



# The Epigenome in Atherosclerosis

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## Abstract

Emerging evidence suggests the growing importance of “nongenetic factors” in the pathogenesis of atherosclerotic vascular disease. Indeed, the inherited genome determines only part of the risk profile as genomic approaches do not take into account additional layers of biological regulation by “epi”-genetic changes. Epigenetic modifications are defined as plastic chemical changes of DNA/histone complexes which critically affect gene activity without altering the DNA

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sequence. These modifications include DNA methylation, histone posttranslational modifications, and non-coding RNAs and have the ability to modulate gene expression at both transcriptional and posttranscriptional level. Notably, epigenetic signals are mainly induced by environmental factors (i.e., pollution, smoking, noise) and, once acquired, may be transmitted to the offspring. The inheritance of adverse epigenetic changes may lead to premature deregulation of pathways involved in vascular damage and endothelial dysfunction. Here, we describe the emerging role of epigenetic modifications as fine-tuners of gene transcription in atherosclerosis. Specifically, the following aspects are described in detail: (1) discovery and impact of the epigenome in cardiovascular disease, (2) the epigenetic landscape in atherosclerosis; (3) inheritance of epigenetic signals and premature vascular disease; (4) epigenetic control of lipid metabolism, vascular oxidative stress, inflammation, autophagy, and apoptosis; (5) epigenetic biomarkers in patients with atherosclerosis; (6) novel therapeutic strategies to modulate epigenetic marks. Understanding the individual epigenetic profile may pave the way for new approaches to determine cardiovascular risk and to develop personalized therapies to treat atherosclerosis and its complications.

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**Keywords**

Atherosclerotic plaque · Epigenetic therapies · Epigenome · Inflammation · Vascular disease

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## 1 Discovery and Impact of Epigenetics

Although inborn genetic variation can influence disease susceptibility, accumulating evidence supports the notion that cardiovascular diseases (CVD) are heavily affected by non-genetic factors. Indeed, the inherited genome determines only part of the risk profile as genomic approaches do not take into account additional layers of biological regulation by “epi”-genetic changes – defined as acquired modifications to the genome subject to influence by the environment (Handy et al. 2011). The discovery of epigenetic modifications dates back to 1956, when the British developmental biologist Conrad Waddington demonstrated for the first time the inheritance of a characteristic acquired in a population in response to an environmental stimulus (Slack 2002). Waddington’s experimental work showed that embryo fruit flies carrying an identical genetic background displayed different thorax and wing structures when exposed to different temperature or chemical stimuli (Noble 2015). While the term “epigenetics” initially embraced the process by which a fertilized zygote develops into a complex organism, the concept was refined over the next years by the observations that cells sharing the same genetic information can exhibit clear differences in gene expression. Waddington’s work has contributed to understand that heritable traits can associate not only with changes in nucleotide sequence, but also with chemical modifications of DNA or proteins interacting with DNA. Later experiments in 1975 showed that epigenetic changes could be

transmitted to daughter cells, thus regulating gene expression across multiple generations (Gonzalez-Recio et al. 2015). There are several examples outlining the importance of epigenetic processing in health and disease. First, epigenetic regulation of gene expression is a major determinant of cell fate. Epigenetic signals occurring at the levels of DNA and histones have the ability to license regions of the genome while shutting down others, thus favoring specific transcriptional programs which enable the differentiation of genetically identical pluripotent stem cells toward different cell types (i.e. myocytes, endothelial cells, adipocytes) (Brunet and Berger 2014). Along the same line, monozygotic twins who share an identical genetic make-up may display different physical and behavioral traits when raised under different environmental conditions (Fraga et al. 2005). Chronic exposure to different stimuli (i.e., pollution, noise) will enable the induction of specific epigenetic programs eventually leading to different gene expression profiles over the lifetime (Baccarelli and Ghosh 2012). Of note, epigenetic-induced deregulation of longevity genes was shown to accelerate cellular senescence and vascular aging (Sen et al. 2016). On the other hand, specific interventions such as caloric restriction have shown to delay age-dependent onset of diseases mostly via an epigenetic reprogramming. Taken together, epigenetic regulation of gene transcription represents a pivotal mechanism of adaptation to different environmental conditions and is among the most important mechanisms underlying cardiovascular diseases. A deep understanding of the epigenetic machinery may enable the characterization of cell-specific transcriptional programs in patients with atherosclerosis and could offer the tools for personalized therapeutic approaches to prevent cardiovascular diseases. The availability of new technologies for the study of our genome has indeed allowed a deep characterization of chromatin structure and function thus unveiling an array of epigenetic changes at the level of DNA and histones. This important information has enabled to understand inter-individual diversity and has prompted us to strive for an ever greater level of personalization in cardiovascular medicine.

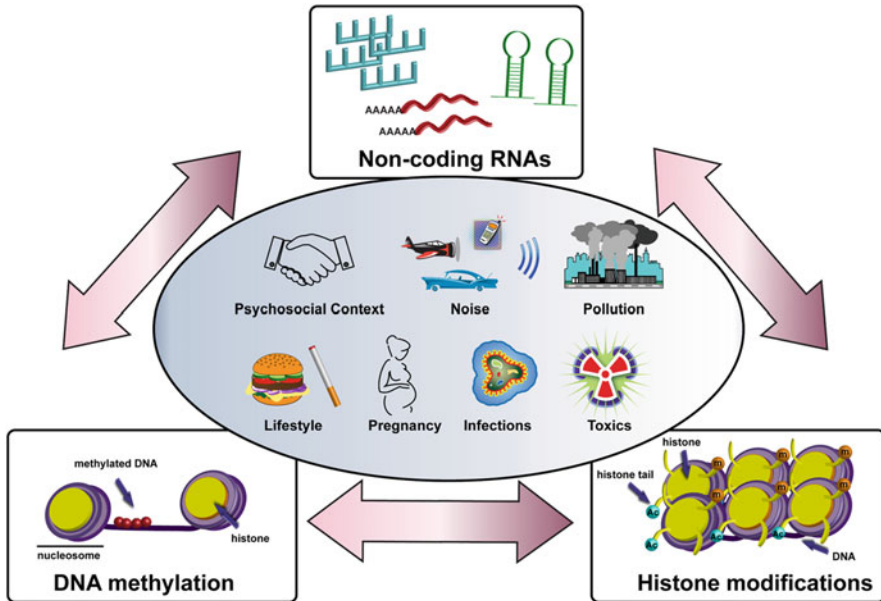
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## 2 Classification of Epigenetic Modifications

Epigenetic modifications fall into three main categories: (1) chemical modifications of DNA (i.e., methylation); (2) posttranslational modifications of histone tails; (3) regulation of gene expression by non-coding RNAs [i.e., microRNAs, PIWI-interacting RNAs, endogenous short interfering RNAs, long non-coding RNAs (lncRNAs)] (Fig. 1). The present chapter will focus on the role of chromatin modifications in atherosclerosis. The function of ncRNAs in this setting is described extensively in a different chapter.

### 2.1 DNA Methylation

Nuclear DNA is tightly wrapped around a core of eight histone proteins (two copies each of H2A, H2B, H3, and H4), generating repetitive units known as nucleosomes.



**Fig. 1** Role of epigenetic inheritance in vascular disease. Environmental factors induce epigenetic changes which are transmitted to the offspring, thus leading to maladaptive transcriptional programs responsible for early vascular damage

These structures enable a very efficient packaging of DNA within the cell nucleus, through the formation of chromatin. Despite its complex three-dimensional structure, chromatin is a very dynamic entity where DNA and histones can be chemically modified. This alters the accessibility of transcriptional factors to genes and, thus, modulates transcription (Goldberg et al. 2007).

DNA methylation plays an important role in the regulation of chromatin structure and gene expression, and therefore, participates in a variety of biological processes (Hamidi et al. 2015), including tissue-specific regulation of gene expression, genomic imprinting, X chromosome inactivation, silencing of transposable elements, and defense against viral sequences (Prasher et al. 2019). This epigenetic modification is relatively stable, it is tissue-specific and can be transmitted to the offspring (Izquierdo and Crujeiras 2019). Methylation of DNA mainly occurs through attachment of methyl group (CH<sub>3</sub>) from S-adenosyl methionine (SAM) to the C5 position in the cytosine-paired-with-guanine (CpG) dinucleotide sequences thus forming 5-methylcytosine (5mC) (Davis and Gallagher 2019). Furthermore, non-CpG methylation (i.e., CHG or CHH region) may also occur and this signature is usually observed in embryonic stem cells (Bernstein et al. 2007).

CpG sequences are generally located into promoter regions of genes, however, they can also be located within gene bodies (Costantino et al. 2015). Promoter methylation is generally associated with transcriptional repression, while gene

body methylation is associated with enhanced transcription (Maunakea et al. 2010). Although both transcriptional repression and activation have been reported, DNA methylation leads – in >95% of cases – to gene silencing (Xiao et al. 2019). Specifically, methylated cytosines are recognized by DNA methyl-binding proteins (MBPs) that prevent the binding of transcription factors to DNA (Prasher et al. 2019). Alternatively, DNA methylation may recruit specific proteins, such as the methyl-CpG binding protein 2 (MeCP2), that specifically bind to methylated regions to foster transcriptional repression (Jones et al. 1998). Besides methylation, other chemical modifications of cytosine have been identified, such as hydroxymethylation, formylation, and carboxylation, but their interrelation with methylation is not completely understood yet (Elia and Condorelli 2019).

DNA methylation is catalyzed by the DNA methyltransferase 1 (DNMT1), 3a (DNMT3a), and 3b (DNMT3b) and is reversed by Tet methylcytosine dioxygenases (TET1, 2, and 3) (Xu et al. 2018). Among these enzymes, Dnmt1 is responsible for the maintenance of methylation patterns in the genome by replicating the hemimethylated CpG sites (Vilkaitis et al. 2005), whereas Dnmt3a/b are considered de novo methyltransferases (Okano et al. 1999). The activity of DNMT1 has been demonstrated to be dependent on a co-factor that recognizes hemi-methylated DNA. The Ubiquitin-like with PHD and Ring Finger Domains 1 (UHRF1), were shown to be essential for maintenance of the methylation status of DNA by directing the recruitment of DNMT1 to the replication forks (Bostick et al. 2007). DNMT3 can also methylate non-CpG sites (at CpA and CHG) and the presence of methylated non-CpG sites in the human genome indicates that there is ongoing de novo DNA methylation at each round of genome replication (Aavik et al. 2019).

DNA demethylation can be achieved by either passive or active mechanisms (Costantino et al. 2015). Passive demethylation can be the result of Dnmt1 inhibition during cell replication (Wolffe et al. 1999) while active demethylation is modulated by DNA demethylases. DNA demethylation may follow two main pathways: the first is dependent on cytosine deamination (AID, APOBEC3G, FTO) while the second is dependent on the oxidation of methylated cytosines (Aavik et al. 2019). The latter reaction is catalyzed by members of the Ten-eleven translocation (TET) proteins family (TET1-3) which convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (Wu and Zhang 2010). TET1 is mostly found in embryonic stem cells, whereas TET2 and TET3 are ubiquitously expressed (Costantino et al. 2015). TET1 is low in normal tissues, whereas TET2 is an abundant protein in most tissues/cell types and TET3 expression is overlapping with TET2 in many tissues (Kohli and Zhang 2013). The observed abundance of TET2 and TET3 enzymes in various tissues indicates that DNA demethylation must have an important role in cellular homeostasis (Aavik et al. 2019). The TET family enzymes represent promising therapeutic targets given their ability to oxidize 5mC thus leading to the reversal of gene repression by DNA methylation (Greco et al. 2016).

## 2.2 Histone Posttranslational Modifications

DNA is packaged into repeating units called nucleosomes by wrapping around multimeric histone proteins. When nucleosomes are organized into tightly packed bundles (heterochromatin), the transcriptional machinery is hampered by a reduction of chromatin accessibility. Conversely, when chromatin is relaxed (euchromatin), DNA is more accessible to transcription factors and gene transcription may occur. Each histone (H) protein is an octamer comprised of 2 sets of H2A, H2B, H3, and H4 proteins with a single histone H1 linker protein between nucleosomes. Each histone subunit has an N-terminal tail containing a lysine (K) residue that protrudes away from the surface of the histone octamer creating an exposed surface (Davis and Gallagher 2019). Histones are amenable to many posttranslational modifications (PTMs), which include methylation, acetylation, ubiquitination, phosphorylation, SUMOylation, GlcNAcylation, carbonylation, and ADP-ribosylation (Zhang et al. 2018). Histone modifications are induced by specific enzymes that act predominantly at histone N-terminal tails primarily involving the amino acids lysine (K) and arginine (R). These modifications can be present on multiple (but specific) sites on histones (Kouzarides 2007).

In addition to DNA modifications, PTMs of histone tails may serve as a repressive or activating signal depending on the specific histone tail, the number of groups added, and the region of chromatin where the modification occurs (Bauer and Martin 2017). Although the biological significance of many PTMs remains to be elucidated, considerable advances have been made in the understanding of lysine acetylation and methylation (Bernstein et al. 2007). The first PTM reported is histone acetylation, an epigenetic signature characterized by the addition of positively charged acetyl groups to amino acid residues on the histones, which neutralizes the negative charges of DNA. This modification reduces the affinity of the histone for DNA and consequently results in the formation of a more relaxed chromatin structure, eventually increasing chromatin accessibility (Nicorescu et al. 2019). Acetylation occurs mainly on lysine residues on histones H3 and H4; this mark mainly associates with activation of transcription by enhancing chromatin accessibility (Gillette and Hill 2015). In this context, bromodomain and extra-terminal proteins recognize histone acetylation marks and initiate the assembly of the transcriptional machinery (Filippakopoulos and Knapp 2014). Acetylation is modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) which are involved in the addition or removal of an acetyl group, respectively (Baccarelli and Ghosh 2012). This modification is driven by recognition and binding of transcription factors able to recruit one of a growing family of HATs, namely CBP/p300, MYST, and GNAT (Kouzarides 2007). Under physiological conditions, lysine residues on histone tails are positively charged and can bind DNA which is a negatively charged molecule due to its phosphate components. The interaction between histone and DNA favors a compact chromatin structure, with scarce accessibility to transcription factors. Conversely, when lysine residues are acetylated they lose their positive charge, thus favoring an open chromatin and, hence, enhanced gene expression (Costantino et al. 2015). HATs catalyze the addition of two-carbon acetyl groups

to lysine residues from acetyl-CoA (Carrozza et al. 2003). On the other hand, removal of acetyl groups from histone residues by HDACs represses gene transcription (Stratton et al. 2019). Specifically, HDACs catalyze removal of acetyl groups from lysine residues and are distributed among four classes (Class I, IIa, IIb, III, and IV) (Haberland et al. 2009).

In contrast to lysine acetylation, a process known to enhance gene expression, histone methylation is a more complex modification which may result in different chromatin states according to the methylated residue and the number of added methyl groups (Cooper and El-Osta 2010). For instance, the lysine residues at positions 4, 9, and 27 on histone H3 can be methylated to different levels (mono-, bi-, and tri-methylation), resulting in different states of chromatin accessibility (Elia and Condorelli 2019). Histone methylation is defined as the transfer of methyl group from S-adenosyl-L-methionine to lysine or arginine residues of histone proteins by histone methyltransferases (HMTs). While lysines may be mono-, di-, or tri-methylated, arginines may be monomethylated or demethylated (symmetrically or asymmetrically) (Bannister and Kouzarides 2011). Histone methyltransferases (HMTs) include the EZH, SETD, PRDM, PRMT, METTL, and MLL enzyme families (Stratton et al. 2019).

Over the last few years, a well-established theory was that histone turnover was the only mechanism regulating histone methylation. More recently, histone demethylases (HDMs) have been reported as a novel mechanism capable of demethylating lysine residues on histone tails (Handy et al. 2011). Indeed, histone methylation is a very dynamic process regulated by histone demethylases, which remove the methyl groups from lysine residues with a high gene specificity (Tsukada et al. 2006). HDMs include members of UTX/Y, JARID1, JMJD, LSD, PHF, and FBXL enzyme families (Stratton et al. 2019).

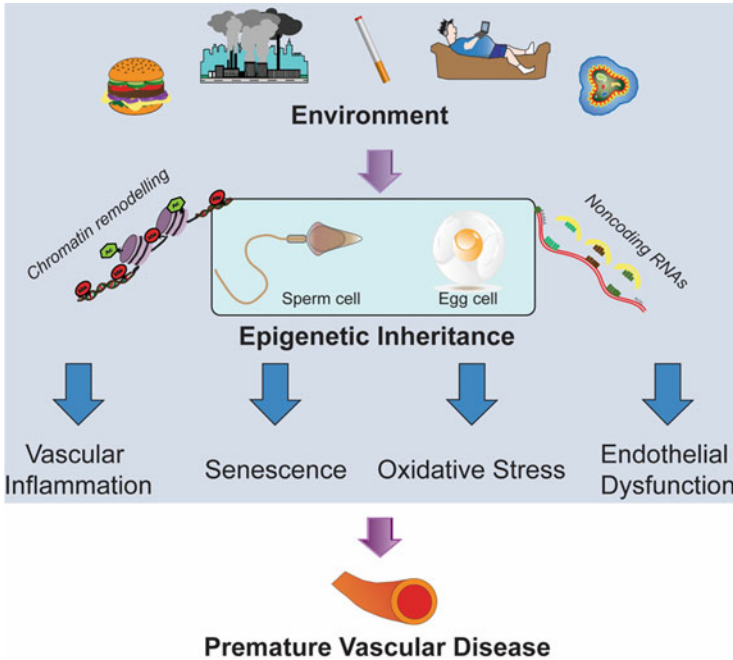
Two important studies have identified the first HDM, lysine demethylase 1 (LSD1) which is able to remove methyl group from H3K4 and H3K9, suggesting that lysine demethylation is subjected to dynamic modulation (Metzger et al. 2005). Several lysine demethylases specific for diverse histone lysine residues have been identified (Costantino et al. 2015). A better comprehension of how such demethylases are regulated during disease states is of paramount importance.

Interestingly, studies have shown that DNA methylase (DNMTs), histone methyltransferase (HMTs), and histone acetyltransferase (HATs) are closely interconnected to regulate chromatin remodelling under specific stimuli (Li et al. 2007). For example, H3K9 methylation induces DNA methylation while CpG methylation favors H3K9m (Cedar and Bergman 2009). Therefore, chromatin modifications may influence each other and can propagate.

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### 3 Epigenetic Inheritance and Vascular Disease

An important aspect to consider when dealing with epigenetics is that epigenetic modifications are heritable. Epigenetic signals acquired during the lifetime are transmitted to the offspring, where they participate to determine substantial changes



**Fig. 2** Epigenetic changes and environmental factors. Exposure to different environmental factors induces several epigenetic modifications. These entail changes of DNA methylation, posttranslational histone modifications, and altered expression of non-coding RNAs. Reproduced with permission from Costantino et al. *Eur Heart J.* 2018;39:4150–4158

in phenotype (Fig. 2) (Heard and Martienssen 2014). In the Överkalix study, conducted in northern Sweden to investigate the physiological effects of various environmental factors on transgenerational epigenetic inheritance, three cohorts born in 1890, 1905, and 1920 were studied until death. The study showed that overeating during a child's slow growth period (SGP), before their prepubertal peak in growth velocity, influences descendants' risk of death from cardiovascular disease and diabetes (Pembrey et al. 2006). If food was not readily available during the father's slow growth period, then cardiovascular disease mortality of the proband was low. By contrast, diabetes mortality increased if the paternal grandfather was exposed to a surfeit of food during his slow growth period (Odds Ratio 4.1, 95% confidence interval 1.33–12.93,  $P = 0.01$ ) (Pembrey et al. 2006). This study was one of the first to demonstrate that a nutrition-linked mechanism through the male line seems to have influenced the risk of developing cardiovascular and metabolic diseases. Periconceptual diet was shown to influence DNA methylation levels with phenotypic consequences. A genome-scale analysis of differential DNA methylation in whole blood after periconceptual exposure to the Dutch Hunger Winter famine revealed changes of differentially methylated regions (Tobi et al. 2014). These changes were preferentially observed at regulatory regions of genes relevant for



metabolic homeostasis, inflammation and longevity, key processes underlying the pathogenesis of atherosclerosis. Specifically, individuals conceived during the Dutch famine showed, six decades later, reduced methylation levels at the promoter of insulin-like growth factor type 2 (IGF-2), a gene regulating of glucose homeostasis, cardiovascular function, and lipid metabolism (Heijmans et al. 2008). Overall, these data suggest that epigenetic modulation of pathways by prenatal malnutrition may promote adverse cardiometabolic phenotypes during childhood. Inheritance of specific epigenetic patterns may contribute to explain the alarming increase in childhood obesity from 32 million in 1990 to around 42 million in 2016 (Brown et al. 2015). Notably, early metabolic alterations in obese children are associated with low-grade inflammation, high oxidative stress levels, impaired nitric oxide bioavailability, endothelial dysfunction, and arterial stiffness (Suglia et al. 2018). Macrophage polarization and adipose tissue inflammation are also important features commonly found in obese children (Singer and Lumeng 2017). Epigenetic modulation of inflammation is supported by the notion that promoter methylation of *TNF- $\alpha$* , pyruvate dehydrogenase kinase 4 (*PDK4*), and leptin (*LEP*) are all reduced in obese as compared to lean children, while methylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1 $\alpha$* ) and proopiomelanocortin (*POMC*) genes are increased (Garcia-Cardona et al. 2014). As a consequence of these alterations, risk of diabetes, dyslipidemia, and silent heart disease are significantly higher in obese as compared to non-obese children (Juonala et al. 2011). Despite emerging evidence indicates the existence of a transgenerational epigenetic inheritance in humans, data still remain inconclusive due to relevant confounders represented by genetic, ecological, and cultural inheritance. Parents and offspring may share the same epigenomic features, but it is challenging to understand whether these features are being transmitted through the germline or are newly established in each generation by the action of shared genes and shared environments (Horsthemke 2018). The most difficult aspect in humans is the demonstration that a specific epigenetic factor in the germ cells is responsible for the phenotypic effect in the next generation. While this phenomenon was clearly described in plants, nematodes, and fruit flies, its occurrence in humans remains controversial. This important aspect of epigenetic regulation is the object of intense investigation and future studies will contribute to clarify the contribution of inheritable epigenetic traits to global vascular risk in humans.

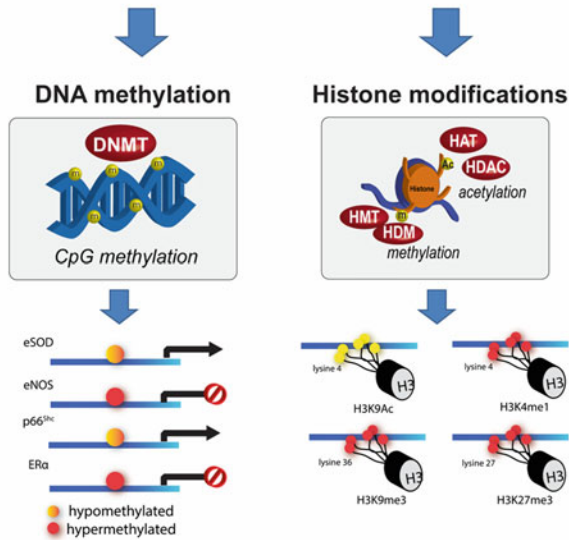
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## 4 Epigenetic Processing in Atherosclerotic Vascular Disease

### 4.1 DNA Methylation

Reduced DNA methylation is an important signature of atherosclerotic lesions both in humans and in experimental models, such as apolipoprotein E knockout mice (*Apoe*<sup>-/-</sup>) and neointima of balloon-denuded New Zealand White (NZW) rabbit aortas (Napoli et al. 2012). Several studies have shown profound changes of atherosclerosis-specific methylated CpGs in human atherosclerotic plaques

## EPIGENETIC LANDSCAPE IN ATHEROSCLEROSIS



**Fig. 3** Alterations of the chromatin landscape in atherosclerosis. *HAT* histone methyltransferase, *HDAC* histone deacetylase, *HMT* histone methyltransferase, *HDM* histone demethylase, *H3* histone 3, *K* lysine residue

(hypomethylation of 3,997 promoter sites = 84%) with a progressive increase in gene methylation as the lesions matured (Aavik et al. 2015). Global hypomethylation of DNA in human aortic lesions was the result of a near-complete demethylation of the subset of CpG islands that were hypermethylated in control aortas (Aavik et al. 2019). Beside global variations in methylation status, alterations in the methylation pattern of specific genes have been causally implicated in the pathogenesis of atherosclerosis. Methylation of genes regulating cellular proliferation, namely genes encoding for estrogen receptor- $\alpha$  (*ER $\alpha$* ) was found in atherosclerotic plaques from human subjects (Fig. 3). Moreover, *ER $\alpha$*  gene was also shown to be methylated in vascular smooth muscle cells (SMCs) in vitro during their phenotypic switch (Aavik et al. 2019). Methylation-dependent increase in proliferation of SMCs may represent an important epigenetic mechanism underpinning atherogenesis. Indeed – under physiological conditions – arterial SMCs are terminally differentiated and replication does not occur. This associates with a low activity of the epigenetic machinery. By contrast, during lesion development, changes in DNA methylation affect transcriptional programs fostering SMCs proliferation. The effects of DNA methylation may be the direct result of gene hypomethylation or can be due to methylation-induced alterations of DNA integrity and function. DNA hypomethylation of 15-lipoxygenase and extracellular superoxide dismutase (*eSOD*) genes were found during SMCs replications (Napoli et al. 2012). Epigenetic remodelling of growth regulatory genes such as platelet-derived growth factor and *c-myc* might also contribute to SMCs proliferation (Hiltunen and Yla-Herttuala

2003). De novo methylation of genes is the most plausible mechanism to explain the dynamic effects of epigenetic processing in atherosclerosis. Intervention studies in cellular models have clearly shown that treatment with the methylation inhibitor 5-azadeoxycytidine heavily affects gene expression in vitro and in vivo (Yang et al. 2010). The importance of de novo methylation is supported by the notion that overexpression of Dnmt, an enzyme which lacks de novo methylation activity, does not reverse genomic hypomethylation in cancer (Zhang and Xu 2017).

The accumulation of reactive oxygen species (ROS) is a key promoter of epigenetic signatures. It has been shown that ROS may foster changes in DNA methylation, and in turn, methylation changes may induce the upregulation of pro-oxidant genes. Reduced methylation of CpG islands at the promoter of the *shc1* gene, encoding for the mitochondrial adaptor p66<sup>Shc</sup>, has been associated with high oxidative stress levels, mitochondrial insufficiency, apoptosis, and endothelial dysfunction (Paneni et al. 2012). Both diabetic mice and humans display reduced DNA methylation of *shc1* promoter in the vasculature and the heart, and this epigenetic signature persists despite intensive glycemic control (Paneni et al. 2012; Costantino et al. 2017). Interestingly, epigenetic reprogramming by selective targeting of *dnmt3b* restores promoter methylation thus repressing p66<sup>Shc</sup> transcription (Costantino et al. 2018a). Other genes implicated in oxidative stress and vascular disease, namely the AP-1 transcription factor JunD and the prolyl-isomerase-1 (Pin1) were also shown to be modulated by specific changes of CpG methylation (Paneni et al. 2013, 2015a). DNA methylation was also reported to affect the expression of inflammatory genes including TNF- $\alpha$ , COX-2, and IL-1 $\beta$  (Stylianou 2019). Genetic deletion of Tet methylcytosine dioxygenase 2 (TET2) in macrophages leads to increased expression of inflammatory cytokines and chemokines in response to native LDL (Peng et al. 2016). A similar increase in plasma interleukin-8 was observed in people with TET2 mutations but not in those without the mutations suggesting that control of DNA methylation by TET2 regulates inflammation in atherosclerosis (Fuster et al. 2017).

Atherosclerosis and ageing are highly interconnected, and age-dependent epigenetic modifications may significantly contribute to vascular disease (Paneni et al. 2017). Aging is generally associated with a progressive decline in global DNA methylation (Hannum et al. 2013). Indeed, DNA methylation content assessed by whole-genome bisulfite is heavily reduced in centenarians as compared with newborns (Heyn et al. 2012). Reduced DNA methylation at the promoter of genes associated with self-renewal was also found in stem cells isolated from aged versus young subjects and associated with functional defects and reduced ability to differentiate (Sun et al. 2014). DNMT3A, TET2, and ASXL1 are key regulators of DNA methylation status and their deregulation is associated with clonal expansion in hematopoiesis and functional defects, a phenomenon known as clonal hematopoiesis of indeterminate potential (CHIP). CHIP was found to be associated with an increased risk of CVD, stroke, coronary calcification, malignancies, and all-cause mortality (Jaiswal et al. 2017). *Dnmt3a* gene was the most recurrently mutated in individuals with CHIP. Hematopoietic tissue-specific conditional *Dnmt3a* deletion in mouse models led to a progressive expansion of long-term HSC with impaired

differentiation by incomplete epigenetic repression of HSC-specific genes (Challen et al. 2011). Hematopoietic tissue-specific Tet2 loss leads to decreased levels of 5hmC, increased stem cell self-renewal, delayed HSC differentiation, and skewed development toward the monocyte/macrophage lineage (Moran-Crusio et al. 2011). Thus, hematopoietic stressors can induce stable epigenetic reprogramming of bone marrow HSCs contributing to clonal hematopoiesis and myeloid skewing. Albeit DNMT3A and TET2 exert opposite functions in terms of DNA methylation, a recent study showed that these chromatin modifiers both repress the erythroid regulator *Klf1* suggesting a model of cooperative inhibition resulting in HSCs transformation (Zhang et al. 2016). Methylation changes at the promoter of eNOS, GATA-2, and GATA-3 during aging were also associated with functional defects and reduced differentiation potential of endothelial progenitor cells (EPCs), whereas reprogramming approaches aimed at restoring promoter methylation of these genes contributed to cell rejuvenation and longevity (Chan et al. 2004). Beside modulating EPCs functionality, DNA methylation directly regulates endothelial cell function mainly via TET2 signaling. TET2 promotes endothelial autophagy and downregulates inflammatory factors such as VCAM1, ICAM1, MCP1, and IL-1 $\beta$  in lesions of high fat fed *ApoE*<sup>-/-</sup> mice (Peng et al. 2016). Moreover, TET2 overexpression decreased the methylation levels of Beclin-1 promoter, thus leading to increased endothelial cell autophagy and decreased inflammatory factors in endothelial cells exposed to oxidized LDL (Li et al. 2015). Epigenetic changes of p66<sup>Shc</sup> and eNOS have also shown to affect endothelial function in vivo as well as in patients with obesity and type 2 diabetes (Costantino et al. 2017, 2019).

## 4.2 Histone Posttranslational Modifications

Growing evidence indicates that posttranslational modifications of histones, mainly at lysine and arginine residues, significantly affect chromatin accessibility thus enabling cell-specific transcriptional programs implicated in the pathophysiology of atherosclerotic vascular disease (Jiang et al. 2018) (Fig. 3). Chromatin modifications play a pivotal role in regulating vascular inflammation by epigenetic modulation of nuclear factor kappa-B (NF- $\kappa$ B) signaling. Several cardiovascular risk factors have shown to modulate histone marks and gene expression. In primary human aortic endothelial cells, hyperglycemia induces a specific mono-methylation of histone 3 at lysine 4 (H3K4m1) thus enabling chromatin accessibility at the level of NF- $\kappa$ B p65 promoter (El-Osta et al. 2008). Hyperglycemia-related H3K4m1 is induced by the mammalian methyltransferase SETD7, which is emerging as a key player in the regulation of vascular inflammation and oxidative stress (Okabe et al. 2012). Several stimuli, including reactive oxygen species are able to increase SETD7 activity. Indeed, overexpression of scavenger enzymes (e.g., uncoupling protein-1) blunted SETD7-dependent H3K4m1 and subsequent activation of NF- $\kappa$ B p65 and downstream inflammatory genes, namely MCP-1, VCAM-1, and ICAM-1 (El-Osta et al. 2008). Of note, SETD7 expression is increased in peripheral blood monocytes from patients with type 2 diabetes, and correlated with endothelial function and

oxidative stress levels, as assessed by flow-mediated dilation (FMD) of the brachial artery and urinary levels of isoPGF<sub>2α</sub>, respectively (Paneni et al. 2015b). Moreover, SETD7 induction positively correlated with the expression of proatherosclerotic genes including COX-2, iNOS, and VCAM-1 (Paneni et al. 2015b). Chromatin immunoprecipitation experiments revealed that SETD7 specifically binds a specific region of the human NF-κB p65 promoter thus fostering the expression of inflammatory genes. Beside SETD7, other chromatin modifiers such as the methyltransferase Suv39h1 and LSD1 have shown their ability to modulate NF-κB-dependent inflammatory genes (IL-6 and MCP-1) by reducing the levels of dimethylation (H3K9me2) and trimethylation of H3 at lysine 9 (H3K9me3) in SMCs (Brasacchio et al. 2009). Suv39h1 gain- and loss-of-function approaches in experimental models of type 2 diabetes showed that H3K9me2 and H3K9me3 can be dynamically modulated thus reverting the SMCs phenotype. Histone acetyltransferases, such as CBP/p300 and p/CAF, were found to be critically involved in the NF-κB signaling and upregulation of TNF-α and COX-2, master regulators of the atherosclerotic process (Deng et al. 2004). Mechanistically, p300 interacts with the p65 (RELA) subunit of NF-κB in the nucleus to displace repressive p50–HDAC1 interactions (Ashburner et al. 2001). This results in increased acetylation of both p65 itself and histones in proximity of NF-κB promoter. The p65–p300 interaction has been found to be critical for the inflammatory activation of both macrophages and endothelial cells (Jiang et al. 2018). The relevance of this epigenetic route is supported by the notion that inhibition of p300 by curcumin enhances macrophage cholesterol efflux in human and mouse macrophages in vitro, and exerts an anti-inflammatory effect by suppressing NF-κB activity (Khyzha et al. 2017). Deregulation of histone deacetylases, namely sirtuins, also represents a major underpinning of vascular disease and atherosclerosis via modulation of oxidative stress, endothelial function, inflammation, mitochondrial bioenergetics, and cellular metabolism (Winnik et al. 2015). SIRT1 activity is reduced in patients with obesity and significantly contributes to endothelial dysfunction (Masi et al. 2020). By contrast, pharmacological activation of SIRT1 by resveratrol restores endothelial function and NO availability and attenuates dyslipidemia and obesity-induced metabolic alterations in human subjects (Carrizzo et al. 2013). Overexpression of SIRT1 in endothelial cells increases their sprouting and migration, whereas SIRT1 depletion impairs angiogenic response, mainly via FLT1 and CXCR4 (Potente et al. 2007). SIRT1 was also found to regulate the transcription of the mitochondrial adaptor p66<sup>Shc</sup>, thus leading to oxidative stress and endothelial dysfunction (Zhou et al. 2011; Mohammed et al. 2020). Furthermore, pharmacological activation of SIRT1 by visfatin has shown to attenuate oxLDL-induced senescence of EPCs through the PI3K/Akt/ERK pathway (Ming et al. 2016). SIRT3 and SIRT6 have also recently emerged as an essential regulator of endothelial cell senescence and angiogenesis, key alterations commonly observed in atherosclerosis (Costantino et al. 2018b). Deregulation of chromatin modifying enzymes SUV39H1, JMJD2C, and SRC-1 in visceral fat arteries from obese patients was associated with increased di-(H3K9me2) and trimethylation (H3K9me3) as well as acetylation (H3K9ac) of histone 3 lysine 9 (H3K9) (Costantino et al. 2019). This epigenetic pattern was found

to promote transcriptional signatures involved in endothelial ROS generation and vascular dysfunction. Interestingly, *in vivo* editing of SUV39H1, JMJD2C, and SRC-1 blunted obesity-related endothelial dysfunction in obese mice (Costantino et al. 2019). Other histone deacetylases have shown to affect the atherosclerotic phenotype, although often with conflicting results. HDAC3, HDAC5, and HDAC7 were shown to repress KLF2 expression in human endothelial cells, while their overexpression fostered a proatherogenic phenotype (Kwon et al. 2014). In mice, HDAC3 and HDAC9 deletion in macrophages favors the upregulation of pro-inflammatory signatures eventually leading to a proatherosclerotic phenotype (Stratton et al. 2019). Consistently, *ApoE*<sup>-/-</sup> mice with endothelial specific deletion of HDAC3 displayed increased neointimal formation, suggesting an atheroprotective role of endothelial HDAC3 (Zampetaki et al. 2010). Along the same line, *Ldlr*<sup>-/-</sup> mice treated with the HDAC inhibitor Trichostatin A exhibited increased plaque size and enhanced macrophage infiltration (Choi et al. 2005). In rats, HDACs are upregulated in aortic SMCs under mitogenic stimulation and their inhibition alters the expression of cell cycle genes, resulting in diminished SMC proliferation, which might be expected to be atheroprotective (Findeisen et al. 2011).

Specific chromatin modifications occurring in macrophages, namely increased activity of the demethylase JMJD3, were also found to act as pivotal regulator of pro-inflammatory cytokines (i.e., TNF $\alpha$ ), by erasing the H3K27me3 mark (Yan et al. 2014). Further work is needed to test whether chemical inhibition of JMJD3 has a protective effect in atherosclerotic mouse models. Technologies to probe H3K27me3 and other histone PTMs genome-wide in small samples will be vital to fully understand the cause and consequence of epigenetic changes that occur during atherogenesis.

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## 5 Chromatin Signatures as Epigenetic Biomarkers in Atherosclerosis

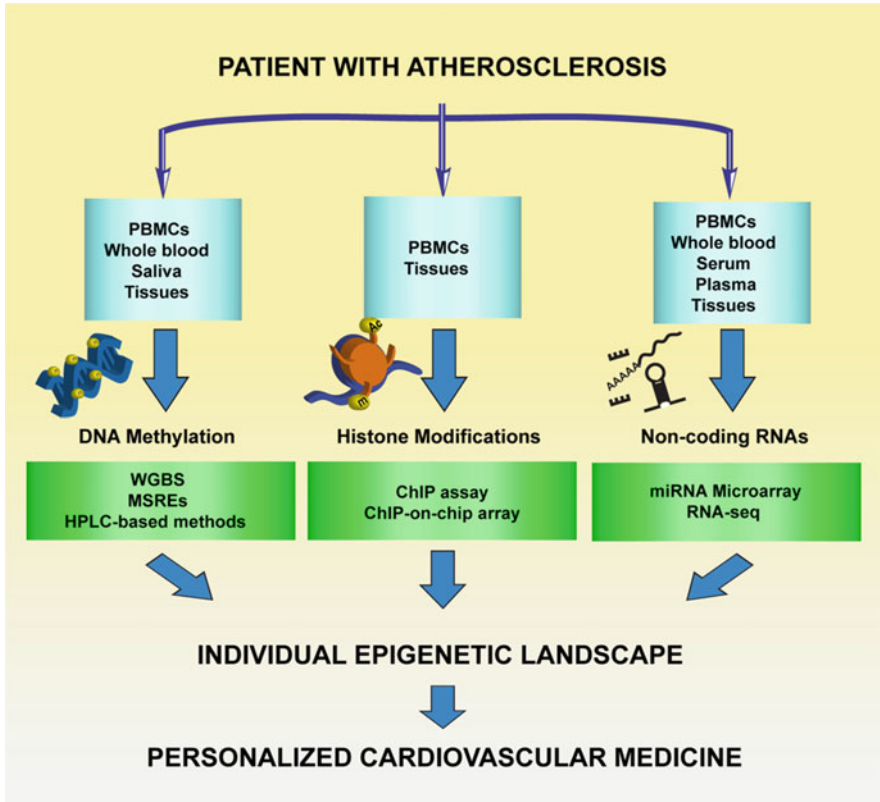
Epigenetic signals acquired during the life course can be employed as potential biomarkers of cardiovascular disease. Indeed, modifications of the epigenetic landscape may reflect dynamic changes in expression of genes relevant to vascular homeostasis. A global reduction of DNA methylation was reported in patients with coronary atherosclerosis. The evaluation of the methylation status of LINE-1 due to its high representation in the human genome (55%) and its high methylation levels can be used to evaluate global methylation changes (Aavik et al. 2019). Hypomethylation of the IL-6 promoter, leading to its upregulation and systemic inflammation, was also associated with a higher risk of coronary heart disease (Zuo et al. 2016). The promoter of two atheroprotective genes, namely the estrogen receptor  $\alpha$  (ESR1) and estrogen receptor  $\beta$  (ESR2), was hypermethylated in atherosclerotic vascular tissues as well as in cultured vascular SMCs (Napoli et al. 2012). Age-related mutations of key epigenetic regulators including DNMT3A, TET2, and ASXL1 in hematopoietic stem cells (HSCs) is emerging as a major mechanism underlying clonal expansion of HSCs, a phenomenon known as clonal

hematopoiesis of indeterminate potential (CHIP) (Li et al. 2018). Notably, the presence of CHIP was associated with an increased risk of CVD and all-cause mortality (Jaiswal et al. 2017). In the northern Sweden population health study, individuals with a history of MI showed differential DNA methylation at 211 CpG-sites representing genes related to CVD, cardiac function, cardiogenesis and recovery after ischemic injury (Rask-Andersen et al. 2016). Hence, epigenetic information may explain the alterations in cardiovascular gene expression trajectories and offer biomarkers for the follow-up of these patients. Overall, available evidence suggests that the epigenome represents a fundamental biological layer in the regulation of gene expression, and epigenetic information may contribute to advance CV risk stratification. The “epigenetic landscape” may provide a real post-genomic snapshot offering the tools to build individual maps of CV risk (Costantino et al. 2018c). Hence, epigenetic changes accumulated during the lifetime may be employed to customize diagnostic and therapeutic approaches in primary and secondary prevention of CVD. Individual epigenetic maps will be invaluable to define new risk scores predicting beyond traditional calculators. This innovative approach will be invaluable for the development of personalized therapies in patients with atherosclerosis (Fig. 4).

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## 6 Epigenetic Drugs

Targeting epigenetic modifications is a highly promising approach to restore gene expression and to rescue or prevent alterations of vascular phenotype (Masi et al. 2020; Landmesser et al. 2020). There are several examples of how specific interventions can be employed to modify the landscape of DNA/histone modifications (Table 1). Emerging evidence indicates that circulating angiogenic cells (CACs), endothelial progenitor cells (EPCs) and, more in general, bone marrow-derived stem cells are critically involved in vascular repair and maintenance of vascular homeostasis via different mechanisms which include local engraftment and paracrine actions. Epigenetic mechanisms substantially contribute to the functional decline of these cells during life (Costantino et al. 2018b). Indeed, DNA methylation is decreased at the promoter of genes associated with self-renewal, whereas promoters of genes regulating differentiation are generally hypermethylated (Sun et al. 2014). Pharmacological blockade of the DNA methyltransferase by RG108 has shown to rejuvenate BM-derived stem cells by resetting the expression of senescence-related genes (Oh et al. 2015). These findings may open perspectives for the *ex vivo* epigenetic reprogramming of BM-derived cells for the treatment of atherosclerosis and ischemic heart failure. However, pharmacological editing of global DNA methylation lacks specificity and may be associated with undesirable effects, such as autoimmune diseases (lupus erythematosus was reported in 25–30% of patients) (Chen et al. 2017). The relevance of epigenetic drugs is outlined by the notion that several of these compounds are being tested in clinical trials for the treatment of cancer, neurological and cardiovascular diseases (Costantino et al. 2015). More than 20 years ago, Decitabine (5-aza-2'-deoxycytidine) was found to



**Fig. 4** Approaches to detect epigenetic biomarkers in patients with atherosclerosis. *PBMCs* peripheral blood mononuclear cells, *WGBS* whole-genome bisulfite sequencing, *MSREs* methylation sensitive restriction enzymes, *HPLC* high-performance liquid chromatography, *ChIP* chromatin immunoprecipitation, *miRNAs* microRNAs, *RNA-seq* RNA sequencing. Modified from Costantino et al. *Eur Heart J.* 2018;39:4150-4158

reactivate *ESR1* and *ESR2* expression in cancer cells and used to treat myelodysplastic syndromes. However almost 20 years later this drug was reported to blunt inflammation in endothelial cells in a mouse model of atherosclerosis (Dunn et al. 2014). The FDA-approved drug Vorinostat (suberoylanilide hydroxamic acid), a histone deacetylase inhibitor, has shown to prevent eNOS uncoupling, NF- $\kappa$ B signaling, and oxidative stress in experimental diabetes (Advani et al. 2011). Vorinostat also promotes the autophagic flux, a process which is defective in atherosclerosis (Xie et al. 2014). In experimental models of atherosclerosis, the HDAC inhibitor sodium butyrate was shown to blunt NF- $\kappa$ B signaling and inflammatory molecules, namely TNF- $\alpha$ , IL-6, VCAM-1, and ICAM-1 (Hu et al. 2014). Chronic treatment with the SIRT1 activator resveratrol improves endothelial function and insulin sensitivity in patients with obesity and T2D (Pollack and Crandall 2013). Metformin and glucagon-like peptide 1 (GLP-1), widely-used anti-diabetic



**Table 1** Epigenetic drugs with potential application for the prevention and treatment of atherosclerosis

Drug	Epigenetic mechanism	Main finding
RG108	DNMT inhibitor	RG108 resets the expression of senescence-related genes in BM-derived stem cells
Azaticidine (5-AZA)	DNMT inhibitor	Blunts the upregulation of inflammatory genes in endothelial cells
(SAHA)/Vorinostat	HDAC inhibitor	Prevents eNOS uncoupling and activation of NF-kB signalling in the setting of diabetes. Reactivates autophagy
Sodium butyrate	HDAC inhibitor	Suppresses NF-kB signaling and transcription of NF-kB-dependent inflammatory molecules
Resveratrol	HDAC activator	Improves endothelial function in obese and diabetic patients by activating SIRT1
Metformin	HDAC activator	Preserves the expression of genes implicated in beta cell function and insulin signaling via activation of SIRT1
GLP-1	HDAC activator	Modulates SIRT1 activity thus preserving insulin signaling
Curcumin	HAT inhibitor	Reduces the expression of proatherogenic cytokines and attenuates atherosclerotic lesion size in ApoE and LDLR double knockout mice. Curcumin supplementation in diabetic patients ameliorates proteinuria, reduces pro-fibrotic cytokines and improves microangiopathy.
Apabetalone	BET inhibitor	Stimulates reverse cholesterol transport by inducing ApoAI expression and increasing HDL levels. It has also shown to decrease systemic inflammation in humans, as assessed by C-reactive protein as well as to act as an anti-thrombotic agent.

*DNMT* DNA methyltransferase, *HAT* histone acetyltransferase, *HDAC* histone deacetylase, *HMT* histone methyltransferase

medications, have also shown to modulate SIRT1 activity, thus affecting histone acetylation and transcription of genes implicated in beta cell function and preservation of insulin signaling (Costantino et al. 2015). The histone acetyltransferase p300 inhibitor curcumin has shown to reduce lipopolysaccharide-induced expression of key proatherogenic cytokines such as MCP-1, IL-1 $\beta$ , and TNF- $\alpha$  in primary human monocytes as well as to reduce macrophage polarization (Moss and Ramji 2016). Chronic treatment (4 months) with curcumin reduced atherosclerotic lesion size in ApoE and LDLr double knockout mice (Hasan et al. 2014). Moreover, a randomized double-blind trial involving 240 individuals with T2D reported a decrease in CVD risk with 6 months of curcumin dietary supplementation, exemplified through a lower pulse wave velocity and improved metabolic profile (Chuengsamarn et al. 2014). In T2D patients, chronic supplementation with curcumin (1–2 months) ameliorated proteinuria, reduced pro-fibrotic cytokines (i.e., TGF- $\beta$  and IL-8), and improved microangiopathy (Khajehdehi et al. 2011). Current limitations in the use of curcumin include its poor bioavailability due to a very rapid excretion from the body (Lopresti 2018). The use of liposomal curcumin, nanoparticles, and a curcumin phospholipid complex represents current strategies to boost the clinical applicability

of this compound. Several other compounds including folates, apicidin, PPAR $\gamma$  agonists, and valproic acid have shown the ability to revert chromatin modifications in cardiometabolic states (Napoli et al. 2012). Recent evidence indicates that apabetalone (RVX-208) – an epigenetic regulator targeting bromodomain and extra-terminal (BET) proteins – stimulates reverse cholesterol transport by inducing ApoAI expression and increasing HDL levels (Ghosh et al. 2017). This drug has also shown to decrease systemic inflammation in humans, as assessed by C-reactive protein as well as to act as an anti-thrombotic agent (Schooling and Zhao 2019). Pooled analysis of short-term non-randomized studies showed fewer cardiovascular events among patients treated with apabetalone as compared to placebo (Nicholls et al. 2018). The recent phase III BETonMACE trial, designed to investigate the impact of apabetalone on cardiovascular outcomes in 2425 patients with diabetes after an ACS, failed to meet the primary endpoint (cardiovascular death, non-fatal myocardial infarction, or stroke) (Ray et al. 2020). However, the drug showed to significantly impact on secondary endpoints. Undoubtedly, larger clinical trials are needed to better explore the safety and efficacy of apabetalone for the treatment of cardiovascular disease.

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## 7 Conclusions

Evidence discussed here suggests that epigenetic processing plays a central role in the pathogenesis of atherosclerosis. Specific epigenetic patterns, characterized by modifications of DNA/histone complexes as well as by ncRNAs, have shown to promote transcriptional programs involved in inflammation, oxidative stress, and endothelial dysfunction. Plastic changes may also contribute to the phenotypic switch of SMCs, a key factor involved in the pathophysiology of atherosclerosis. A careful analysis of the individual epigenetic landscape may furnish novel biomarkers of atherosclerotic vascular disease. An important challenge is the demonstration that epigenetic signals are causally linked to vascular disease phenotypes. The causality of presumed epigenetic events and atherosclerotic phenotypes can be assessed by using different strategies. One possibility would be using longitudinal studies whereby the epigenome can be profiled before the onset of the disease and during follow-up. Alternatively, specific tools, such as the framework of Mendelian randomization which explores the causal link between exposure, epigenetic marks and outcome, can be employed to discriminate between epigenetic phenomena and epi-phenomena. Ongoing epigenomic studies will help to unveil the complex relationship among genetics, epigenetics, and CVD, and will contribute to characterize the incremental value of epigenetic information over established clinical and genetic scores. Epigenetic information can also be used to develop new mechanism-based therapies. The growing understanding of chromatin architecture and metabolism has led to the design of specific molecules able to modulate chromatin accessibility by enhancing or repressing epigenetic marks on DNA/histone complexes. Noteworthy, some of these drugs have been already approved for the treatment of several conditions including cancer, neurological and cardiovascular disease. Taken

together, epigenetic information could advance individualized risk assessment and personalized therapeutic approaches in patients with cardiometabolic disturbances.

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