)** Evaporation (45 °C) – Addition of deionized water – Storage stability (25 °C, 14 days) – Thermal stability (4–60 °C) – pH stability: aqueous phase adjusted at pH 2–7 – Salt stability: addition of NaCl or $CaCl_2$ (0–200 mmol/L)	– Optimized formulation [PS (1%)/SL ratio of 1:4, 0.75% SPI, 16 mL of ethanol]: particle size of 93 nm, PDI of 0.179, ζ-potential of −29.3 mV, EE of 97%. – High storage stability (no crystallization). – Moderate stability toward pH, T and ionic strength. – Good redispersion behavior in water. – PS bioaccessibility: unencapsulated < encapsulated (18% vs. 71%).	Li et al. (2022)

Steps for nanodispersion/nanocapsules elaboration: (1) PS dissolution, (2) aqueous phase preparation, (3) emulsification, (4) organic phase evaporation
D average particle diameter, D_z particle size distribution, *EE* encapsulation efficiency, *LAC* loading capacity, *PDI* polydispersity index, *PS* phytosterols, *SC* sodium caseinate, *SL* soybean lecithin, *SLA* sucrose laureate, *SOL* sucrose oleate, *SP* sucrose palmitate, *SPI* soy protein isolate, *SS* sucrose stearate, *WPC* whey protein concentrate, ζ-potential zeta-potential, φ organic volume fractions

system under different pH and ionic strength conditions.

Regarding nanocapsules (Table 20.3), all studies focused on the use of unesterified PS as the core material. In the study by Cao et al. (2016), an emulsification-evaporation method was used to successfully obtain PS nanoparticles with different food proteins as encapsulation materials. SC had the highest potential to form PS nanoparticles with smaller sizes and higher EE among all the tested proteins [whey protein concentrate (WPC), SPI, and SC]. Apart from the protein type, the particle characteristics of the nanoparticles (such as particle size, EE, and/or LAC) varied according to the protein concentration in the aqueous phase and the organic volume fraction. The freeze-dried PS nanoparticles formulated with SC showed good redispersion behavior in water and low PS crystallinity. Storage resulted in a gradual decrease in LAC of PS, especially at high temperatures (>25 °C).

In the study by Li et al. (2021a), instead, an SC/dextran conjugate was prepared (promoting the Maillard reaction under controlled dry heating conditions) to be employed as an emulsifier. PS nanoparticles encapsulated by SC or SC/dextran were prepared using the emulsion evaporation method. The EE of PS in SC/dextran nanoparticles was higher (5.22–78.81%) than that in SC nanoparticles (2.78–73.5%). SC/dextran nanoparticles occurred as relatively loose aggregates, whereas SC nanoparticles were present as compact and dense structures. DSC analysis showed that PS encapsulation significantly reduced its crystallinity. Therefore, SC/dextran conjugates prepared via the Maillard reaction are more suitable for use as wall materials for PS nanoencapsulation. Gan et al. (2022), on the other hand, successfully prepared SC/pectin-based nanoparticles (SCP) using emulsification evaporation and complex coacervation techniques. In SCP-PS, the best mass ratio of SC/pectin was 2:1. Under these conditions, PS were successfully loaded into SCP-PS with high EE (91%) and LAC (21%). The nanoparticle size was approximately 240 nm, and the PDI was less than 0.3. X-ray diffraction analysis showed that nanoencapsulation caused a PS change, from an ordered crystalline state to a disordered amorphous state. After storage, the results demonstrated that SCP-PS nanoparticles were stable against dissociation and thus could provide excellent PS encapsulation and protection. Recently, Li et al. (2022) prepared PS nanoparticles (PSNP) using a combined emulsification-evaporation high-pressure homogenization method. The organic phase was prepared by dissolving PS and SL in anhydrous ethanol. The best formulation parameters were PS:SL (1:4), SPI (0.75%), and ethanol 16 mL. Once the emulsions were prepared, they were subjected to evaporation high-pressure homogenization using 6 passes at 90 MPa. The PSNPs had an average particle size of 93.35 nm, a PDI of 0.179, a ζ-potential of 29.3 mV, and an EE of 97.3%.

Table 20.4 shows the applications of NLCs as PS encapsulation systems. As indicated previously (Sect. 20.3.3), an improvement in LAC and EE can be achieved when a mixture of solid and liquid lipids is used as the coating material. The more disorganized crystalline structure is obtained with these types of blends, the higher number of bioactive compounds is able to accomodate. In this sense, Soleimanian et al. (2018) confirmed that the development of NLCs with a mixed solid lipid matrix (propolis wax and glyceryl behenate) also favors the formation of less organized crystalline lattices. Thus, imperfections in the crystal lattice increased the incorporation of β-sitosterol, leading to higher LAC and EE with respect to those obtained with NLCs formulated only with propolis wax. The authors used response surface methodology to obtain the optimal formulation for NLCs containing β-sitosterol. As shown in Table 20.4, formulations with lower β-sitosterol and higher liquid lipid content (from pomegranate seed oil) showed smaller particle size and higher EE. According to the authors, high concentrations of β-sitosterol (15–17% of the lipid phase content) decreased the EE, due to its accumulation on the NLC droplet surface with consequent crystal formation; β-sitosterol accumulation on the droplet surface was already observed in an emulsion system (Engel and Schubert 2005). In contrast,

Table 20.4 Review of the most relevant results of nanostructured lipid carriers (NLCs) used for PS encapsulation

Disperse phase	Aqueous phase	Encapsulation methodology	Main results	Ref.
Model systems				
– Bioactive compound: β-sitosterol (2.9–17.1%). – Solid lipid: PW **or** PW:GB (1:1). – Liquid lipid: PSO (1.7–58.3%). – Lipid phase content: 10%.	Tween 80/lecithin (ratio 1: 0.25) (6%)	**(1)** Stirring (85 °C, 5 min) **(2)** Intense stirring (85 °C) **(3)** Hot high-speed homogenization (14,000 rpm, 85 °C, 10 min) **and** ultrasonication (250 W, 85 °C, 8 min, on for 2 s at intervals of 2 s) **(4)** Ice bath (30 min) – Lyophilization (−70 °C, 0.001 bars, 24 h)	– ↑PS: ↑particle size and ↑PDI. – ↑%Liquid lipid: ↓particle size. – Optimized formulation (10% PS and 50% PSO): particle size of 97 and 105 nm, PDI of 0.19 and 0.20, ζ-potential of −26 mV, and EE of 90% and 97% for PW and PW:GB formulations, respectively.	Soleimanian et al. (2018)
– Bioactive compound: β-sitosterol (10%). – Solid lipid: PW **or** PW:GB (1:1). – Liquid lipid: PSO (50%). – Lipid phase content: 10%.	Tween 80/lecithin (ratio 1: 0.25)	**(1)** Stirring (85 °C, 5 min) **(2)** Intense stirring (85 °C) **(3)** Hot high-speed homogenization (14,000 rpm, 85 °C, 10 min) **and** ultrasonication (50% amplitude, 100 W, 8 min, on for 2 s at intervals of 2 s, 250 W) **(4)** Ice bath (30 min) - Lyophilization (−70 °C, 0.001 bars, 24 h) – Thermal stability (60–90 °C, 20 min) – Salt stability (0–200 mM NaCl) – pH stability (aqueous phase adjusted to values from 2 to 8)	– Thermal stability, β-sitosterol solubility and miscibility: PW:GB > PW. – Thermal treatment >70 °C: ↑particle size and ↓β-sitosterol content. – pH stability: acidic conditions ↑particle size; pH > 6 no effects. – Stable to electrolyte concentrations. – Crystallization: PW: GB < PW.	Soleimanian et al. (2019)
– Bioactive compound: PS (30% **or** 50%). – Solid lipid: fully hydrogenated CA **or** CR **or** different mixtures of CA and CR (30% **or** 50%). – Liquid lipid: HOSO (20–70%). – Lipid phase content: 10%.	Tween 80 (2%)	**(1)** Melting (130 °C). Conditioned at 5 °C (24 h) and at 25 °C (24 h) **(2)** Homogenization (20,000 rpm, 3 min) **(3)** High-pressure homogenization (800 bar, 3 cycles) **(4)** At 5 °C for 24 h and at 25 °C for 24 h – Lyophilization (−25 °C, 0.370 mbar, 24 h) – Storage stability (60 days)	– Particle size of 148–342 nm, PDI of 0.28–0.48 and ζ-potential from −22 to −30 mV. – Formulation with 30% PS: best hydrodynamic diameter, PDI, ζ-potential and particle size. – ↑HOSO content: ↓particle size – Encapsulation: ↑thermal resistance.	da Silva Santos et al. (2019)
– Bioactive compound: PS (0.5%). – Solid lipid: fully hydrogenated PO **or** CR. – Liquid lipid: SO. – Lipid blends: simple **and** interesterified blends of SO (50%) and PO or CR (50%).	Soybean lecithin (2%)	**(1)** Stirring (85 °C) **(2)** Homogenization (15,000 rpm, 5 min) **(3)** High-pressure homogenization (700 bar, 2 cycles) **(4)** At 5 °C for 24 h – Storage stability (30 days)	– After 30-d storage: D_{32} of 167–330 nm, ζ-potential from −30 to −44 mV. – Encapsulation with interesterified blends vs. simple blends: ↓particle size, ↓crystallinity, ↑stability, EE and ↑LAC.	da Silva et al. (2022)

(continued)

Table 20.4 (continued)

Disperse phase	Aqueous phase	Encapsulation methodology	Main results	Ref.
Food				
– Bioactive compound: β-sitosterol. – Solid lipid: glyceryl palmitostearate. – Liquid lipid: MCT. – Co-surfactant: PEG.	Tween 80	**(1)** Stirring (85 °C) **(2)** Homogenization (20,000 rpm, 85 °C) **(4)** Refrigeration – Addition into butter (mixed with a homogenizer, 7000 rpm) – Butter storage stability (90 days)	– β-sitosterol-loaded NLCs characteristics: 165 nm, PDI of 0.21, ζ-potential of −13.5 mV and EE of 97%. – Storage: no effect on PDI, no negative effect on acid and peroxide values.	Bagherpour et al. (2017)

Steps for nanostructured lipid carriers' (NLCs) elaboration: (1) fusion of the lipid fraction, (2) formation of the emulsion, (3) nanoemulsification, (4) crystallization of the lipid phase

CA canola oil, *CR* crambe oil, D_{32} surface diameter, *EE* encapsulation efficiency, *GB* glyceryl behenate, *HOSO* high-oleic sunflower oil, *LAC* loading capacity, *MCT* medium chain triacylglycerols, *PDI* polydispersity index, *PEG* polyethylene glycol, *PO* palm oil, *PS* plant sterols, *PSO* pomegranate seed oil, *PW* propolis wax, *SO* soybean oil, ζ-potential zeta-potential

the increase in pomegranate seed oil (50% of the lipid phase content) favored the solubilization of β-sitosterol and reduced its crystallization.

In a subsequent study (Soleimanian et al. 2019), the stability of the optimized NLC formulation to thermal treatment, salt concentration and pH was evaluated. Both heating at temperatures above 70 °C and pH 2 resulted in an increase in the NLC particle size, while the electrolyte concentration did not impact the NLC stability. Thus, there are certain limitations for the incorporation of these delivery systems in food formats, such as acid products.

In another study, the effect of the lipid matrix composition on the properties of NLCs used for PS encapsulation was studied (da Silva Santos et al. 2019). A higher thermal resistance of NLCs with respect to lipid matrices used for their development (high-oleic sunflower oil, high-oleic sunflower oil with fully hydrogenated canola or crambe oils, or their mixture) was observed. In fact, when high concentrations of PS (50% of the lipid phase content) were used, complete incorporation into NLCs was not achieved, which led to decreased stability during 60 days of storage (as observed by Soleimanian et al. 2018). When PS were added at a 30% concentration, their complete solubilization and a more pronounced reduction in their crystallinity were attained in the matrix with a mixture of solid and liquid lipids. In addition, NLCs formulated with canola and crambe oils separately showed improved PS incorporation.

In NLCs formulated with soybean oil as liquid lipid and palm or crambe oils as solid lipid, a loss of encapsulated PS during storage occurred, as described in the previously mentioned works, due to their crystallization and accumulation on the surface of the encapsulation system. However, it was observed that PS crystallization is reduced when using interesterified instead of simple lipid blends. According to the authors, the interesterification process produces a greater heterogeneity of triacylglycerols that: (1) have a lower melting point, thus favoring the incorporation of the liquid lipid and therefore PS solubilization (as observed by Soleimanian et al. 2018) and (2) result in a lipid matrix with a higher degree of imperfections. All this may have contributed to the higher EE and LAC of PS observed in the presence of the interesterified blends. Although the use of palm oil resulted in smaller particle sizes, the NLCs obtained with crambe oil showed greater stability against processes such as flocculation and coalescence, as well as better encapsulation properties.

As shown in Table 20.4, the application of NLCs in food is limited. Bagherpour et al. (2017) developed β-sitosterol-loaded NLCs with a high EE for their incorporation into butter.

Enrichment with the encapsulated PS did not adversely affect the acid and peroxide values of the butter, and optimal stability during storage was also observed.

Table 20.5 reports a series of studies on PS encapsulation that used spray-drying technology. Di Battista et al. (2015) evaluated the effect of different surfactant sources and concentrations on the yield, EE and properties of PS microcapsules using arabic gum and maltodextrin as wall materials. It was observed that microencapsulation with sodium lauryl sulfate resulted in an adequate process yield, albeit lower than that obtained using Tween 20; however, a smaller particle size and higher EE were achieved. In a subsequent work (Di Battista et al. 2017), response surface methodology was applied to find the optimal formulation for PS encapsulation, in terms of PS concentration, wall material composition, and spray-drying conditions. This approach led to an improvement in process yield (84% vs. 55%), EE (76% vs. 50%), PS retention (76% vs. 40%), and smaller particle size (5.0μm vs. 5.9μm) with respect to those obtained in their previous work. The addition of PS at lower concentrations (2% vs. 8%) favored the formation of microparticles with a smaller size, which is probably related to the reduction in the viscosity of the feed solution, thus favoring the formation of smaller droplets during the process that resulted in improved EE. It was also observed that the atomization air flow rate significantly impacted the process yield, as higher flow rates reduced the contact time between the particles and the drying air stream, thus decreasing the process yield. Similar results were obtained during encapsulation with different combinations of WPI, inulin and chitosan and different concentrations of Tween 80 as a surfactant (Tolve et al. 2019). In this case, when only WPI and the lowest concentration of Tween 80 were used, a feed solution with lower viscosity was obtained, so higher process yields and LAC were achieved.

Recently, an optimization of the spray-drying process for PS encapsulation was published (Alvarez-Henao et al. 2023). The authors reported that the different viscosities of the feed solution obtained using SPI, maltodextrin or arabic gum as encapsulating agents affected the process yield. Therefore, formulations elaborated with arabic gum resulted in solutions with lower viscosities than those obtained with SPI, thus increasing the encapsulation yield as the drying process was facilitated (Di Battista et al. 2017). However, the use of SPI resulted in a higher EE, due to its greater emulsifying capabilities and, in addition, a good synergistic effect on this efficiency was observed from the interaction between arabic gum and SPI.

Spray-drying technology has also been used for the co-encapsulation of E-PS, fish oil and limonene using WPI in different combinations with SC (Chen et al. 2013a) or soluble corn fiber (Chen et al. 2013b) as wall materials. Regarding spray-drying conditions, Chen et al. (2013a) observed that the outlet temperature had a direct effect on PDI. Elevated outlet temperatures (>80 °C) could favor protein denaturation and aggregation, influencing solubility and particle size. In fact, reconstituted emulsions with narrower droplet size distributions were obtained with a drying temperature of 70 °C. Similarly, it was observed that high inlet temperatures (180 °C) negatively affect the EE by excessive evaporation, thus resulting in rupture of the capsule membrane and release of the core phase. Increasing the PS content with respect to the wall material led to an increase in particle size and distribution, which agrees with what had been observed in other encapsulation methods already mentioned. An accelerated storage test (45 °C, 30% RH and saturated oxygen conditions for 7 days) showed that encapsulation is an effective protection mechanism against oil oxidation.

In PS-enriched oil microcapsules developed with WPI (Tolve et al. 2018a), the presence of inulin reduced the formation of protein aggregates produced by the high outlet temperatures of the drying process. In contrast, high inlet temperatures favored the process yield, which is the opposite behavior observed by Chen et al. (2013a). The authors associated this trend with a higher drying efficiency, which might have decreased the adherence of the microcapsules in the drying chamber. In the same study, higher EE

Table 20.5 Summary of the main results obtained using spray drying for PS encapsulation

Core material	Wall material	Surfactant	Encapsulation methodology	Main results	Ref.
Model systems					
PS (6.7%)	AG (15%) **and** MD (5%)	Tween 20 **or** SLS (0.1–2.7%)	**(1)** Stirring (50 °C, 30 min) **(2)** Continuous agitation (1 h) **(3)** Homogenization (25,000–35,000 rpm, 9 min) **(4)** T_{in}: 160 °C, T_{out} < 120 °C, Q_{atom}: 601 L/h, Q_{feed}: 2 mL/min, drying air volumetric flow rate: 35–38 m^3/h	– Process yields at 2% of surfactant: Tween 20 > SLS (65% vs. 55%). – Best formulation (SLS at 2%): EE of 50%, PS retention of 40% and particle size of 5.9μm.	Di Battista et al. (2015)
PS (2–8%)	AG/MD at ratios from 1:19 to 19:1	SDS (2%)	**(1)** Stirring (50 °C, 30 min) **(2)** Continuous agitation (1 h) **(3)** Homogenization (25,000–35,000 rpm, 9 min) **and** sonication (60 s) **(4)** T_{in}: 110–160 °C, Q_{atom}: 30–60 mm, Q_{feed}: 5–15%	– Optimized formulation (2% PS, 15% total solids, and AG/MD ratio of 2.06) and conditions (T_{in}: 160 °C, Q_{atom}: 42 mm, Q_{feed}: 7%): process yield of 84%, EE of 72%, PS retention of 76% and mean particle size of 5.0μm.	Di Battista et al. (2017)
PS (7%)	WPI (8%), inulin (with 1% **or** without) and chitosan (with 0.125% **or** without)	Tween 80 (1.25% **or** 2.50%)	**(1)** Stirring (until complete dissolution) **(2)** Continuous agitation (1 h) **(3)** Homogenization (20,000 rpm, 15 min) **(4)** T_{in}: 125 °C with T_{out}: 60–80 °C **or** T_{in}: 155 °C with T_{out}: 80–100 °C, Q_{feed}: 0.5 L/h, Q_{atom}: 600 L/h	– Uniform microcapsules (average size < 25μm). – Formulation WPI without inulin or chitosan, Tween 80 at 1.25% and T_{in} of 155 °C: highest process yields (44%) and LAC (25%). – No differences between results using a lab spray dryer vs. a pilot plant scale one.	Tolve et al. (2019)
Food					
Fish oil **or** fish oil:E-PS: limonene (6:1:1)	WPI/SC ratio of 4:1 (wall to core ratios of 1:1–4:1)	–	**(1)** Stirring (55 °C, 60 min) **(3)** Homogenization (13,500 rpm, 1 min) **and** high-pressure homogenization (80 MPa, 4 passes) **(4)** T_{in}: 160–180 °C, T_{out}: 70–80 °C, Q_{feed}: 15–20 mL/min – Accelerated storage (45 °C, 30% RH, 7 days)	– Best conditions (wall to core ratio of 4:1, T_{in}: 170 °C, T_{out}: 70 °C): Oil recovery >90%. – Accelerated storage ↑ PV and AnV: protection of the encapsulation against oil oxidation. – Retention of EPA and DHA: Co-encapsulation > encapsulated oil.	Chen et al. (2013a)
Fish oil: E-PS: limonene (6:1:1)	WPI/SCF ratio of 1:1 (wall to core ratio of 1:2)	–	**(1)** Stirring (55 °C, 60 min) **(3)** Homogenization (13,500 rpm, 1 min) **and** high-pressure	– Emulsions: narrow-size particle distribution and monodispersity. – Particle microstructure: spherical and regular shape for spray-dried; irregular, sharp and	Chen et al. (2013b)

(continued)

Table 20.5 (continued)

Core material	Wall material	Surfactant	Encapsulation methodology	Main results	Ref.
			homogenization (80 MPa, 4 passes) **(4)** T_{in}: 170 °C, T_{out}: 70 °C, atomizer speed: 15,000–2000 rpm **or** lyophilization (−51 °C, 0.120 mbar, 48 h) – Accelerated storage (45 °C, 30% RH, 7 days)	broken for freeze-dried. – EE, volatile retention, PV and AnV: spray-dried > freeze-dried. – Redispersion properties and oil phase oxidation: similar for spray-dried and freeze-dried.	
PS (5%) in SO **or** PS (10%) in SO and asolectin	WPI (6.75%), inulin (1 **or** 2%), chitosan (0 **or** 0.125%) (wall to core ratio of 4:1)	–	**(1)** Stirring (until complete dissolution) **(2)** Dissolution in SO at 70 °C **(3)** Homogenization (8000 rpm, 4 min) **(4)** T_{in}: 125 °C **or** 185 °C, T_{out}: 67 °C **or** 115 °C, Q_{feed}: 2 mL/min	– Encapsulation with WPI:inulin:chitosan formulation: spherical and uniform particles (average size < 50 nm), EE of 85%, LAC of 0.95 g/g powder, surface oil content of 9.7% and water activity (0.2–0.4). – Low oxidation stability: PV of 101.7 meq O_2/kg up to 125.6 meq O_2/kg oil after storage.	Tolve et al. (2018a)
PS (7%)	WPI (8%)	Tween 80 (1.25%)	**(1)** Stirring (until complete dissolution) **(2)** Continuous agitation (1 h) **(3)** Homogenization (20,000 rpm, 10 min) **(4)** T_{in}: 155 °C, Q_{feed}: 0.5 L/h, Q_{atom}: 600 L/h – Fortification of dark chocolates (64–85% cocoa) with 0–15% of encapsulated PS. – Storage (18 °C, 3 months)	*Particle characterization*: – Size < 30μm, water activity of 0.3, moisture of 4.5%, PV of 0.46 meq O_2/kg fat, LAC of 24.7%. *Addition of PS microcapsules to dark chocolate samples*: – Slight ↑$D_{[4:3]}$ and ↑D_{90}. – Slight ↑PV during storage. – PS bioaccessibility: from 13.3% to 3.3% (gastric phase) and from 6.5% to 8.4% (intestinal phase).	Tolve et al. (2018b)
PS in SO (1:1)	AG (0–100%), MD (0–100%) **and** SPI (0–100%) Wall material: PS ratio of 3:1	Tween 20 (1%)	**(1)** Stirring (50 °C, 30 min) **(2)** Homogenization (3500 rpm, 3 cycles, 1 min) **(3)** Continuous agitation (5 min) and homogenization (5000 rpm, 10 min) **(4)** T_{in}: 165 °C, Q_{atom}: 42 mm, Q_{feed}: 10%, drying air and aspirator 80%	– Optimized formulation (SPI:AG:MD ratio of 21:78:1): process yield of 66%, EE of 74%, moisture of 9%, particle size of 24μm. – Presence of AG improved encapsulation yield. – Presence of SPI improved EE.	Alvarez-Henao et al. (2023)

Preparation steps for spray-drying process: (1) wall materials and surfactant dissolution in distilled water, (2) PS dispersion, (3) core-wall material homogenization, (4) spray-drying conditions

AG arabic gum, *AnV* p-anisidine value, $D_{[4:3]}$ volume moment mean, D_{90} 90th percentile size, *DHA* docosahexaenoic acid, *E-PS* esterified phytosterols, *EE* encapsulation efficiency, *EPA* eicosapentaenoic acid, *LAC* loading capacity, *MD* maltodextrin, *PS* plant sterols, *PV* peroxide value, Q_{atom} atomization air flow rate, Q_{feed} feed flow rate, *RH* relative humidity, *SC* sodium caseinate, *SCF* soluble corn fiber, *SDS* sodium dodecyl sulfate, *SLS* sodium lauryl sulfate, *SO* soybean oil, *SPI* soybean protein isolate, T_{in} inlet temperature, T_{out} outlet temperature, *WPI* whey protein isolate

were obtained with increasing total solids concentration; in this case, the presence of chitosan and inulin could have increased the viscosity of the feed solution, thus decreasing oil migration to the surface. However, relatively high PVs were found even just after production. The high temperatures reached during emulsion elaboration, together with a possible pro-oxidant effect of PS at the concentrations used, have been pointed out as possible factors responsible for the results obtained.

In another study, PS-loaded microcapsules elaborated with WPI and Tween 80 were used for the enrichment of dark chocolates with different cocoa contents (Tolve et al. 2018b). Despite a 3-month storage (shelf-life of the product), PVs below 2.5 meq O_2/kg of fat were found, and it was shown that the addition of microencapsulated PS did not affect consumer acceptance.

Complex coacervation has been reported as a successful method for *Echium* oil and PS co-encapsulation. The use of gelatin-cashew gum as wall materials resulted in microcapsules with a particle size three times lower than those elaborated with a gelatin-arabic gum combination (Comunian et al. 2017). The authors observed that both wall materials (arabic and cashew gums), as well as the addition of sinapic acid as a cross-linking agent for encapsulation, provided greater oxidative stability to the oil compared to the non-encapsulated oil; such differences could be partly attributed to the different microcapsule morphologies (spherical morphology with gelatin-arabic gum vs. an agglomerated undefined shape in the cashew gum formulation), which depended on the wall material combination used, as reported in a subsequent study (Comunian et al. 2018). Although the sinapic acid cross-linking reaction is higher with cashew than with arabic gum (promoting a greater retention of the core material during the encapsulation process), better results were observed in terms of oxidative stability and PS protection during 30 days of storage when using the gelatin-arabic gum combination. Microcapsules with both wall material combinations were added into yogurt without impacting the final overall quality of the product (Comunian et al. 2017). SPI and pectin have also been used as wall materials for PS encapsulation by complex coacervation (Li et al. 2021b). In this case, PS dispersed into conjugated linoleic acid showed the highest EE (88%) when the SPI:pectin ratio was 5:1. According to the authors, the higher number of hydrophobic sites provided by the increased pectin concentration could justify the improved PS encapsulation. At the proposed ratio, complete entrapping of PS in its amorphous state was achieved, and a favorable oxidation stability of the core material was demonstrated by the relatively low PV (<6 meq O_2/kg of fat) found after 5 weeks of storage at 37 °C.

Electrospinning has been applied few times for PS encapsulation. Paaver et al. (2016) developed β-sitosterol-loaded nanofibers using chitosan as the carrier polymer and trifluoroacetic acid (TFA) or hexa-fluoro-2-propanol (HFIP) as solvent systems. The authors observed that the electrospinning process affected the solid state of β-sitosterol, thus ensuring its amorphous state and increasing its solubility. The solvent system used for the electrospinning process influenced the mean diameter (TFA: 218 nm, TFA + HFIP: 150 nm) and morphology of the formed fibers, which were related to the different viscosities of the feed solutions. On the other hand, Mousavi et al. (2021) determined a positive effect of gelatin addition to chitosan solution (used as carrier polymer) for the formation of stigmasterol-loaded fibers using aqueous acetic acid as solvent system. The addition of gelatin caused an increase in the viscosity of the chitosan solution, leading to the formation of more homogeneous fibers with a mean diameter of 217 nm. Stigmasterol loading at 0.04% decreased the fibers' mean diameter to 208 nm, while concentrations above these levels had a negative impact on their morphology and spinnability. It is interesting to note that, despite the great advantages of electrospinning as an encapsulation method, it also presents some difficulties for commercial applications in the food industry that should be considered. One of the most important is the possible presence of traces of organic solvent outside or inside the fiber, which could be detrimental to consumer health and safety (Ubeyitogullari et al. 2022).

SC-CO_2 technology has been used as a drying method for the formation of polysaccharide-based aerogels, as well as an impregnation method to insert PS in these aerogel networks (Ubeyitogullari et al. 2022). Impregnation of PS into starch aerogels has been shown to be a suitable method to reduce PS crystallinity, as the aerogel network is able to act as a mold and physical barrier to prevent the formation of large PS crystals. For wheat starch aerogels, the PS impregnation process by SC-CO_2 was optimized in terms of temperature and cooling rate to achieve the highest impregnation capacity (99 mg of PS/g nanoaerogel), smallest particle size (59–87 nm) and improved PS distribution (Ubeyitogullari and Ciftci 2017). Impregnation of PS increased from 126 to 195 mg/g when the aerogel was elaborated with corn starch (Ubeyitogullari et al. 2019). The higher amylose content resulted in the formation of aerogels with a larger surface area (220 m^2/g vs. 62 m^2/g) and pore volume (0.36 cm^3/g vs. 0.27 cm^3/g), which improved the impregnation process compared to wheat starch formulations.

Regarding SLNs, only one study has applied it for PS encapsulation (Guo et al. 2022). The hot melt emulsification method was employed to obtain PS-loaded SLNs, and the effect of different lipid matrices (glycerol monostearate, glycerol distearate, or glycerol tristearate) on the encapsulation process was evaluated. A uniform particle size (ranging from 100 to 300 nm, depending on the lipid matrix), ζ-potential higher than 30 mV, and satisfactory encapsulation properties (EE and LAC >98% and 7.6%, respectively) were observed in all formulations proposed. As expected, the inclusion of PS in SLNs significantly reduced their crystallinity. A good resistance of the nanoparticles to the early stages of digestion (oral and gastric) was established, while a higher degradation of SLNs was observed after the simulated intestinal stage with the subsequent release of PS. The release behavior of free fatty acids and PS from the encapsulation system was also influenced by the lipid composition, being higher in formulations with glycerol monostearate. Encapsulation of PS with a hydroxypropyl-β-CD inclusion complex was studied by Meng et al. (2012). Process parameters (such as the type of solvent, the β-CD:PS ratio, PS content, temperature, and reaction time) were optimized to achieve high EE (92–98%) and complexes with high water solubility. β-sitosterol-hydroxypropyl-β-CD inclusion complexes obtained by grinding also showed conversion to their amorphous form and a reduction in the melting enthalpy of β-sitosterol due to their inclusion in the CD cavity (Yu et al. 2018). The authors estimated that approximately 97% of the β-sitosterol was encapsulated by the hydroxypropyl-β-CD complex, which is similar to the EE values obtained by Meng et al. (2012). Moreover, an improvement in the reduction of intracellular lipid accumulation was observed with the inclusion of β-sitosterol into the complex with respect to its nonencapsulated form.

20.4.2 Encapsulated PS Bioaccessibility

For the food industry, the amount and bioavailability of PS are of interest from both nutritional and functional standpoints. The bioavailability of a compound can only be measured by in vivo methods, which are considered as reference standards. Nevertheless, the difficulties related to experimental design, costly equipment, time-consuming procedures, ethical constraints and data interpretation limit their use. On the other hand, bioaccessibility is defined as the fraction of a compound released from the food matrix along the gastrointestinal tract and is considered potentially available for absorption. It can be assessed by in vitro methods, generally quicker and simpler, which in turn do not have ethical constraints and simplify the physiological process of digestion (Faubel et al. 2022).

Bioaccessibility aspects have been faced in different types of PS encapsulation systems. In nanodispersed formulations with stigmasterol as the core material, Feng et al. (2021) noted that the in vitro release of stigmasterol was limited under gastric conditions, whereas it was enhanced in the intestinal environment. When pectin was used for

stabilizing nanodispersions, it limited stigmasterol release in simulated gastric fluid, thus confirming that pectine could improve nanodispersion stability and could be used to achieve controlled release of bioactive compounds.

Regarding nanocapsules, Cao et al. (2016) found that encapsulation in SC nanoparticles significantly improved the bioaccessibility of PS compared with unencapsulated PS (29.2% vs. 17.8%). The reduced size and crystallinity of PS crystals, in fact, may be largely responsible for the improved bioaccessibility. In the study by Li et al. (2021a), instead, it was observed that, under acidic gastric conditions, the PS release rates from SC and SC/dextran nanoparticles were 8.6% and 4.8%, respectively. After 7 h of intestinal digestion, the release rate of PS from SC/dextran nanoparticles (52.2%) was significantly higher than that from SC nanoparticles (32.7%). In another study, Gan et al. (2022) prepared SCP nanoparticles that successfully improved PS bioaccessibility by at least 43.8% compared to unencapsulated PS, thus confirming that the presence of pectin is able to improve PS bioaccessibility, as previously reported by Feng et al. (2021). Recently, Li et al. (2022) prepared PSNP and noticed that, after 3 h of in vitro digestion, the PS bioaccessibility in nanoparticles (70.8%) was significantly higher than that of unencapsulated PS (18.2%). PSNP size increased to 199.1 nm after lyophilization, resulting in a bimodal distribution. Encapsulated PS solubility in water was 2.12 mg/mL, which was 155 times higher than that of unencapsulated PS.

In nanofibers, the in vitro release of stigmasterol was evaluated by the dialysis bag diffusion technique (Mousavi et al. 2021). After mimicking the stomach and small intestine environment, higher stigmasterol release occurred in nanofibers without chitosan than in those prepared with chitosan:gelatin (25:75) (96% vs. 46%). These values corresponded to 10% and 71% of the initial loaded content, respectively.

Regarding NLCs, the release behavior of microcapsules developed for PS co-encapsulation with *Echium* oil was evaluated in gastrointestinal fluids (Comunian et al. 2017). The authors observed a higher core release in the gastric fluid when sinapic acid was used as cross-linking of the complex coacervation process, while no clear trend could be established for intestinal fluid. In another study on complex coacervation with SPI and pectin, a high resistance to gastric acid conditions was established for PS-loaded microcapsules (PS release of 7.6%), while a release of approximately 70% was achieved after 7 h in simulated intestinal fluid (Li et al. 2021b). The limited release of bioactive compounds observed in the gastric conditions could be related to the pH value of the coacervation formation, which was very close to that of gastric fluid (2.5 vs. 2.0); in contrast, at pH 8 (intestinal conditions), thermostatic attraction between SPI and pectin disappeared, thus causing the release of the core material. As mentioned in the previous section, impregnation of PS in wheat starch aerogels was presented as a suitable method to reduce their crystallinity (Ubeyitogullari and Ciftci 2016). This resulted in a consequent increase in their solubility in both water (37-fold) and gastrointestinal fluids (14.5% and 5.5% for gastric and intestinal fluids, respectively). PS nanometric size and amorphous form achieved by their incorporation into the aerogel network are key factors for increasing PS solubility. According to the authors, the higher solubility and release of PS in an acidic medium (gastrointestinal fluid) compared to those detected at neutral pH conditions (intestinal fluid) were due to more intense starch hydrolysis and thus to a higher hydrolysis of the aerogel matrix. PS bioaccessibility obtained after three-step gastrointestinal digestion was also improved by PS impregnation into corn or wheat starch aerogels (20-fold higher than non-impregnated PS) (Ubeyitogullari et al. 2019). Although higher impregnation capacities were achieved with corn starch formulations, PS were less bioaccessible compared to wheat starch aerogels (14% vs. 28%); in fact, wheat starch hydrolysis was higher than that of corn starch during gastrointestinal digestion, thus improving PS release from aerogel nanopores and increasing their bioaccessibility. In food matrices, an

improvement in PS bioaccessibility by the impregnation process was also observed with respect to the nonencapsulated ingredient (by threefold in granola bars with different fat content formulations and by eightfold in pudding) (Ubeyitogullari and Ciftci 2019). The authors also found a clear effect of the food matrix on PS bioaccessibility, since all granola bar formulations presented the highest bioaccessibility values (53–92% vs. 19%). Most likely, the higher content of proteins and emulsifiers with respect to those of the pudding formulation improved PS solubilization during in vitro digestion in granola bars. In dark chocolate enriched with PS-loaded microcapsules obtained by spray drying, much lower PS bioaccessibility was obtained for both gastric and intestinal phases (<13%) (Tolve et al. 2018b).

20.5 Conclusions and Future Perspectives for Industrial PS Encapsulation

The design of new delivery systems for the encapsulation of PS is an emerging concern due to the increasing market demand for PS-enriched food products. As detailed in this chapter, most studies related to encapsulated PS are focused on the effects of diverse wall materials or system compositions on the parameters that determine the characteristics (particle size, shape, physical state, interfacial properties, and charge), functionality and physicochemical stability of the particles obtained. However, studies dealing with the application of encapsulated PS into food products are scarce compared to food models.

Beyond the use of food-grade ingredients and processing methods that ensure consumer safety, other factors should be considered in future developments when selecting the most suitable PS delivery systems to be applied in the food industry:

- The production process must be optimized to achieve commercial viability by using cost-effective systems or methodologies with reliable large-scale production. In this sense, more in-depth research on the use of innovative food-grade encapsulants is of interest to reduce production costs (McClements 2015).
- Studies on the compatibility of encapsulation systems with the food matrix are necessary to confirm the absence of adverse effects that may be detrimental to the stability/properties of the final product (processes such as aggregation or sedimentation) or to consumer acceptability (appearance, mouthfeel) (McClements 2015; Tolve et al. 2020).
- Interaction with the food ingredients, as well as the manufacturing, processing and storage processes applied to the final product, may affect the stability of the PS-loaded particles. Therefore, the stability of both the encapsulated PS and the delivery system, before and once added into the food format, must be clearly defined (Mohammadi et al. 2020; Zhang et al. 2022).
- Apart from the advantages and disadvantages of the different encapsulation systems and the nature of the core-wall material composition, one key aspect is the selection of the most suitable encapsulation method according to the food application. For this reason, it is necessary to determine their specific effectiveness for each food matrix proposed for PS enrichment (McClements 2018).
- To date, most studies have used general parameters to characterize and assess the physicochemical stability of the encapsulation systems without dealing with the actual oxidative stability of the encapsulated PS. Since PS are considered beneficial for their effects on human health, it is necessary to guarantee that their biological activities are preserved after encapsulation and digestion, so it is highly advisable that, in future studies, the presence of the sterol oxidation products is determined.
- Once the optimal encapsulation system for the defined application has been achieved, the controlled release of PS in the gastrointestinal tract should be ensured, and its biological functionality as a hypocholesterolemic compound should be analyzed (by both in vitro

and in vivo methods) (McClements and Xiao 2012). In addition, microbiota modulation (at the colonic level) by encapsulated PS contained in the food matrix is another field to be studied.

– Safety aspects of PS delivery systems should also be investigated more in depth. Due to their small particle size and high surface area/volume ratio, nanoparticles may be able to cross certain biological barriers, thus leading to possible toxicological effects. In this sense, *in vitro* cell culture models could be employed to test the interactions with the novel developed delivery systems. In addition, more research on long-term safety and possible interactions with human cells should be considered (da Silva Santos et al. 2019).

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In Vitro Evaluation of the Effects of 7-Ketocholesterol and 7β-Hydroxycholesterol on the Peroxisomal Status: Prevention of Peroxisomal Damages and Concept of Pexotherapy

Imen Ghzaiel, Samah Maaloul, Mohamed Ksila, Amira Namsi, Aline Yammine, Meriam Debbabi, Asma Badreddine, Wiem Meddeb, Vivien Pires, Thomas Nury, Franck Ménétrier, Laure Avoscan, Amira Zarrouk, Mauhamad Baarine, Olfa Masmoudi-Kouki, Taoufik Ghrairi, Raoudha Abdellaoui, Boubker Nasser, Sonia Hammami, Mohamed Hammami, Mohammad Samadi, Anne Vejux, and Gérard Lizard

I. Ghzaiel
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Faculty of Medicine, Laboratory 'Nutrition, Functional Food and Vascular Health' (LR12ES05), University of Monastir, Monastir, Tunisia

S. Maaloul · R. Abdellaoui
Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms (LR16IRA03), Arid Regions Institute, University of Gabes, Medenine, Tunisia

M. Ksila
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Neurophysiology, Cellular Physiopathology and Valorisation of Biomolecules (LR18ES03), Department of Biology, Faculty of Sciences, University Tunis El Manar, Tunis, Tunisia

A. Namsi · A. Yammine · M. Debbabi · W. Meddeb · V. Pires · T. Nury · M. Baarine · A. Vejux · G. Lizard (✉)
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France
e-mail: gerard.lizard@u-bourgogne.fr

A. Badreddine
Bio-PeroxIL Laboratory, EA7270, University of Bourgogne & Inserm, Dijon, France

Laboratory of Biochemistry, Neuroscience, Natural Resources and Environment, Faculty of Science and Technology, University Hassan I, Settat, Morocco

F. Ménétrier
Centre des Sciences du Goût et de l'Alimentation, AgroSup Dijon, CNRS, INRAE, Université Bourgogne Franche-Comté, Dijon, France

L. Avoscan
Agroécologie, AgroSup Dijon, CNRS, INRAE, University Bourgogne Franche-Comté, Plateforme DimaCell, Dijon, France

A. Zarrouk
Faculty of Medicine, Laboratory 'Nutrition, Functional Food and Vascular Health' (LR12ES05), University of Monastir, Monastir, Tunisia

Faculty of Medicine, University of Sousse, Laboratory of Biochemistry, Sousse, Tunisia

O. Masmoudi-Kouki · T. Ghrairi
Laboratory of Neurophysiology, Cellular Physiopathology and Valorisation of Biomolecules (LR18ES03), Department of Biology, Faculty of Sciences, University Tunis El Manar, Tunis, Tunisia

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Abstract

7-Ketocholesterol and 7β-hydroxycholesterol are most often derived from the autoxidation of cholesterol. Their quantities are often increased in the body fluids and/or diseased organs of patients with age-related diseases such as cardiovascular diseases, Alzheimer's disease, age-related macular degeneration, and sarcopenia which are frequently associated with a rupture of RedOx homeostasis leading to a high oxidative stress contributing to cell and tissue damages. On murine cells from the central nervous system (158N oligodendrocytes, microglial BV-2 cells, and neuronal N2a cells) as well as on C2C12 murine myoblasts, these two oxysterols can induce a mode of cell death which is associated with qualitative, quantitative, and functional modifications of the peroxisome. These changes can be revealed by fluorescence microscopy (apotome, confocal microscopy), transmission electron microscopy, flow cytometry, quantitative reverse transcription polymerase chain reaction (RT-qPCR), and gas chromatography-coupled with mass spectrometry (GC-MS). Noteworthy, several natural molecules, including ω3 fatty acids, polyphenols, and α-tocopherol, as well as several Mediterranean oils [argan and olive oils, Milk-thistle (*Sylibum marianum*) and *Pistacia* lenticus seed oils], have cytoprotective properties and attenuate 7-ketocholesterol- and 7β-hydroxycholesterol-induced peroxisomal modifications. These observations led to the concept of pexotherapy.

B. Nasser
Laboratory of Biochemistry, Neuroscience, Natural Resources and Environment, Faculty of Science and Technology, University Hassan I, Settat, Morocco

S. Hammami · M. Hammami
Faculty of Medicine, Laboratory 'Nutrition, Functional Food and Vascular Health' (LR12ES05), University of Monastir, Monastir, Tunisia

M. Samadi
LCPMC-A2, ICPM, Department of Chemistry, University Lorraine, Metz Technopôle, Metz, France

Keywords

7-Ketocholesterol · 7β-Hydroxycholesterol · Oxysterol · Oxiapoptophagy · Peroxisome · Pexotherapy

Abbreviations

7KC	7-Ketocholesterol
7β-OHC	7β-Hydroxycholesterol
Abcd transporter	ATP-binding cassette sub-type D transporter
Acox1	Acyl-CoA oxidase 1
AMD	Age-related macular degeneration
AO	Argan oil
DHA	Docosahexaenoic acid
DHAP-AT	Dihydroxyacetone-phosphate acyltransferase
ELOVL	Fatty acid elongase
GC-MS	Gas chromatography-coupled with mass spectrometry
GPx	Glutathione peroxidase
Mfp2	Peroxisomal multifunctional protein-2
PLSO	*Pistacia* lenticus seed oil
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
VLCFA	Very-long chain fatty acid

21.1 Introduction

Oxysterols are bioactive lipids that result from the oxidation of cholesterol, which can be formed either by auto-oxidation or enzymatically, or by both processes (Mutemberezi et al. 2016). They are involved in numerous diseases, in particular, those linked to age-related diseases, due to their increase or decrease (Zarrouk et al. 2014; Testa et al. 2018; De Medina et al. 2022). The biological activities of oxysterols, which are constituents of the oxysterome (set of oxysterols present at a given time) (Guillemot-Legris and

Muccioli 2022), are therefore the resultant of the oxysterols simultaneously present. However, this aspect does not exclude the study of their highly variable individual biological activities over a wide range of concentrations. 7-Ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-OHC), mainly formed by cholesterol auto-oxidation (Anderson et al. 2020; Ghzaiel et al. 2022b), were among the first oxysterols studied because of their well-established involvement in cardiovascular diseases (Vejux et al. 2008; Vejux and Lizard 2009). These two oxysterols are indeed present in increased quantities in oxidized LDL (oxLDL) and in atheromatous lesions (Samadi et al. 2021). Their oxidative and inflammatory activities as well as their capacity to induce cell death by apoptosis in the cells of the vascular wall (endothelial cells, smooth muscle cells and macrophages) widely contribute to the development of the atheromatous plaque with often a fatal issue. At the moment, histological analogies have been highlighted between the appearance of atheromatous lesions and the drusen (localized between the Bruch membrane and the basement membrane of retinal pigment epithelial cells), which contain high 7KC levels, and which are identified in patients with age-related macular degeneration (AMD) (Malvitte et al. 2006). The high level of 7KC in drusen suggests an involvement of this oxysterol in the development of AMD (Pariente et al. 2019). Furthermore, in advanced Alzheimer's disease, enhanced levels of 7KC and 7β-OHC have also been observed in plasma as well as post-mortem in brain lesions (Testa et al. 2016). In addition, increases in 7KC and 7β-OHC have been found in the plasma of sarcopenic patients aged over 65 years (Ghzaiel et al. 2021). It is important to note that during lipid peroxidation, cholesterol oxidation occurs chronologically after fatty acid oxidation (Noguchi et al. 1998). Therefore, increased levels of 7KC and 7β-OHC indicate significant oxidative stress. Therefore, the prevention of oxidative stress at the systemic and/or local level in cardiovascular disease, AMD, and sarcopenia seems to be essential to treat these diseases. In this context, the targets on which it is necessary to act are multiple and include: (1) the inhibition of pro-oxidant enzymes such as NADPH oxidase which exist under several isoforms (Pedruzzi et al. 2004); (2) the activation of pro-oxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase] (Nury et al. 2020); and (3) the prevention of mitochondrial and peroxisomal activities, whose dysfunctions participate in the disruption of the RedOx balance (Trompier et al. 2014; Leoni et al. 2017).

Thanks to the use of several cellular models, it is now well demonstrated that 7KC and 7β-OHC act on these different targets by promoting oxidative stress by increasing the overproduction of superoxide anions ($O_2^{\bullet -}$) via NADPH oxidase, by decreasing the efficiency of the antioxidant system and by disturbing mitochondrial and peroxisomal activity (Vejux et al. 2020; Nury et al. 2021a). While the effects of 7KC and 7β-OHC are well established at the mitochondrial level (reduction of glycolysis and the citric acid/Krebs cycle, decrease in oxidative phosphorylation and ATP production, fall in mitochondrial potential ($\Delta\Psi m$), contribution to apoptosis by the release of cytochrome c, overproduction of $O_2^{\bullet -}$, and decrease in the expression and activity of antioxidant enzymes), the effects of these two oxysterols at the peroxisomal level are still little explored and therefore not well known whereas their involvement is suspected in cardiovascular diseases and Alzheimer's disease (Lizard et al. 2012; Zarrouk et al. 2020). The peroxisome is a mostly circular organelle (0.1–1 μM in diameter), devoid of DNA, formed by protein import from the endoplasmic reticulum and closely linked to the mitochondria both topographically and functionally (Schrader and Fahimi 2008; Lismont et al. 2015). Indeed, many of the membrane transporters of the peroxisome are ABCD transporters (ATP-binding cassette sub-type D) and require ATP to function (Kemp et al. 2011; Morita and Imanaka 2012). The peroxisome is involved in the β-oxidation of VLCFA and branched fatty acids, in the synthesis of docosahexaenoic acid (DHA; C22:6 n-3) and plasmalogens, and in the synthesis of cholesterol (Wanders and Waterham 2006a; Kawaguchi and Morita 2016; Charles et al. 2020). Plasmalogens

have a major role in the regulation of inflammation, oxidative stress and cell death, and their involvement in aging and some age-related diseases is widely documented (Hossain et al. 2020, 2023). The peroxisome is also involved in phagocytosis, cytokine production, degradation of eicosanoids, and regulation of the RedOx balance (Fransen et al. 2012; Lismont et al. 2015; Di Cara et al. 2019).

Using 7KC and 7β-OHC on different cell lines (158N murine oligodendrocytes, murine neuronal N2a cells, murine microglial BV-2 cells, and murine myoblasts C2C12 cells), we established an experimental approach to determine the effects of these oxysterols on the peroxisome by using several criteria. The effects on peroxisomal topography and morphology were addressed by transmission electron microscopy and fluorescence microscopy (conventional, apotome, confocal); for the latter approach, the peroxisomes were revealed by indirect immunofluorescence with an anti-Abcd3 antibody (Debbabi et al. 2017a). The effects on the amount of peroxisome per cell were determined by flow cytometry (Nury et al. 2018; Ghzaiel et al. 2022a) by measuring the expression of the peroxisomal transporter Abcd3, which is a transporter of pristanic acid, dicarboxylic acid, and bile acid intermediates, and which is also considered as a suitable marker of the number of peroxisomes per cell and/or of the peroxisomal mass (Gray et al. 2014; Tawbeh et al. 2021). The effects on peroxisomal function were addressed (1) on the one hand by measuring by RT-qPCR the expression of the genes of peroxisomal transporter (Abcd1, Abcd2, Abcd3) and enzymes (Acox1), peroxisomal multifunctional protein-2 (Mfp2) involved in peroxisomal β-oxidation (Wanders 2014) as well as in plasmalogens synthesis (dihydroxyacetonephosphate acyltransferase (DHAP-AT), alkyl-DHAP synthase) (Wanders and Waterham 2006b; Kanzawa et al. 2012; Honsho and Fujiki 2023), and (2) on the other hand by quantifying by gas chromatography-mass spectrometry (GC-MS) the amount per cell of VLCFAs (C24:0; C24:1; C26:0, C26:1) metabolized in the peroxisome (Wanders and Waterham 2006a) as well as the rate of cellular plasmalogens, whose first two enzymes (DHAP-AT, alkyl-DHAP synthase) involved in their synthesis, are located in the peroxisomal membrane (Brites et al. 2004; Nury et al. 2018). These different approaches have also made it possible to identify several molecules (synthetic and natural, as well as oils often of Mediterranean origin) that attenuate the cytotoxicity of 7KC and 7β-OHC while opposing qualitative, quantitative, and functional peroxisomal modifications (Debbabi et al. 2016, 2017b; Badreddine et al. 2017; Ghzaiel et al. 2022a). This attenuation of peroxisomal dysfunctions by different synthetic or natural molecules has given rise to the notion of pexotherapy.

21.2 Evaluation of the Effects of 7-Ketocholesterol and 7β-Hydroxycholesterol on the Peroxisomal Status

21.2.1 Effects of 7-Ketocholesterol and 7β-Hydroxycholesterol on the Peroxisomal Topography and Morphology

In the experimental strategy developed to evaluate the impact of molecules on the peroxisome, different microscopic techniques can be used. These techniques include transmission electron microscopy (TEM) and fluorescence microscopy [conventional fluorescence microscopy, structured fluorescence microscopy (apotome), and confocal microscopy].

The visualization of peroxisomes by TEM requires the use of diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2). In this particular experimental condition, the peroxisomal catalase activity is revealed, and the peroxisomes, which are stained in black, are visualized (Trompier et al. 2014; Ghzaiel et al. 2022a). The livers of 9- to 10-week-old C57 Black/6 male mice were used as a positive control for the detection of peroxisomes by TEM (Fig. 21.1a, b). Without DAB and H_2O_2, the peroxisomes are not detected. This experimental condition does not affect the detection of other cell components and permits the identification of all organelles. The

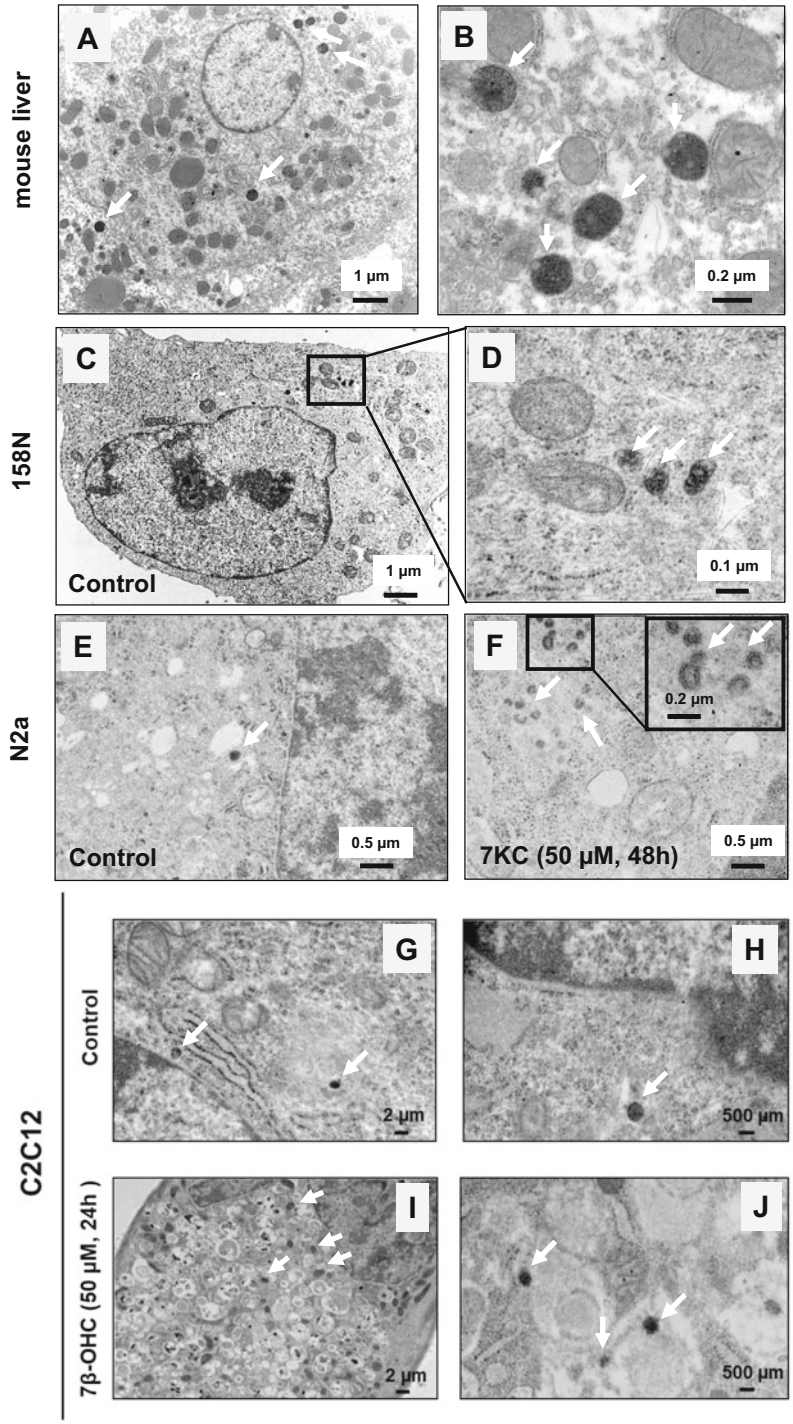

Fig. 21.1 Visualization of peroxisome by transmission electron microscopy on mouse liver, nerve cells (158N oligodendrocytes, murine neuronal N2a cells), and murine C2C12 myoblasts cultured with or without 7-ketocholesterol- or 7β-hydroxycholesterol. White arrows point toward peroxisomes observed in different cell types. The livers of 9- to 10-week-old C57 Black/6 male mice were used as positive control for the detection of peroxisomes (Trompier et al. 2014; Nury et al. 2018, 2020). (**a, b**) Peroxisome in mouse liver; (**c, d**) in 158N oligodendrocytes, some peroxisomes closely located to mitochondria were identified (**d**); (**e, f**) peroxisomes in N2a cells; comparatively to untreated cells (control) (**e**), the morphological aspects of peroxisomes were modified in 7KC (50 µM, 48 h)-treated cells (**f**); in C2C12 murine myoblasts round and regular peroxisomes were observed in the cytoplasm of untreated cells (control) (**g, h**); in 7β-OHC-treated cells most of the peroxisomes were located in vacuoles (**i, j**)

visualization of peroxisomes by TEM gives information on the morphological aspect and size of the peroxisomes, and also information on the peroxisomal topography: distribution in the cytoplasm, interaction with other organelles such as mitochondria and endoplasmic reticulum, localization or not in vacuoles (this latter aspect provides ultrastructural information on pexophagy) and oxiapoptophagy (Nury et al. 2021b). On murine nerve cells (158N, BV-2, and N2a) or on murine myoblasts (C2C12) treated with 7KC or 7β-OHC, similar ultrastructural changes were observed at oxysterol concentrations inducing cell death (oxiapoptophagy on 158N, BV-2, and N2a cells; caspase-independent mode of cell death on C2C12 cells) (Nury et al. 2020) (Fig. 21.1c, j). In those conditions, comparatively to untreated cells (control), morphologically altered peroxisomes were often observed under treatment with 7KC or 7β-OHC (Nury et al. 2018) (Fig. 21.1e, f), and their cytoplasmic distribution was often modified such as in 7β-OHC-treated C2C12 cells: in these cells several peroxisomes were present in vacuoles (Ghzaiel et al. 2022a) (Fig. 21.1, g–j).

Conventional fluorescence microscopy, realized on a right or inverted microscope, can also be used to reveal the peroxisomes detected by immunofluorescence with an antibody raised against the peroxisomal transporter Abcd3 or catalase (Trompier et al. 2014) or other peroxisomal proteins (Acox1, Mfp2) (Baarine et al. 2009). This approach makes it possible to estimate the effect of molecules, such as oxysterols, on the topography of peroxisomes and their quantity per cell. However, this approach is approximative and needs to be completed either by observations in structured fluorescence microscopy (apotome) or in confocal microscopy, whose excellent resolution in z-makes it possible to apprehend the peroxisomal distribution in different planes for further 3D reconstruction and reliable quantifications. These last two methods also make it possible to evaluate mitochondria and peroxisomes simultaneously under excellent conditions (Nury et al. 2018; Namsi et al. 2019). Data obtained on untreated (control) as well as on 7KC- and 7β-OHC-treated C2C12 murine myoblasts by structured fluorescence microscopy (apotome), after mitochondria staining with Mito Tracker Red and detection of peroxisome with an antibody raised against Abcd3 and revealed with Alexa-488, are shown in Fig. 21.2: topographic modifications of peroxisomes and mitochondria are clearly observed. This approach with an apotome is also well appropriated to simultaneously evaluate the mitochondria and the peroxisomes in neurites (axones and dendrites) (Namsi et al. 2019). Thus, on differentiated N2a cells obtained under treatment with octadecaneuropeptide (ODN, 10^{-14} M), the peroxisomes and the mitochondria stained in green and red, respectively, can be observed in the neurites in structured fluorescence microscopy and when these two organelles were closely located a yellow fluorescence was observed (Fig. 21.2). By confocal microscopy, observation in the *x*-*y* plan can be coupled with observations along the *z*-axis, allowing a 3D reconstruction to precise the distribution of peroxisomes in the cells; this approach has been successfully used on 158N cells (Fig. 21.3). Since catalase is localized both in the cytoplasm and in the peroxisome, it is less used than Abcd3 (specifically present at the peroxisomal membrane level) to visualize the peroxisome (Baarine et al. 2009; Trompier et al. 2014). Data obtained on human neuronal cells (SK-N-BE) by confocal microscopy show a cytoplasmic distribution pattern of catalase which evocates the distribution of Abcd3 (Fig. 21.3). TEM, structured fluorescence microscopy (apotome) and confocal microscopy are well adapted complementary methods to study the morphology of peroxisomes and their topography as well as their interaction with other organelles, especially mitochondria.

21.2.2 Effects of 7-Ketocholesterol and 7β-Hydroxycholesterol on the Peroxisomal Mass

The effects of 7KC and 7β-OHC, in a concentration range 12.5–50 μM, are rather well described on the endoplasmic reticulum, the lysosomes, and

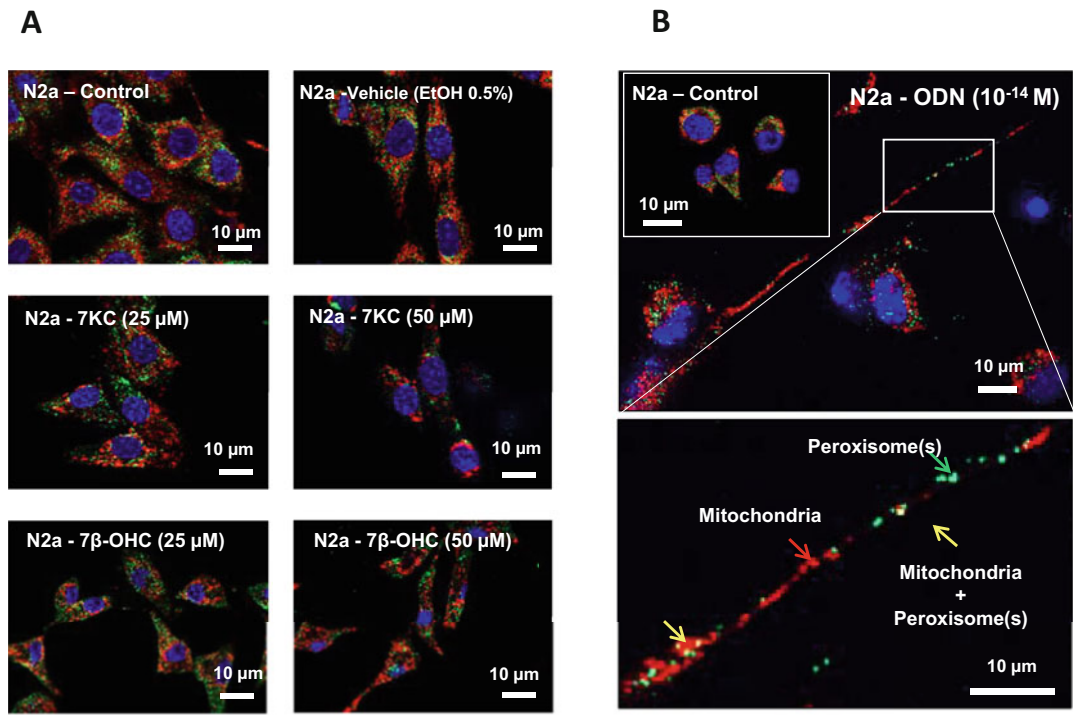

Fig. 21.2 Simultaneous visualization of peroxisomes and mitochondria by illumination microscopy (Apotome) on undifferentiated cells cultured with or without 7-ketocholesterol- or 7β-hydroxycholesterol and on differentiated N2a cells: mitochondria were revealed by Mito Tracker Red and peroxisome by indirect immunofluorescence with antibodies raised against Abcd3. The fluorescence procedure was performed as follows on N2a cells as previously described (Debbabi et al. 2017a; Namsi et al. 2019). After mitochondria staining with Mito Tracker Red, the peroxisomes were revealed with a rabbit polyclonal antibody raised against Abcd3 (Ref: 1152365; Pierce/Thermo Fisher Scientific) which was revealed with a goat anti-rabbit 488-Alexa antibody (Santa Cruz Biotechnology, Santa Cruz, USA). The nuclei were stained with Hoechst 33342 (2 µg/mL). Cells were mounted in Dako fluorescent medium. Cells were stored in the dark at +4 °C until examination with structured illumination microscopy (Apotome 3 imaging system, Zeiss, Jena, Germany). Green dots: peroxisomes; red dots: mitochondria. (**a**): undifferentiated N2a cells cultured with 7-ketocholesterol (7KC: 25–50 µM) for 24 h or 7β-hydroxycholesterol (7β-OHC: 25–50 µM) for 24 h; (**b**): differentiated N2a cells; N2a were previously cultured for 24 h in conventional culture medium; the cells were further cultured for 48 h in medium without FBS in the absence (control) or presence of octadecaneuropeptide (octadecaneuropeptide (ODN); 10^{-14} M) as previously described. Along the neurites, several mitochondria (red fluorescence) and peroxisomes (green fluorescence) were detected. Yellow spots (colocalization of mitochondria and peroxisomes) were also identified. Green arrows point toward peroxisomes; red arrows point toward mitochondria; yellow arrows point toward colocalized peroxisomes and mitochondria. The images were realized with ZEN imaging software (Zeiss)

the mitochondria (Nury et al. 2021a). Morphological and functional alterations of these organelles were reported in the presence of these two oxysterols. On the other hand, the effects of these molecules on the peroxisome are still poorly known. To quantify the effects of 7KC and 7 β-OHC on the peroxisomal mass, the peroxisomes were detected on different cell types by indirect immunofluorescence with a rabbit polyclonal antibody raised against Abcd3 (Debbabi et al. 2017a). Quantification was performed by flow cytometry. Under these conditions, a decrease in peroxisomal mass was observed; this decrease was strongly counteracted in the presence of α-tocopherol (200–400 µM) whatever the cell type considered (Vejux et al. 2020). Data shown are those obtained on C2C12 murine myoblasts: untreated cells (control) and

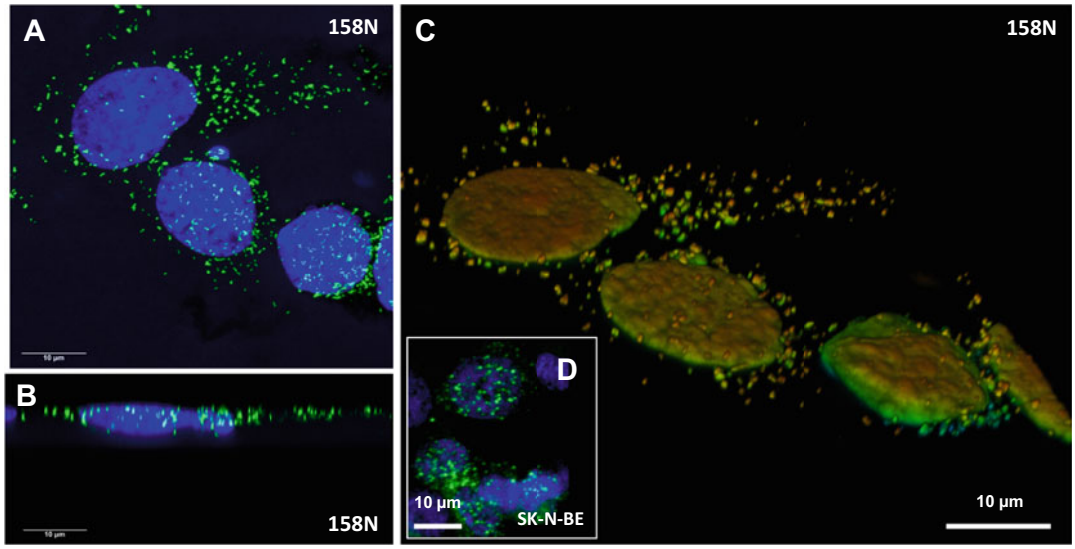

Fig. 21.3 Simultaneous visualization of mitochondria and peroxisomes by confocal microscopy on N2a and SK-N-BE cells. The immunofluorescence procedure was performed on murine N2a and human SK-N-BE cells cultured on glass slides as previously described (Trompier et al. 2014; Debbabi et al. 2017a). The peroxisomes were revealed either with a rabbit polyclonal antibody raised against Abcd3 (Ref: 1152365; Pierce/Thermo Fisher Scientific) or a rabbit polyclonal antibody raised against catalase (Ref: ab16771, Abcam, Paris, France) which were revealed with a goat anti-rabbit 488-Alexa antibody (Santa Cruz Biotechnology). The nuclei were stained with Hoechst 33342 (2 µg/mL). Cells were mounted in Dako fluorescent medium and stored in the dark at +4 °C until examination by confocal microscopy (Confocal Laser Scanning Microscope TCS SP8, Leica, Wetzlar, Germany). The images were realized with LASX (Leica)

cells cultured in the presence of 7β-OHC associated or not with α-tocopherol (Fig. 21.4). At the moment, α-tocopherol, which is known to strongly attenuate 7KC- and 7β-OHC-induced cell death on different cell types, also strongly attenuates the decrease in peroxisomal mass measured with the anti-Abcd3 antibody and revealed by a secondary antibody coupled to Alexa-488. Similar results were obtained with 7KC. This observation led to the notion of pexotherapy, which can be defined as the ability of a natural or synthetic molecule to prevent quantitative and qualitative peroxisomal alterations (Ghzaiel et al. 2022a).

21.2.3 Effects of 7-Ketocholesterol and 7β-Hydroxycholesterol on the Peroxisomal Activity

Concerning peroxisome function, peroxisomal damages (alteration of peroxisomal β-oxidation) can favor the accumulation of very-long chain fatty acids (VLCFA; C ≥ 22) (Savary et al. 2012), which can contribute to amplifying cell dysfunctions (Nury et al. 2020). In C2C12 cells, the analysis of the effects of 7β-OHC (50 µM) associated or not with α-tocopherol (400 µM) on VLCFA levels supports cytotoxic effects of 7β-OHC on peroxisomal activity and cytoprotective effects of α-tocopherol at the peroxisomal level. In untreated cells (control) and vehicle (EtOH: 0.1 and 0.5%)-treated cells, no significant differences were found; similar levels of VLCFA (C22:0, C24:0, C26:0) were found (Fig. 21.5a, b). The level of VLCFAs was determined by gas chromatography coupled with mass spectrometry (GC-MS). When C2C12 cells were exposed to 7β-OHC, a significant increase in VLCFAs was detected, and these latter were significantly reduced when 7β-OHC was associated with α-tocopherol (Ghzaiel et al. 2021, 2022a) (Fig. 21.5a, b). However, enhanced elongase activity could also be involved in the increased

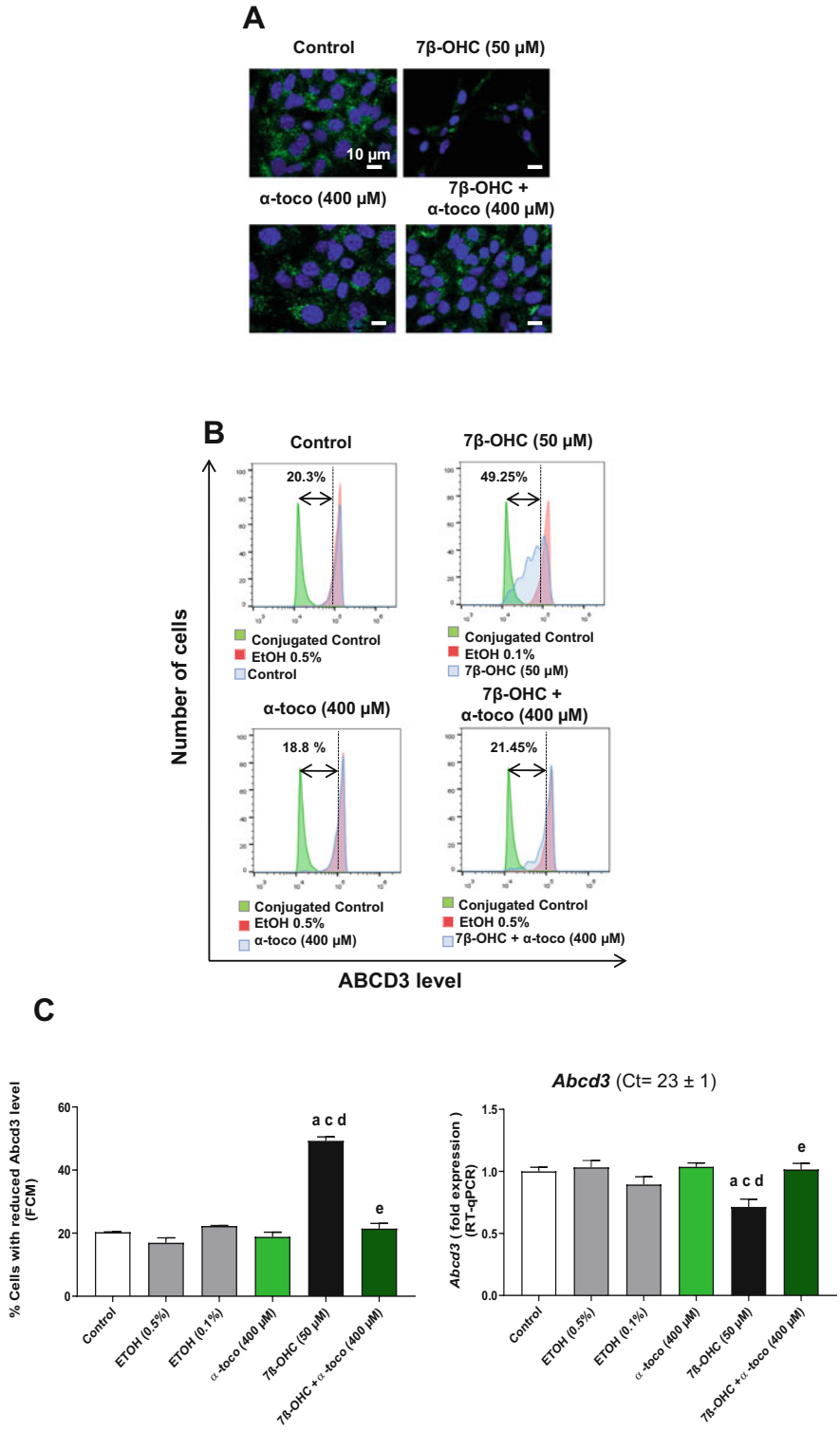

Fig. 21.4 Effect of 7β-hydroxycholesterol on the level of the major peroxisomal membrane transporter (Abcd3) used to evaluate the peroxisomal mass. C2C12 cells were incubated for 24 h with or without 7β-OHC (50 μM) in the presence or absence of α-tocopherol (400 μM) (Ghzaiel et al. 2022a). The protective effect of α-tocopherol (400 μM) against 7β-OHC was analyzed by: (**a**) structured illumination microscopy (apotome); the nuclei were

level of VLCFAs (Jakobsson et al. 2006; Kihara 2012). At the moment, seven enzymes, ELOVL 1–7 (Fatty Acid Elongases 1–7), localized in the endoplasmic reticulum, have been identified. ELOVL1 is considered to control VLCA synthesis up to C26:0, and ELOVL1 is the most potent elongase for C24:0 and C26:0, however, depending on the cell type, similar elongase activity has been reported with ELOVL3 and ELOVL6. The data obtained support an increase in the elongase activity index which could correspond to ELOVL1, 3, and 6 activity; ratio (C24:0/C22:0), and ratio (C26:0/C22:0) under treatment with 7β-OHC; these different elongase activity indexes were also strongly attenuated when 7β-OHC was associated with α-tocopherol (Ghzaiel et al. 2021) (Fig. 21.5d).

21.2.4 Impact of 7KC and 7β-Hydroxycholesterol on the Expression of Peroxisomal Genes Associated with Peroxisomal Biogenesis, Peroxisomal β-Oxidation, and Plasmalogen Synthesis

To address 7KC and 7β-OHC-mediated changes in peroxisomal gene expression, RT-qPCR was used not only to quantify the expression of the peroxisomal *Abcd3* transporter gene but also to measure the expression of other peroxisomal genes such as those associated with peroxisomal biogenesis (*Pex5, Pex13, Pex14*), peroxisomal β-oxidation (*Abcd1, Abcd2, Acox1, Mfp2, Thiolase A*) (Wanders and Waterham 2006a), and the first two steps of plasmalogen synthesis (*DHAP-AT, alkyl-DHAP synthase*) (Brites et al. 2004). Depending on the cell model used (murine nerve cells: 158N, N2a, and BV-2; murine myoblasts C2C12), the expression of some genes is either decreased or not modified under treatment with 7KC and 7β-OHC. Interestingly, when 7KC and/or 7β-OHC led to a reduction in peroxisomal gene expression, the addition of α-tocopherol always normalized this expression, demonstrating the potent cytoprotective effects of α-tocopherol against the peroxisomal toxicity induced by 7KC and 7β-OHC (Badreddine et al. 2017; Nury et al. 2018; Ghzaiel et al. 2022a). These findings underscore the crucial role of α-tocopherol in preventing peroxisomal dysfunction caused by 7KC and 7β-OHC.

21.3 Prevention of 7-Ketocholesterol- and 7β-Hydroxycholesterol-Induced Peroxisomal Changes: Interest of Nutrients (ω3 Fatty Acids, Polyphenols) and Edible Oils (Argan and Olive Oils, Milk-Thistle (Sylibum Marianum) and *Pistacia* Lenticus Seed Oils)

Among the nutrients that oppose the toxicity of 7KC and 7β-OHC, α-tocopherol has shown efficacy on many cell types of different species. This tocopherol, which is a major component of Vitamin E, is constituted of four tocopherols (α-,

Fig. 21.4 (continued) stained with Hoechst 33342 (2 μg/mL); (**b**) flow cytometry (FCM). (**c**): the percentages of C2C12 cells with reduced Abcd3 levels were determined by FCM, and *Abcd3* gene expression was quantified by RT-qPCR; the data are presented as the mean ± SD of two independent experiments performed in triplicate. A multiple comparative analysis between the groups, taking into account the interactions, was carried out using an ANOVA test followed by a Tukey's test. A *p*-value less than 0.05 was considered statistically significant. The statistically significant differences between the groups, which are indicated by different letters, take into account the vehicle used. (*a*): comparison versus control; (*b*): comparison versus ethanol (ETOH: 0.5%); (*c*): comparison versus ETOH (0.1%); (*d*): comparison versus α-tocopherol (α-toco: 400 μM); (*e*): comparison versus 7β-OHC (50 μM). No significant differences were observed between the untreated (control) and vehicle-treated cells. Ct cycle threshold

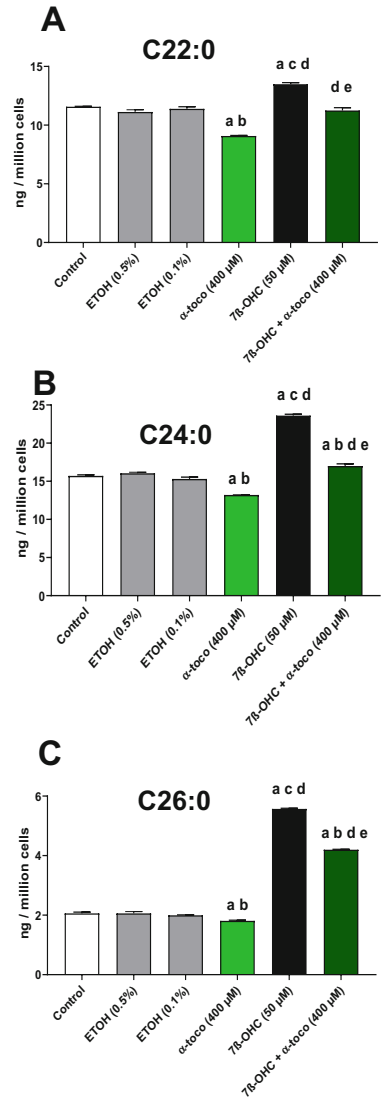

Fig. 21.5 Effect of 7β-hydroxycholesterol with and without on very-long chain fatty acid (VLCFA) levels. C2C12 cells were incubated for 24 h with or without 7β-OHC (50 µM) in the presence or absence of α-tocopherol (400 µM). The level of VLCFA ($C \geq 22$) was determined by GC-MS: C22:0 (**a**), C24:0 (**b**), C26:0 (**c**) (Ghzaiel et al. 2021). Data are the mean ± SD of two independent experiments. A multiple comparative analysis between the groups, taking into account the interactions, was carried out using an ANOVA test followed by a Tukey's test. A *p*-value less than 0.05 was considered statistically significant. The statistically significant differences between the groups, which are indicated by different letters, take into account the vehicle used. (*a*): comparison versus control; (*b*): comparison versus ethanol (ETOH: 0.5%); (*c*): comparison versus ETOH (0.1%); (*d*): comparison versus α-tocopherol (α-toco: 400 µM); (*e*): comparison versus 7β-OHC (50 µM). No significant differences were observed between the untreated (control) and vehicle-treated cells

D

	Elongase activity index	
	C24:0 / C22:0	**C26:0 / C22:0**
Control	1,36 ± 0,01	0,18 ± 0,00
EtOH 0.5%	1,44 ± 0,01	0,19 ± 0,00
EtOH 0.1%	1,34 ± 0,03	0,18 ± 0,00
α-toco (400 µM)	1,45 ± 0,00	0,20 ± 0,00
7β-OHC (50 µM)	1,75 ± 0,00 a c d	0,41 ± 0,00 a c d
7β-OHC + α-toco (400 µM)	1,51 ± 0,01 a e	0,37 ± 0,01 a b d e

β-, γ-, and δ-tocopherol) and four tocotrienols (α-, β-, γ-, and δ-tocotrienol) (Rimbach et al. 2002), is particularly opposed to topographical, morphological and functional changes in peroxisomes induced by these two oxysterols and can be considered as the leader in pexotherapy (Nury et al. 2021a; Ghzaiel et al. 2022a).

However, other nutrients (oleic acid, polyphenols) as well as several oils, mostly of Mediterranean origin (argan and olive oils, Milk-thistle, and *Pistacia lentiscus* seed oils), also strongly attenuate the toxicity of 7KC and 7β-OHC as well as the associated peroxisomal modifications (Yammine et al. 2020; Rezig et al. 2022).

- Thus, oleic acid (C18:1 n-9/C18:1 cis-9), also prevents 7KC-induced oxidative stress and cell death (such as oxiapoptophagy) on 158N, N2a, and BV-2 cells. On BV-2 cells, oleic acid as well as α-and γ-tocopherol were able to prevent the decrease in Abcd3 protein levels, which allows the measurement of peroxisomal mass, and in mRNA levels of Abcd1 and Abcd2, which encode for two transporters involved in peroxisomal β-oxidation (Debbabi et al. 2016). It is suggested that oleic acid could contribute to the inactivation of 7KC by esterification. Indeed, on U937 cells treated with 7KC-oleate, comparatively to 7KC, no cytotoxic effect was observed (Monier et al. 2003). Similar observations were realized when C2C12 murine myoblasts were treated either with 7KC-oleate or 7β-OHC-oleate (Ghzaiel I., PhD Thesis, Univ. Bourgogne, 2022).
- Among polyphenols, known for their health benefits, quercetin (QCT), trans-resveratrol (RSV), and apigenin (API) also prevented peroxisomal dysfunction in 7KC-treated N2a cells (Yammine et al. 2020). These three polyphenols prevented the impact of 7KC by counteracting the decrease in ATP-binding cassette subfamily D member (Abcd3) at the protein and mRNA levels, as well as the decreased expression of genes associated with peroxisomal biogenesis (*Pex13*, *Pex14*) and peroxisomal β-oxidation (*Abcd1*, *Acox1*, *Mfp2*, *Thiolase A*). 7KC-induced decrease in Abcd1 and Mfp2, two proteins involved in peroxisomal β-oxidation, was also attenuated by RSV, QCT, and API.
- As Milk-thistle seed oil (MTSO) contains high amounts of α-tocopherol, oleic acid as well as low amounts of polyphenols (Meddeb et al. 2017), this led us to evaluate its cytoprotective activities on 7KC- and 7β-OHC-treated cells. On 158N cells, MTSO opposes oxidative stress and cell death induced by 7KC and 7β-OHC (Badreddine et al. 2020; Zarrouk et al. 2019). On C2C12 murine myoblasts, in the presence of 7β-OHC, comparatively, to untreated cells, important quantitative and qualitative peroxisomal modifications were identified: (a) a reduced number of peroxisomes with abnormal sizes and shapes, mainly localized in cytoplasmic vacuoles, were observed; (b) the peroxisomal mass was decreased as indicated by lower protein and mRNA levels of the peroxisomal Abcd3 transporter; (c) lower mRNA level of Pex5 involved in peroxisomal biogenesis as well as higher mRNA levels of Pex13 and Pex14, involved in peroxisomal biogenesis and/or pexophagy, was found; (d) lower levels of Acox1 and Mfp2 enzymes, implicated in peroxisomal β-oxidation, were detected; (e) higher levels of very-long chain fatty acids, which are substrates of peroxisomal β-oxidation, were found. These different cytotoxic effects were strongly attenuated by MTSO, in the same range of order as with α-tocopherol (Ghzaiel et al. 2022a). The cytoprotective results obtained with MTSO prompted us to evaluate the cytoprotective activities of other oils used in the Mediterranean diet.
- Olive oil also highly attenuates the toxicity of 7KC (oxiapoptophagy, oxidative stress) on 158N cells (Badreddine et al. 2017; Zarrouk et al. 2019).
- With argan oil (AO), important cytoprotective effects were also observed on 158N. Under treatment with 7KC, AO significantly attenuates loss of cell adhesion, cell growth

inhibition, increased plasma membrane permeability, mitochondrial and lysosomal dysfunction, as well as oxiapoptophagy induction (Badreddine et al. 2017). Marked effects on the peroxisome were also observed: thus, argan oil significantly counteracts the decreased expression of *Abcd1* and *Abcd3* observed under treatment with 7KC (Badreddine et al. 2017). Based on data obtained on BV-2 cells, it is suggested that Schottenol and Spinasterol, two major phytosterols of AO and cactus seed oil (El-Mostafa et al. 2014; El Kharrassi et al. 2014), could protect cells from oxidative stress and of its harmful consequences for peroxisomal functions (Essadek et al. 2023).

- With *Pistacia* lenticus seed oil (PLSO), on C2C12 murine myoblasts, the cytotoxic effects of 7β-OHC were also strongly reduced (Ghzaiel et al. 2021). Thus, at the peroxisomal level, PLSO strongly attenuates (1) the topographical and morphological changes revealed by illumination microscopy (apotome) and TEM, (2) the decrease of peroxisomal mass revealed by lower levels of Abcd3 protein and mRNA measured by flow cytometry and RT-qPCR, and (3) the decrease of peroxisomal β-oxidation revealed by an intracellular accumulation of C24:0 and C26:0 quantified by GC-MS.

21.4 Conclusions

Both 7KC and 7β-OHC modify the topography, mass, structure, and activity of peroxisomes on several cell types. However, the detrimental effects can be attenuated by several nutrients and Mediterranean oils. This has led to the development of pexotherapy, where natural and synthetic molecules, as well as specific oils, are used to prevent peroxisomal damage. The techniques that have been developed for studying peroxisomal status can be applied in both experimental and clinical contexts, providing a better understanding of peroxisomes, which are still poorly understood in both physiological and pathological contexts.

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Phytosterols: Potential Therapeutic Effects and Challenges in Food Industry

22

Leila Rezig and Yosser Zina Abdelkrim

Abstract

Increases in serum total and low-density lipoprotein (LDL) cholesterol are known as hypercholesterolemia, and it is a significant risk factor for the emergence of cardiovascular illnesses. Any action strategy for lowering serum cholesterol is supported by lifestyle changes. Phytosterols are organic substances from the triterpene family. Phytosterols can lower serum LDL cholesterol levels because of their structural resemblance to cholesterol. Phytosterols are used to enrich or fortify a broad spectrum of food products. Phytosterols are quickly oxidized, just like cholesterol and unsaturated fatty acids. The utilization of free phytosterols for the manufacture of functional meals is highlighted in this chapter, which also focuses on the therapeutic effects of phytosterols and their technological concerns in the industrial field.

Keywords

Phytosterols · Bioavailability · Therapeutic effects · Human health · Fortification in food matrices

Abbreviations

BOPP	Biaxially oriented polypropylene
CONT	Control chocolates
GRAS	Generally recognized as safe
LDL	Low-density lipoprotein
MDA	Malondialdehyde
PHAN	Chocolate with phytosterols and antioxidants
PHYT	Chocolate with phytosterols
POPs	Phytosterols oxidation products
PS	Phytosterols
PV	Peroxide value

22.1 Introduction

Plant sterols, often known as phytosterols, are organic substances from the triterpene family. Phytosterols have similar physicochemical properties as cholesterols. The difference is that the C-24 of phytosterols contains methyl or ethyl

L. Rezig (✉)
University of Carthage, National Institute of Applied Sciences and Technology, LR11ES24, LIP-MB 'Laboratory of Protein Engineering and Bioactive Molecules', Tunis, Tunisia

High Institute of Food Industries, University of Carthage, Tunis, Tunisia

Y. Z. Abdelkrim
Laboratory of Molecular Epidemiology and Experimental Pathology (LR11IPT04/LR16IPT04)/Laboratory of Epidemiology and Ecology of Parasites, Institut Pasteur de Tunis - University Tunis El Manar, Tunis, Tunisia

Process Engineering Department, Institut Supérieur des Études Technologiques de Bizerte, Direction Générale des Études Technologiques, Tunis, Tunisia

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groups. More than 250 phytosterols differ with the substitution on the side chains of C-4 and C-24, the degree of unsaturation of the side chain and ring, and the combination of alcoholic hydroxyl groups at the C-3 position with other compounds. Brassicasterol, Campesterol, Stigmasterol, and β-sitosterol are the main chemical structures among them with four ring structures and C-17 side chains (Li et al. 2022). The absence of the double bond in phytostanols, which are phytosterols in their saturated form, makes them distinct from them. Fatty acids derived from vegetable oils can be used to esterify both phytosterols and stanols. The resultant esters can be added to various processed foods since they are liquid or semi-liquid materials with characteristics that are similar to those of edible fats and oils in terms of both chemical and physical properties. While phytosterols play significant metabolic roles in plant cells, cholesterol is a crucial component of animal cell membranes. These substances play a crucial part in controlling the fluidity and permeability of membranes. Phytosterols are also precursors of brassinosteroids and are implicated in embryogenesis (Merah and Mouloungui 2019). However, they are entirely obtained from diet and cannot be generated endogenously in humans. These molecules are biosynthesized by a series of 30 enzyme-catalyzed processes. The mevalonate pathway and the sterol-specific enzymatic pathways might be considered the two main stages of the whole synthesis process. Squalene, which is produced from acetyl-CoA through a sequence of processes, is the major precursor.

This compound changes into 2,3-oxidosqualene, which then changes into cycloartenol. Then a series of oxidative and demethylation events, including the isomerization of the double bond and methylation at C24, start. Brassicasterol, stigmasterol, campesterol, stigmasterol, campestanol, and sitostanol are the primary phytosterols. The structures of cholesterol, β-sitosterol, stigmasterol, and campesterol are shown in Fig. 22.1 (Rezig et al. 2022). Low-density lipoprotein (LDL) cholesterol levels are important because phytosterols work to lower them. A daily intake of 2–3 g of phytosterols can reduce LDL cholesterol by 10–15% (Racette et al. 2010). Additionally, phytosterols are involved in the prevention of colon, breast, and prostate cancer as well as having anti-inflammatory, anti-diabetic, and anticarcinogenic properties. Because of their positive effects on human health, phytosterols are frequently employed as functional food components (Jesch and Carr 2017). They are regarded as GRAS (generally recognized as safe). The term "functional foods" is relatively recent, having only been developed in the last century. It was first used in Japan in the middle of the 1980s to describe food items that had been enriched or fortified with natural ingredients that had a particular physiological preventive or health-promoting effect (Hasler 1998). The creation of foods enriched with phytosterols is crucial since the amount of phytosterols naturally found in food does not meet the amount that is required to be consumed to see a decrease in LDL blood cholesterol. However, the phytosterols' incorporation into food products is challenging because of their susceptibility to oxidation, water insolubility, and chalky taste. Against this background, the purpose of this chapter was to provide an overview about the bioavailability of phytosterols, their therapeutic effects, and the use of free phytosterols for the formulation of functional foods, taking into account technological issues in the food industry field.

22.2 Bioavailability of Phytosterols

It is crucial to know the pharmacokinetics and to evaluate the bioavailability of phytosterols in order to study the positive effects of these compounds on human health. For example, the absorption efficiency in the body of sitosterol and campesterol is about 0.51–1.9% (Borel 2003). Many factors affect the bioavailability of phytosterols as well as intestinal transporters, their different molecular types, and genetic factors. Li et al. summarize the effects of phytosterols on cholesterol absorption and metabolism in terms of its bioavailability (Li et al. 2022). The authors reported that the intestinal absorption rate of phytosterol (less than 5%) is

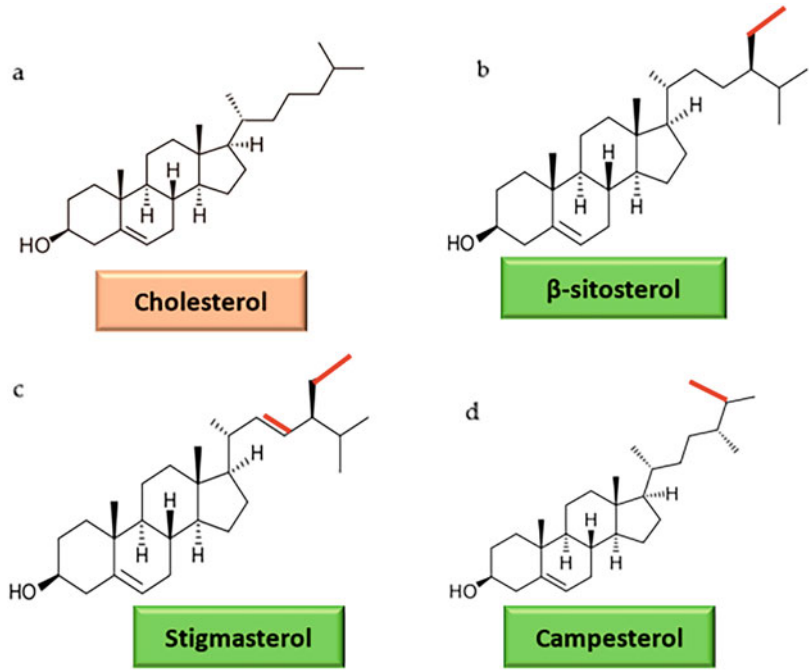

Fig. 22.1 (**a**) Structure of cholesterol, (**b**) structure of β-sitosterol (ethyl group represents in red line), (**c**) structure of stigmasterol (ethyl group and double bond represented by red line), and (**d**) structure of campesterol (methyl group represented by red line) (Rezig et al. 2022).

much less than that of cholesterol (50–60%). In the digestive tract, phytosterols could be present in a bound or a free form: phytosterols bound to the sterol transporter Niemann-pick C1-like 1 located in the intestinal cell membrane in the intestinal lumen, are absorbed by intestinal epithelial cells; free phytosterols are excreted into the intestinal lumen by ATP-binding cassette G5/8 in the intestinal cells. Few phytosterols can avoid this pathway and circulate through lipoproteins. Most of the phytosterol molecules circulate in LDL particles (70–80%). The bioavailability of phytosterols is also influenced by several other proteins and genes which can affect directly or indirectly their transport across intestinal cells (e.g., genetic mutations in NPC1L1 and apolipoprotein E (ApoE) 3/4) (Li et al. 2022).

22.3 Therapeutic Effects of Phytosterols

Phytosterols have been intensively investigated for the treatment and management of diabetes, parasitic diseases (e.g., Leishmaniases), numerous cancers, cardiovascular disorders, and atherosclerosis (Majid Shah et al. 2019; Mandlik Ingawale and Namdeo 2021; Makhmudova et al. 2021; Sharmila and Sindhu 2017; Babu and Jayaraman 2020; Jones et al. 2018). In this section, we will discuss different examples of therapeutic potential effects of plant sterols.

22.3.1 Anti-*Leishmania* Effects

Leishmaniases are a series of neglected tropical diseases caused by parasitic protozoa from more than 20 different species of *Leishmania*. The development of anti-*Leishmania* molecules constitutes an urgent need to fight the disease. Different drug isolation sources and drug targeting strategies are used. Naturally occurring plants offer a potential source for screening of anti-leishmanial activities, considering that many of the bioactive compounds from plants constitute part of active principles of medicaments. Majid Salah et al. evaluated the anti-leishmanial potential of β-sitosterol, isolated from *Ifloga spicata Sch. Bip.*, against *Leishmania tropica*

promastigotes using biological experiments and docking and molecular insights against leishmanolysin (GP63) and trypanothione reductase (TR) receptors. β-sitosterol exhibited an IC50 value of 9.2 μg/ml, and binding scores of 6.966 and −7.5659 with tested targets, respectively. The study confirmed that β-sitosterol has anti-leishmanial potential; however, further studies are still needed for natural anti-leishmanials compounds against *Leishmania* (Majid Shah et al. 2019).

Moreover, oxygenated sterol derivatives were synthetized and tested for their anti-leishmanial activities. Three oxygenated sterols compounds showed anti-leishmanial activities: 3-acetoxy cholest-5(6)en-7-ol, cholest-5(6)en-3,7-diol, and cholest-4(5)en3,6-dione. Compounds were found to inhibit the proliferation of *Leishmania donovani* and *Leishmania major* extracellular promastigotes, to inhibit the replication of intracellular amastigotes in macrophages by more than 30% at 0.125 μg/ml (Ghosh et al. 2016). Other studies showed anti-leishmanial activity against *Leishmania* parasites of synthesized oxysterols and nitrogenous sterols. 7-Aminomethylcholesterol (Bazin et al. 2006) and 7-aminocholesterol (Bazin et al. 2006; Abdelkrim et al. 2022) were active on both extracellular promastigotes and intramacrophage amastigotes. The authors suggested a mode of action interacting with sterol metabolism or translation pathway (Bazin et al. 2006, Abdelkrim et al. 2022).

22.3.2 Cholesterol Control and Cardiovascular Disease Prevention

The most common manifestation among cardiovascular diseases, which caused 17.9 million deaths in 2019 according to the World Health Organization (WHO 2021), is coronary heart disease. Important levels of cholesterol blood constitute the major cardiovascular factor of coronary heart disease. It is possible to adjust this factor by lifestyle modifications which include a healthy diet leading to lipid-lowering therapy. Thus, phytosterols (or plant sterols) are progressively used as supplements or functional foods to keep plasma cholesterol concentrations under control because of their cholesterol-lowering efficacy and consequently cardiovascular risk control (Poli et al. 2021). Gylling et al. reported the effect of plant sterols and plant stanols in the management of dyslipidemia and prevention of cardiovascular disease. The authors suggest that plant sterols do not affect cellular cholesterol homeostasis, although further studies are needed to confirm this. Some studies indicate mild anti-inflammatory effects of phytosterols/stanols, but confirmation at physiological concentrations of plant sterols, which are not toxic, is needed (Gylling et al. 2014).

In vitro and in vivo studies, and clinical trials about the effect of phytosterols in the reduction of cellular cholesterol levels were reviewed by Makhmudova et al. (2021). Incubation assays of HepG2 and CaCo-2 cell lines in vitro with phytosterols reduced cellular cholesterol levels (Ho and Pal 2005; Fahy et al. 2004). The sitosterol induced the necrotic death of mice-derived macrophages which is supposed to lead to plaque necrosis, plaque rupture, and eventually to cardiovascular events (Bao et al. 2006). In another study, Genser et al. conducted a meta-analysis of epidemiological studies which did not support an association between moderate fluctuations of serum concentrations of plant sterols particularly sitosterol and campesterol, and cardiovascular disease risk (Genser et al. 2012). Further investigations need to be conducted to highlight the controversy about the correlation between cardiovascular diseases and phytosterol consumption.

22.3.3 Anticancer Effects

Many studies have investigated the association between dietary phytosterols and cancer risk, but the results have been contradictory. Jiang et al. support the hypothesis that high phytosterol intake is inversely related to risk of cancer. They conducted a meta-analysis of literature to study the relative risk for the highest versus the lowest

intake for total phytosterols, β-sitosterol, campesterol, stigmasterol, β-sitostanol, and campestanol. The analysis demonstrated a linear association for campesterol and a nonlinear association for total phytosterol intake in a dose-response analysis (Jiang et al. 2019).

A case control study conducted by Huang et al. demonstrated that dietary phytosterol intake, β-sitosterol, campesterol, and campestanol is inversely associated with colorectal cancer risk in a Chinese population. It was found to be associated with a 50% reduction in colorectal cancer risk; an inverse association was found between the consumption of β-sitosterol, campesterol, campestanol, and colorectal cancer risk. However, stigmasterol intake was related to an increased risk of colorectal cancer. No statistically significant association was found between β-sitostanol and colorectal cancer risk (Huang et al. 2017).

The antigenotoxic and anticancer role of β-sitosterol against renal carcinogen was evaluated through an experimental and in silico study, which concluded that β-sitosterol has a strong potential against genotoxic as well as suppress neoplastic transformation in experimental renal cancer (Sharmila and Sindhu 2017).

Further studies with prospective designs to investigate the important anticancer effects of dietary phytosterols are required.

22.4 Enrichment of Phytosterols in Food Matrices in Food Industry

More and more individuals are seeing the connection between diet and diseases including coronary artery disease, diabetes, obesity, and even some types of cancer. Additionally, there is a rise in the number of elderly people actively managing their health issues by monitoring their meals. It is also preferred to "prevent" rather than "treat"; hence, authorities and healthcare organizations are under significant pressure to reduce hospitalization and healthcare expenditures. For instance, eating foods enriched with phytosterols may enable the UK to save $150 million annually on healthcare expenses (Gray et al. 2003). Problems with technological solutions that are both commercially and economically viable are crucial to the development of successful functional items. In this aspect, a company's growth and market share depend on its ability to differentiate its products.

For that purpose, and as an initial phase, it is important to confirm which laws in the country where production and marketing must occur regulate the newly invented products and whether the addition of specific bioactive components in a given food matrix is permitted. From a technological standpoint, it is necessary to: Assess the interaction between the functional compound and food matrix; choose the amount of compound to be added; assess the potential impact on the product or on the compound of the various operations to which the product will be subjected; and validate the final product from a chemical, physical, and sensorial point of view, as well as conduct in vivo tests to demonstrate that the product currently has positive effects on human health. In fact, there are situations when adding any bioactive molecule to a food matrix is not feasible because the compound may be incompatible, produce strange smells or scents, or significantly alter the product's organoleptic properties. The chemicals may also be subject to the effects of gastric acids in the gastrointestinal tract or they may be harmed during the manufacturing process. The bioavailability of these substances is one of the elements that must constantly be taken into account. To put it in different terms, how much of this molecule does the body truly absorb (Tolve et al. 2020)?

A recent research conducted by Vaghini et al. (2016) demonstrated that the type of matrix in which phytosterols are incorporated affects how bioavailable they are. To determine how much of this substance transferred from the food matrix to the mixed micelles in the intestinal chyme, the digestion process was simulated. Greater incorporation into the mixed micelles and consequently greater displacement of cholesterol from those micelles result from a higher bioavailability percentage. According to Alvarez-Sala et al. (2016), other substances used as ingredients in food formulation may affect the solubility and

bioavailability of phytosterols. It was also noted that the type of food matrix (such as its nutritional composition, presence of additives or other bioactive compounds, etc.), the number of portions consumed daily, and the time of ingestion all had an impact on the effectiveness of phytosterols on the reduction of LDL cholesterol levels. It was found that some foods—like milk and yoghurt—allow for a higher reduction in LDL cholesterol and that eating them is most effective at mealtimes rather than upon awakening on an empty stomach in the morning. It was also discovered that the presence of other bioactive substances may enhance the effects of phytosterols.

It is important to point out that Finland was the pioneer in 1995 in the production of the first phytosterols-fortified food, the Benecol margarine, as a food enriched with plant stanol fatty acid esters. Since then, a wide variety of different functional foods enhanced with phytosterols have been created and are currently offered everywhere (Moreau 2015). These bioactive components can be added to a variety of matrices, but the most popular ones include dairy products, spread, bread goods, meat products, as well as low-lipid matrices like orange juice (Demonty et al. 2009).

Olfa et al. (2019) studied the effects of supplementation of yoghurt with phytosterols (1.6%) and/or lactulose (6%) on its quality during refrigerated storage. In this context, the physicochemical, rheological and sensorial properties, starter culture contents, and oxidative stability of the fortified yoghurt samples were determined and compared with control yoghurt. Phytosterols used in this study consisted of β-sitosterol, stigmasterol, and campesterol at ratio of (~80%), (~12%), and (~8%), respectively. pH values of all yoghurts decreased during 28 days of storage period at 4 °C. On the other hand, Dornic acidity increased for all prepared yoghurts, over the storage period. The reduction of pH values and increase in Dornic acidities were due to the growth of acid-forming bacteria which produced lactic acid during the storage period (Oshadi et al. 2015; Amal et al. 2016). It was worth pointing out that in phytosterols yoghurt, acidity and pH variations were the lowest over the storage period; followed by yoghurt with mixed lactulose and phytosterols. These results were in agreement with those of Izadi et al. (2015) who reported that titrable acidity values were lower in yoghurt enriched with phytosterols (1.4%); whereas, when milk fat content varied, acidity and pH values are not affected (Helal et al. 2018). These findings may be due to the addition of oil in water emulsion in enriched yoghurt which induces differences within the food environment influencing the metabolism of microorganisms during storage. In terms of syneresis, the syneresis levels increased in all samples over the storage period. In fact, the evolution of pH and Dornic acidity could change global charge of casein, it becomes negative, hence, capillary water is kept between casein molecules. Moreover, it is noteworthy that syneresis value of phytosterols yoghurt ($58.92 \pm 0.03\%$) after 28 days was significantly lower ($p < 0.05$), than control yoghurt ($60.11 \pm 0.01\%$). Such a result could be due to the interactions between fat and protein network (Izadi et al. 2015; Lucey and Singh 1997); wherein, phytosterols made copolymer with casein to enhance the gel of yoghurt and reduce the syneresis levels leading to an improvement of the yoghurt quality. Besides, differences in counts of both *Streptococcus thermophilus* and *Lactobacillus bulgaricus* during yoghurt storage, with and without added phytosterols, at the same periods were not significant ($p > 0.05$). Therefore, yoghurt bacteria growth was unaffected by phytosterols. It was owing to microbial metabolism; mainly, the capacity of some lactic acid bacteria to assimilate cholesterol (Lye et al. 2010; Monu et al. 2008). The evolution of lipid oxidation in fortified yoghurts with phytosterols during refrigerated storage was also monitored.

In this context, peroxide and thiobarbituric acid reactive substance values were used to investigate either primary or secondary oxidation of phytosterols. Olfa et al. (2019) found that the initial peroxide value (PV) of yoghurt sample enriched with phytosterols was about 0.61 ± 0.01 meq O_2/kg of fat. This value increased significantly ($p < 0.05$) during storage at 4 °C to reach 4.47 ± 0.01 meq O_2/kg. In regard to the secondary products of phytosterols'

secondary oxidation, malondialdehyde (MDA) production increased with storage time to reach 0.87 ± 0.02 mg MDA/kg. Such a feature was also observed in several previous studies conducted on yoghurt. In fact, Semeniuc et al. (2016) and Citta et al. (2017) reported that in all types of yoghurts tested, MDA formation increased during storage time shelf life. According to Serra et al. (2008), lipid peroxidation depends on pH, temperature, material of packaging, and milk quality.

Dark chocolate containing phytosterol esters was also developed by Botelho et al. (2014) to reduce cholesterol in individuals. For that purpose, Phytosterols derived from vegetal oils esterified with canola oil fatty acids were used. The phytosterol mixture contained 46 g/100 g b-sitosterol, 26 g/100 g campesterol, 17 g/100 g stigmasterol, and 11 g/100 g of other minor phytosterols. Cocoa powder, cocoa liquor, palm oil, polydextrose, rice protein, cocoa butter, erythritol, maltitol, hazelnut paste, soy lecithin, polyglycerol polyricinoleate, nut flavor, sucralose, and nut flavor were used to produce control chocolates (CONT). Phytosterols esters were utilized in place of palm oil in the PHYT (chocolate with phytosterols) and PHAN (chocolate with phytosterols and antioxidants) formulations to produce the filler. Ascorbic acid and α-tocopherol were also included in the filling composition of the PHAN chocolates (0.90 mg/100 g of chocolate). An industrial pilot plant produced one batch of Belgian pralines. All of the fats were first weighed and added to the mixer to melt at 45 °C. Following the addition of the dried components, the melted fats were conched by a runner mill at 60 °C/6 h to encourage the evaporation of the water and unfavorable tastes. To reach an average particle size of 23 μm, the mixture was refined at 40–55 °C. All samples were manually heated to a temperature of 29 °C on a cold marble surface. To receive the filling, the chocolate was molded in plastic molds that were 14 cm in length and 13 mm in height. After cooling, a thin layer of chocolate was poured into the mold, followed by 15 g of filling. To prevent the detrimental temperature effect on lipid oxidation throughout the coaching and tempering process, phytosterols and antioxidants were added to the filling. A second, thin layer of chocolate was added to cover the filled chocolate when the filling had cooled to room temperature. Each bar (weighing 30 g) therefore contained 15 g of shell and 15 g of filler. The filled chocolates were finally demolded, placed in a metallic 60 mm BOPP (biaxially oriented polypropylene) commercial pack under normal atmospheric conditions, and kept between 20 °C and 30 °C for 5 months. Samples were solely utilized to assess oxidative stability when stored at 30 °C. Every month, samples were collected, and all parameters were examined.

After 60 days at 20 °C and after 30 days at 30 °C, a hydroperoxide production peak was seen. PS-enriched samples had greater hydroperoxide values than control samples, which could be attributable to the PHYT's higher concentration of alpha-linolenic acid. After 90 days of storage, all chocolate bars became lighter and softer. These physical modifications did not affect their sensory acceptance, either. Additionally, PS bioactivity was preserved during storage because up to 5 months, no appreciable changes in the PS esters were seen. After 90 days of storage, all chocolate bars became lighter and softer. These physical modifications did not affect their sensory acceptance, either. Additionally, PS bioactivity was preserved during storage because up to 5 months, no appreciable changes in the PS esters were seen. However, considerable PS oxidation took place in the PHYT bars; the main phytosterol oxidation products (POPs) were sitostanetriol, 6-ketositosterol, 6β-hydroxycampesterol, and 7-ketocampesterol. The POPs/PS ratio was low (0.001).

Therefore, the dark chocolate bars developed for this study maintained their potential utility after 5 months of room temperature storage, offering an alternative as a functional food.

In terms of the stability of phytosterols in foods, Rudzińska et al. (2014) studied the evolution of the oxidation phenomena in margarines enriched in phytosterols/phytostanols. Margarine samples were purchased in local stores in Poland and stored on open Petri dishes in a 0.5 cm layer at 4 and 20 °C for up to 18 weeks. Analyzed margarines contained four phytosterols:

brassicasterol, campesterol, sitosterol, avenasterol, and two phytostanols: sitostanol and campestanol. The content of phytosterols and phytostanols in margarines changed from 79 mg/g in a control sample to 63 mg/g and 55 mg/g in samples stored for 18 weeks at 4 °C and 20 °C, respectively. At the end of storage, contents of sitostanol decreased by 23% and 30%, while the amounts of oxidized sterols increased by 35% and 100%, respectively, for both temperatures. Hydroxy derivatives dominated among all oxidized phytosterols and their content increased threefold at the end of storage. Additionally, the storage temperature had a big impact on POP production: Phytosterols oxidized almost 1.5 times faster at 20 °C than they did at 4 °C for refrigeration. Therefore, it is critical to consider storage conditions to prevent the development of phytosterols oxidation products (POPs) that are harmful to human health. Different food matrices, such as fruit juices, have been the subject of studies on the stability of phytosterols, such as the one conducted by González-Larena et al. (2012). It should be noted that the type of storage packaging utilized may also have an impact on how stable a food product containing phytosterols is. As shown by Semeniuc et al. (2016), it is possible to alter the packaging to lessen light contact in order to better preserve the qualities of a yoghurt beverage enhanced with phytosterols. In this particular instance, a little plastic container with a triple black layer and an entirely opaque intermediate layer served as the appropriate packing material. Because light contact was avoided and photodegradation processes were stopped, it was able to stop lipid oxidation in this manner. Furthermore, a method that protects the bioactive components added to the food matrices could be employed up to now to guarantee that the phytosterols do not change during the production processes or during their conservation. This process is called microencapsulation.

22.5 Conclusions

The usage of phytosterols in the creation of functional foods is growing as a result of the numerous scientific studies that demonstrate the positive effects of these compounds on human health. In light of this, it is critical to safeguard phytosterols against unfavorable environmental factors (such as moisture, oxidants, light, and temperature) in order to increase their stability and the shelf life of the finished product. It should also be remembered that phytosterols are rapidly oxidized, just like cholesterol and unsaturated fatty acids. Therefore, by developing novel procedures, phytosterols may be encapsulated to improve their applicability in food systems. Considering that numerous research have already recommended and proposed their application, further efforts are needed to enable the possibility of using microencapsulated phytosterols for the formulation of functional foods.

Conflict of Interest The authors declare no conflict of interest.

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