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Review article

DNA methylation processes in atherosclerotic plaque

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ABSTRACT

Underlying mechanisms of cardiovascular diseases (CVD) have been investigated for over 100 years and novel molecular level mechanisms in the pathophysiology are still continuously being discovered. Genetic polymorphisms (SNPs = single nucleotide polymorphisms) have explained about one tenth of the CVD risk, but polymorphisms fail to account for gene-environment interactions i.e. explain the dynamics of epigenome modifications in CVD. Accumulating evidence suggests that epigenetic modifications are actively reshaping pathological processes (e.g. dedifferentiation of smooth muscle cells, accumulation of senescent cells) in CVD. Senescence of vascular cells in ageing arteries not only counteracts regenerative processes but also exacerbates atherogenesis. Epigenome modifications include changes in DNA methylation, histone code and expression of non-coding RNAs. DNA methylation is a major epigenetic regulator modulating cell-type specific gene expression in mural cells, but there is some controversy regarding how to interpret the role of DNA hyper- and hypomethylation in CVD pathology. DNA hypomethylation (loss of methyl cytosines) appears to predominate in atherosclerosis, while a few genes become more methylated (i.e. hypermethylated) as the disease progresses in medium-sized and large arteries. The actual time-course of atherosclerosis-linked changes in genomic DNA methylation is still poorly studied. This review highlights recent novel findings which link alterations in DNA methylation to atherogenesis and points out new potential approaches for novel treatments.

1. Introduction

CVD remain the leading cause of death and morbidity worldwide, although the mortality from ischemic heart diseases and ischemic stroke has declined in some parts of the world over the past few decades [1,2] (Herrington 2016, Benjamin 2017). CVD can be caused by a number of factors including hyperlipidemia, hypertension, diabetes, and smoking. The pathogenesis follows a common path from fatty streak formation on the abluminal side of arteries to characteristic complex lesions. The rupture of complex lesions leads to partial or total occlusion of the affected artery, which can cause severe morbidity or even death. Proliferation of smooth muscle cells (SMCs) in response to injury [3], accumulation of immune cells [4], oxidized lipids [5], inflammation and thrombosis [6] have been considered to play major roles in the formation of atherosclerotic lesions [7,8]. Recent molecular level studies of the CVD pathophysiology have revealed some novel mechanisms in the development of atherosclerotic lesions. Particularly, epigenetic mechanisms have been shown to continuously reshape interactions between human genome and environment. Epigenetic control of gene expression involves three levels of regulation, which comprise a) direct chemical modifications of DNA (methylation/demethylation of cytosines), b) post-translational modifications of histones, and c) non-coding RNA-dependent mechanisms [9–12]. Recent advances in DNA methylation studies have identified specific changes in DNA methylation that reflect the rate at which we age biologically [13]. Ageing is a known risk factor for CVD and differentiation between

Abbreviations: 5 mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fmc, 5-formylcytosine; 5caC, 5-carboxylcytosine; BER, base-excision repair; BM, bone marrow; CGI, CpG island; CSE, cystathionine -γ-lyase; CHIP, clonal hematopoiesis of indeterminate potential; DNMT, DNA methyl transferase; H3K4me1, histone 3 lysine 4 monomethylation; Hcy, homocysteine; HHcy, hyperhomocysteinemia; HSC, hematopoietic stem cell; HSPC, chematopoietic stem and progenitor cell; HDAC, histone deacetylase; HMT, histone methyl transferase; MBD, methyl cytosine binding domain; MPG, methylpurine DNA glycosylase; NuRD, nucleosome remodeling deacetylase; NZW, New Zealand White; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SMC, smooth muscle cell; TDG, thymine-DNA-lycosylase; TET, ten-eleven translocation

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the age-related epigenetic changes (e.g. in DNA hypomethylation) and those specifically linked to CVD pathology is not trivial as removal of methyl marks from DNA may lead to spurious transcription from internal (intronic) promoter sites [14]. Spurious transcription from intronic promoters may cause unexpected outcomes (e.g. through activation of miRNA expression) since 65% of miRNA genes have been located to introns [15]. Moreover, the open state of demethylated DNA may lead to chromosomal DNA instability, loss of chromatin and cell senescence [16,17]. Age-related DNA demethylation contributes to vascular health also via a newly discovered mechanism of clonal hematopoiesis of indeterminate potential (CHIP) [18]. CHIP is related to a 10-fold increased risk of acute myeloid leukemia and mortality in people older than 70 years with normal blood counts, but this higher risk of death appears to be caused also by increased CVD [19,20]. Understanding the newly discovered links between genetic and epigenetic mechanisms of inheritance and plaque rupture will pave a road to novel therapeutic treatments of atherosclerosis. Epigenetic processes are reversible which makes them attractive drug targets. The scope of current overview is limited to novel findings about DNA methylation in atherogenesis including the role ncRNAs and miRNAs in the regulation of DNA methylation. We skip most of the other aspects of epigenetic inheritance (e.g. DNA methylation synergism with histone modifications, trained immunity) in atherogenesis as several excellent reviews on these topics have been recently published [21,22].

2. Pathogenesis of atherosclerosis

Atherosclerosis is a complex disease and over 200 factors have been associated with the disease development [23]. However, the majority of cases can be explained by a much smaller number of risk factors such as hyperlipidemia, hyperhomocysteinemia, smoking, male gender, obesity, diabetes, ageing and sedentary lifestyle. Hyperlipidemia appears as the main risk factor, and low lipid levels in blood predict a lowered risk for disease even if other risk factors are present [24]. Hyperlipidemia is only partially explained by genetics. Collectively, genetic polymorphisms explain only a small fraction (about 10%) of the cumulative CVD risk [25]. Furthermore, there are also people with no known risk factors, but who still develop CVD [26]. So, environmental factors (e.g. environmental toxins, maternal obesity, famine, stress, intermittent hypoxia at sleep) may epigenetically predispose to atherosclerosis development. It is impossible to predict whether the epigenetic mechanisms of atherogenesis will explain the residual inheritability, but it can be envisaged that the discovery of ties between genome and environment brings new insights how to treat and even prevent CVD.

According to response to injury hypothesis [3], atherosclerosis development is initiated at sites of repeated injuries to endothelium (= > endothelial dysfunction), which is characterized by common features like reduced levels of nitric oxide in arterial wall, increased secretion of endothelin 1 (ET1), angiotensin II (ANGII), and thromboxane. Decreased NO production may lead to endothelial cell apoptosis [27] and ANGII upregulation upon ageing has been demonstrated to prevent regeneration of endothelium [28]. In human arteries, gradual intimal thickening leads to accumulation ("lipid retention") of modified low-density lipoprotein (LDL) particles in sub-intimal extracellular space [5,29]. Macrophages scavenging excessive LDL become foam cells, which in turn, leads to lesion formation [30]. Gradual lesion formation (= neointimal hyperplasia) may also develop in response to intravascular treatments of occluded arteries. After local injury (e.g. balloon angioplasty, bypass grafting, endarterectomy) arterial SMC lose their contractility, start to proliferate and may occlude the artery. Cytokine secretion by dedifferentiated SMC stimulate lesion growth and recruited immune cells build up a local inflammatory environment. Atherogenesis is not just a lipid accumulation disease but involves local and systemic inflammatory processes. Consequently, atherosclerosis is often considered an inflammatory disease [7] and increased levels of inflammatory cytokines in blood stream predict future complications (rupture of plaques, thrombosis, fibrosis, vascular wall calcification). It is not clear what exactly causes an increase in circulating cytokines like MCP1, IL-8, VCAM1, ICAM1 [31] and VEGF [32]. Important stimulators of systemic inflammatory signals are modified LDL [33], monocytosis and monocytes infiltrating arterial wall [34], dietary fat mediated release of LPS into blood stream [35], TOLL receptors recognizing self RNA [36] and hypomethylated self DNA [37]. In humans intermittent hypoxia (sleep apnea) induces systemic inflammation and has been recognized as an important risk factor of cardiovascular health [38]. Generally, inflammation appears to be present throughout the course of atherosclerosis development and as discussed below, alterations in DNA methylation pattern is an adaptive response to inflammation and also a mechanism supporting the inflammatory state. However, it should be emphasized that without elevated lipid levels significant atherosclerosis rarely develops in human arteries.

3. DNA methylation and regulation of gene expression

Gene expression regulation by DNA methylation is a dynamic process involving de novo methylation, 5 mC recognition, active and passive demethylation [39].

De novo DNA methylation is carried out at symmetric CpG sites in an early stage of embryonal development [40] by DNMT3A/3B and methylation pattern is maintained intact during successive cell replications by DNMT1. This established viewpoint does not completely hold true any more as there is experimental evidence that maintenance of DNA methylation patterns needs also DNMT3A/3B, while DNMT1 can carry out de novo methylation [41]. Furthermore, DNMT3 can methylate non-CpG sites (at CpA and CHG) and the presence of methylated non-CpG sites in human genome indicates that there is ongoing de novo DNA methylation at each round of genome replication [41]. De novo DNA methylation is essential to atherosclerosis development and up to 25% of methylated cytosines are found in non-CpG context [42]. Patterns of DNA methylation are dynamic in atherosclerotic tissues and oxidation of 5 mC to 5hmC by TET1-3 (Ten-Eleven Translocation 1–3) plays a role in enhancer activation and chromatin unfolding [43]. DNA methylation patterns are established in mutual relationships between the activities of DNA and histone methylation enzymes. Synergy has been detected between DNMT3A/3B and H3K9 methylases (SETDB1, SUV39H1/2, EHMT1/2) as well as between DNMT3A/3B and H3K36 methylases (SETD2), but H3K4 monomethylation completely prevents DNA methylation [17]. Table 1 provides a short list of proteins commonly involved in regulation of DNA methylation.

Table 1 also exemplifies the limits of simple classification of the proteins participating in DNA methylation patterning. For example, TDG acts both as a reader and an eraser, and SETDB1 is a 5 mC reader and H3K9 methyl writer (H3K9 methyl transferase). Most of the proteins listed in Table 1 can be classified alternatively into three groups according to the domain responsible for 5 mC recognition (**MBD class**, **Zinc finger class** and **SRA class**). MBD of MeCP2 was the first domain identified to selectively bind 5 mC MeCP2 binds a single 5 mC and has negligible non-specific affinity for DNA [44]. MBD1-4 can cause DNA heterochromatinization, and upon 5 mC binding they recruit HDACs and HMTs. Table 1 lists just a few transcription factors (TFs; e.g.

Table 1
Methyl cytosine writers, readers, and erasers.

Writers	5 mC readers	5hmC readers	5fmC readers	Erasers
DNMT1	BAZ2A	MHS6	EHMT1	AID
DNMT3A	MBD1, 2, 4	PRP8	FOXI3	APOBEC3G
DNMT3B	MeCP2	RPL26	FOXK1, 2	FTO
	SETDB1, 2	UHRF2	FOXP1, 4	TET1
	UHRF1, 2		L3MBTL2	TET2
	ZBTB4, 33, 38		MPG	TET3
	ZFP57		TDG	

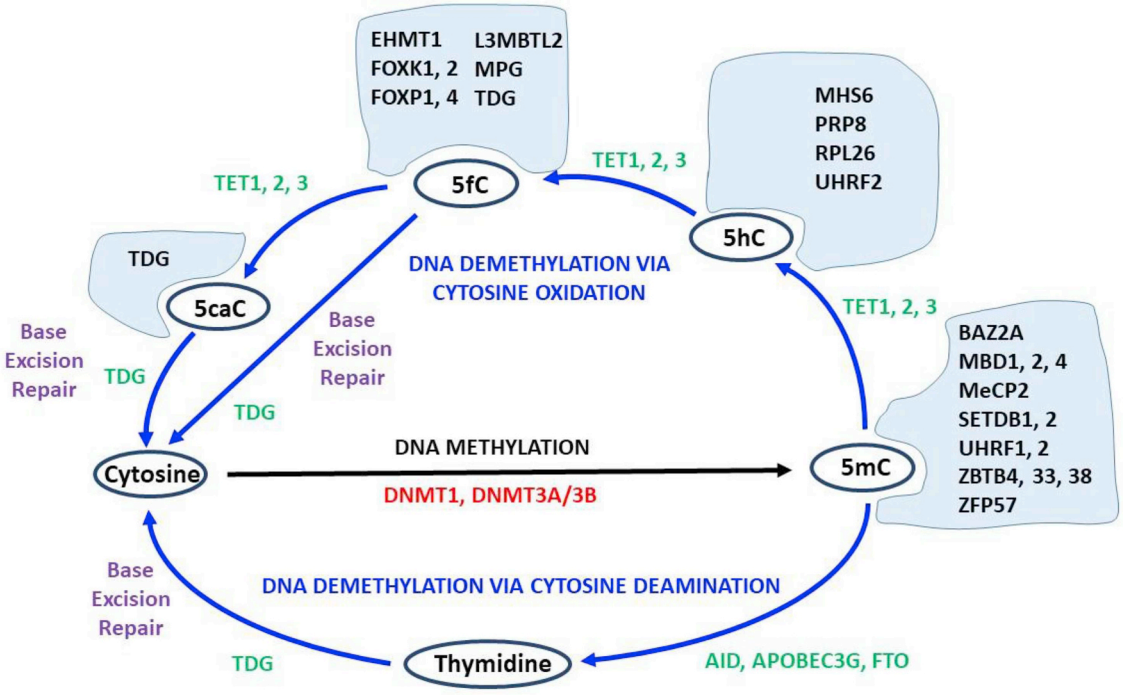

Fig. 1. Two pathways of active DNA demethylation.

MECP2, MBD1, -2, -4, ZBTB4, -33, −38, ZFP57) that have been demonstrated to recognize methylated DNA. Recently, on the basis of systematic efforts a database has been built (http://medreader.org/) revealing that hundreds of TFs can bind methylated DNA [45].

There are two pathways for active DNA demethylation (Fig. 1). The first one is dependent on cytosine deamination (AID, APOBEC3G, FTO) and the other on the oxidation of methyl group present on cytosines (TET1-3). TET 1–3 belong to Ten-eleven translocation (TET) family and are mammalian DNA oxidases that catalyze the conversion of 5 mC to 5hmC and further to 5fc and 5caC [46]. Oxidized methyl cytosines (i.e. 5fc and 5caC) can be directly recognized and excised by TDG-mediated base-excision repair (BER) to generate unmethylated cytosine, leading to active DNA demethylation. TET1 is low in normal tissues, TET2 is an abundant protein in most tissues/cell types and TET3 expression is overlapping with TET2 in many tissues (The Human Protein Atlas). The observed abundance of TET2 and TET3 enzymes in various tissues indicates that DNA demethylation must have an important role in cellular homeostasis. Indeed, macrophages (MΦs) and osteoblastic cells (OCs) undergo highly similar 5hmC and 5 mC changes during differentiation, but the acquisition of difference between MΦs and OCs appears to depend on TET2/TDG ratio. TET2 and TDG downregulation has been shown to impair the acquisition of differentiating histone modification and expression patterns at MΦ-/OC-specific genes [47].

TET enzymes need molecular oxygen for active DNA demethylation, and most likely retain their activity in hypoxic atherosclerotic plaques when the molecular oxygen concentration has dropped below 30 μM [48,49].

Rare proteins bind 5hmC specifically (Table 1), but proteins with a strong preference for 5 fC are more abundant, including transcriptional regulators (FOXK1,2, FOXP1,4, FOXI3), DNA repair factors (TDG, MPG) and chromatin regulators (EHMT1, L3MBTL2, NuRD complex) [53]. Active enhancers in mouse tissues show high levels of 5 fC [54], although 5 fC is present at low numbers in general – 10 copies per million bases in mammalian genome [46].

It has been suggested that the 5hmC modification has a negative role (prevents binding of repressive MBD1, 2, 4 and MECP2), which keeps CGIs unmethylated and enhancers open [55,56].

DNA methylation altered in atherogenesis deranges the expression of hundreds of genes [57]. Most of the human genome is transcribed, when less than 2% of DNA encodes for protein production [58], the rest of the genome encodes for at least 50,000 noncoding RNA genes [59]. Noncoding RNAs (ncRNAs) were earlier considered to be transcriptional noise because of the inherent randomness of transcription initiation by RNApolII and low abundance of most of the ncRNAs [60]. In the past ten years the role of ncRNAs has been widely recognized in different aspects of vascular diseases and ageing [61]. Regulation of ncRNAs may open new opportunities of using e.g. miRNAs as therapeutic targets to treat vascular diseases.

3.1. DNA methylation and hyperhomocysteinemia

Homocysteinemia was linked to CVD almost 50 years ago [62]. Later studies have confirmed the association between high blood Hcy (> 15 μM) and high risk for vascular diseases [63]. High intracellular levels of SAH (S-adenosylhomocysteine) can compete with SAM (S-adenosylmethionine, methyl donor in DNA methylation and other methylations) for binding to DNA methylases and inhibition of DNMTs may lead to passive loss of methylation in replicating DNA [64,65]. Excess SAH has been shown to suppress the function of EZH2 methyltransferase [66]. EZH2 inhibition increased the expression of adhesion molecules and cytokines in human coronary artery endothelial cells via activation of NF-kB, and, thus, elevated concentrations of SAH contributed to inflammatory microenvironment in the arterial wall [66]. EZH2 activity has been shown to control DNA methylation [67], and high levels of intracellular SAH inhibit both histone and DNA methylation in human carotid lesions.

HHcy has not been linked to DNA hypomethylation universally. Genomic DNA isolated from peripheral lymphocytes (137 angiographically confirmed CAD patients vs. 150 controls) demonstrated a positive correlation of global DNA methylation with plasma Hcy [68].

SAH is normally hydrolyzed further to Hcy and adenosine, but accumulation of Hcy shifts the chemical equilibrium towards SAH synthesis [69]. Aberrant DNA methylation has been documented in atherogenesis, both in humans and in animal models [50,70–73].

Sharma et al. have concluded after extensive literature search that elevated levels of Hcy affect the expression of at least 135 genes including ones involved in lipid metabolism, which contribute to atherosclerosis development [74]. Elevated Hcy reduced the expression and activity of DNMT1, increased demethylation of PDGF-A, −C and -D promoters in endothelial cells *in vitro* and upregulation of PDGF was confirmed in the aortic intima of mice with HHcy [75]. Multivariate regression analysis revealed HHcy as a predictor of increased serum PDGF level in patients [75].

Underlying pathogenic mechanisms of HHcy have been broadly studied in vascular disorders but still remain poorly understood [76]. Existing evidence suggests a pathogenic role for HHcy in atherosclerosis correlating high blood levels of Hcy (> 75 μmol/l) with impressive 35% reduction in DNA methylation level of blood lymphocytes [50]. Elevated risk for various forms of vascular disease is associated with heightened plasma Hcy [63,77]. Trials aiming to lower Hcy and prevent CVD (by providing vitamins B6, B9 and B12) appear rational as Hcy plasma levels tend to increase about 1 μmol/L per decade upon ageing [63], but the trials have not brought any benefit [52,78]. Passive demethylation can occur during successive replications due to low activity of DNMT1 or low concentration of SAM or presence of high amounts of SAH in case of HHcy [50]. HHcy has been identified as a risk factor of CVD [51], but the relatively small effect of homocysteine-lowering interventions in reduction of cardiovascular risk [52] can be interpreted as deficiency in DNA and protein methylation leading to malfunction of cellular memory. Overall, describes experimental results lead to the conclusion that high plasma level of Hcy is an epiphenomenon and causality possibly does not exist.

4. DNA methylation in CVD

Changes in DNA methylation have been analyzed by assaying for global methylation, epigenome wide methylation and gene specific methylation. Research material has been usually derived either from blood cells or vascular tissue or both. Genome wide DNA hypomethylation has been commonly linked to CVD [57,79–86], but some authors have concluded that DNA hypermethylation is actually the more characteristic feature [68,87–92]. The causes for the observed discrepancy in DNA methylation results are not immediately obvious, but at least two explanations are worth of considering.

Firstly, atherosclerosis is an ageing disease as numerous studies have linked ageing to general DNA hypomethylation [81,93,94], and possibly that is why the above examples pointing to DNA hypermethylation appear so perplexing. DNA hypomethylation upon ageing is accompanied by focal methylation of CGI sites that are normally not methylated [13,95,96]. As can be expected, there are intra-individual differences in DNA methylation, but even more important is to note the existence of family-wise patterns in age-related DNA methylation alterations, which demonstrate cases of familial hypomethylation, hypermethylation or no changes over extended periods of time [97]. There are plenty of other examples of the genetic determination of epigenome [98,99]. As the atherosclerosis study groups have usually been small, individual variations in age-related changes in DNA methylation are likely to explain some differences in the results when changes in DNA methylation level have been associated with CVD risk.

Secondly, there is another biological issue that may explain the divergent results in CVD risk associations with DNA methylation studies. Namely, DNA hypermethylation in atherosclerotic plaques or peripheral blood cells appears to be less frequent, but more focal than hypomethylation, and the number of hypermethylated sites reaching statistical significance exceeds the number of significantly hypomethylated sites [95]. Demethylated areas cover long stretches of DNA and changes reach statistical significance at any particular CpG only infrequently. As a consequence, when assessing CVD risk the relatively rare sites of hypermethylation reaching statistical significance may appear even 4-fold more numerous than hypomethylated sites [91] and the conclusion has been drawn misleadingly that hypermethylation is the predominating process in atherosclerotic tissue.

Discrepancies have also been observed when the methylation patterns of so-called critical genes in atherogenesis have been analyzed and the methylation status has been confirmed in independent studies only for a half a dozen of genes (*ABCA1, ABCG1, ESR1, F2RL3, IL-6, SOD3*) [100].

4.1. AGEING: physiological vs. chronological age

Progressive loss in DNA methylation has been detected in elderly [81]. Lower level of global DNA methylation was also associated with higher CVD risk in postmenopausal women [101]. Identifiable methylation signatures predicted chronic low grade inflammation and chronic diseases [102]. Growing old involves countless alterations in DNA methylation pattern, but the characteristic changes also serve as an accurate molecular clock. Age-dependent CpG signatures can be defined independently of sex, tissue type, and disease state [13]. Measurements of changes in DNA methylation allow precise estimates of persons biologic age and, consequently, the pace of epigenetic clock allows to predict the lifespan [13]. Horvath's epigenetic clock involves measurements of 353 CpGs. The 193 positively and 160 negatively correlated CpGs get hypermethylated and hypomethylated with age, respectively. Of note, the DNA methylation levels of the 160 negatively related CpGs vary more and the age-related change in methylation measurements is typically small [13]. Specific sites that become hypermethylated upon ageing are found preferentially at CGIs, poised promoters associated with key developmental genes and at Polycomb-group protein targets [13].

General DNA hypomethylation - based marker or, more precisely, the calculated DNA methylation age, predicts all-cause mortality [103,104]. Stroke patients are biologically older than their chronological age and biological age is a predictor of mortality from ischemic stroke [105]. DNA hypomethylation in monocytes increases the risk for CVD. Global hypomethylation of blood cells was defined predominantly by the monocyte DNA hypomethylation [86].

4.2. DNA methylation and clonal hematopoiesis

Hematopoietic stressors (identical to atherosclerotic risk factors e.g. hyperlipidemia, smoking, advanced age, hyperglycemia) disturb the balance in between lymphoid and myeloid lineages by converting bone marrow niches to sites of increased proliferation and preferential production of myeloid cells (Fig. 2). Increased hematopoietic activity has also been detected upon ageing in patients with stable CVD [106]. Increased progenitor potential was evident in hematopoietic stem and progenitor cells (HSPC) isolated from patients compared to controls [106]. Additionally, HSPC demonstrated increased progenitor potential and myeloid differentiation in response to oxidized LDL [106]. Similarly, hypercholesterolemia induced HSPC proliferation in *Ldlr*$^{-/-}$ mice and, interestingly, hypercholesterolemia-primed HSPCs acquired an enhanced propensity to generate myeloid cells [107]. Hyperglycemia induced proliferation and expansion of bone marrow myeloid progenitors and preferentially released inflammatory Ly6-C^{hi}monocytes in to the circulation as demonstrated in diabetic mouse models, associated with increased atherosclerosis [108]. Additionally, T1DM patients with coronary artery disease showed increased monocyte and neutrophil counts [109]. Epidemiological studies have also shown a positive correlation between smoking and clonal hematopoiesis [19]. Mechanistically - aging, hypercholesterolemia, hyperglycemia and smoking seem to reduce the activity of epigenetic regulators including DNMT3A, TET1, TET2 and ASXL1 thus linking epigenetics to observed clonality in hematopoiesis [19,20,110–113].

In steady state, bone marrow hematopoiesis maintains a balance between myeloid and lymphoid cells. Atherosclerotic risk factors contribute to alterations in DNA methylation in hematopoietic stem cells

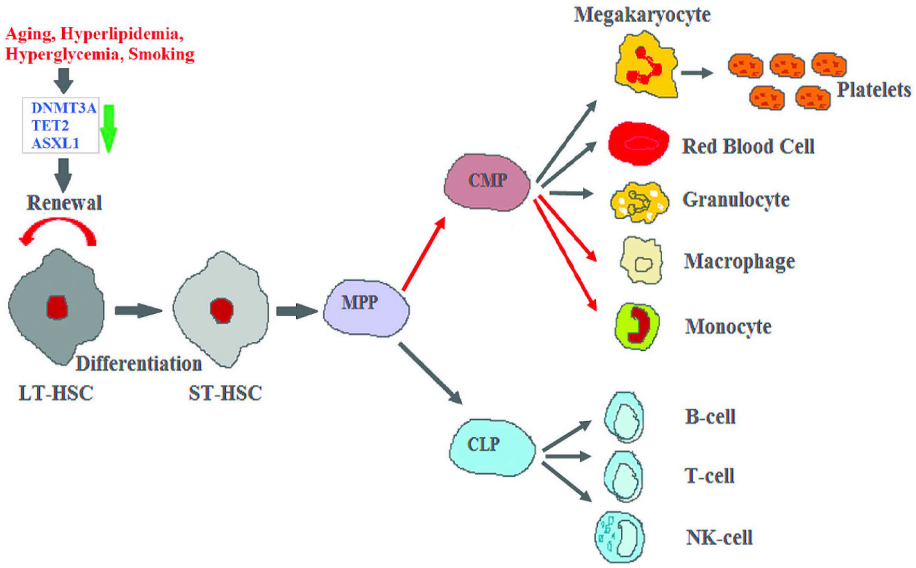

Fig. 2. Basal and clonal hematopoiesis.

leading to loss of HSC quiescence, increased proliferation and preferential increase in myeloid cell production (thick red arrows). Abbreviations: LT-HSC, long term; ST, short term; HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; NK, natural killer.

Recent genetic analyses suggest an increased prevalence of mutations in epigenetic regulators including DNMT3A, TET2 and ASXL1 in HSCs of older individuals leading to clonal expansion in hematopoiesis [20,110]. This phenomenon has been termed 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 [114]. DNMT3A was the most recurrently mutated in individuals with CHIP. In mouse models with hematopoietic tissue specific conditional DNMT3A deletion led to a progressive expansion of long-term HSC with impaired differentiation by incomplete epigenetic repression of HSC-specific genes [115]. Hematopoietic tissue specific Tet2 loss led to decreased levels of 5hmC, increased stem cell self-renewal, delayed HSC differentiation and skewed development toward the monocyte/macrophage lineage [116]. Thus, hematopoietic stressors can induce stable epigenetic reprogramming of bone marrow HSCs contributing to clonal hematopoiesis and myeloid skewing.

4.3. DNA-methylation in blood cells and macrophage dysfunction

In 2009, Bollati et al. [81] reported a drop in Alu repeat methylation in blood samples from elderly. Blood cell DNA hypomethylation has been associated with CVD and stroke, and persons with lower LINE-1 methylation were at higher risk for total mortality [82]. DNA methylation levels are informative concerning the blood white cell composition [117], and the authors did not see an effect of DNA methylation on lipid levels, but on the contrary, differential methylation of DNA was induced by triglycerides, and by LDL. In comparison to age-matched controls, a global decrease in DNA methylation was detected in white blood cells isolated from CVD patients but statistical significance was reached in monocytic and lymphocytic subsets, not in neutrophils [86].

Monocyte recruitment and macrophage differentiation play a critical role in atherosclerosis initiation and progression. Several lines of evidence point to the role of DNA methylation in regulating macrophage function in atherosclerosis. Macrophage-specific Tet2 knockout led to increased inflammatory cytokine and chemokine gene expression and protein secretion in response to native LDL. An analogous increase in plasma interleukin-8 was observed in people with TET2 mutations but not in those without the mutations [114]. Inactivating mutations in TET2 was also shown to aggravate atherosclerosis progression in *Ldlr*$^{-/-}$ mice on high fat diet. Interestingly, analysis of TET2 deficient macrophages showed increased levels of inflammatory chemokines and cytokines contributing to atherosclerosis [111].

Dietary fat induced hypercholesterolemia in *Ldlr*$^{-/-}$ mice showed altered bone marrow (BM) methylation patterns. BM displayed promoter demethylation of *Pu.1* and *Irf8* genes, important for monocyte and macrophage hematopoiesis. Upon BM transplantation to chow diet fed *Ldlr*$^{-/-}$ mice, the recipient mice showed increased plaque size and increased numbers of circulating leukocytes [118]. Hcy, an independent risk factor for atherosclerosis, increased inflammatory cytokine levels in macrophages, accompanied by increased promoter hypermethylation of CSE. DNMT1 inhibition by 5-aza-2′-deoxycytidine treatment could reverse these effects of Hcy on CSE expression [119].

4.4. DNA methylation and VSMC plasticity

VSMCs can adopt phenotypes of many different cell types like myofibroblasts, osteoblasts, chondrocytes and adipocytes. Accumulating evidence suggests that changes in DNA methylation are mediating this phenotypic switch. We demonstrated genomic hypomethylation in advanced human atherosclerotic lesions, lesions of *ApoE*$^{-/-}$ mice and neointima of balloon-denuded New Zealand White (NZW) rabbit aortas [80]. Recent studies have addressed the active role of DNA demethylation regulator TET2 in SMC phenotypic switch. TET2 was downregulated in murine models of vascular injury and human atherosclerotic plaque. Additionally, TET2 and 5-hmC were enriched in contractile, but reduced in dedifferentiated human coronary artery SMCs *in vitro*. TET2 knockdown inhibited expression of procontractile genes (MYOCD and SRF), with concomitant transcriptional upregulation of KLF4. TET2 overexpression reversed these effects by inducing a contractile phenotype [120]. The role of DNA methylation in SMC phenotypic switch has been studied in ApoE$^{-/-}$ mice. In this study, authors addressed regulatory mechanism of DNA methylation in SMC and its role in atherosclerosis by using DNMT inhibitor, 5-aza-2′-deoxycytidine (5-Aza-dC). 5-Aza-dC treatment significantly suppressed DNMT activity, concomitantly decreased global 5 mC contents and attenuated atherosclerotic lesions. Atherosclerosis increased promoter hypermethylation of TET2 in a DNMT1 dependent manner. 5-Aza-dC treatment ameliorated TET2 promoter hypermethylation leading to global DNA hydroxymethylation and 5hmC enrichment at the promoter of myocardin [121].

These experiments demonstrate that the contractile phenotype of

SMC is dependent both on DNMT1 activity and on active hypomethylation. Extensive hypomethylation exhausts the replicative/regenerative potential of SMC upon ageing [122,123] and SMC in atherosclerotic aortas enter replicative senescence which is similarly characterized by global DNA hypomethylation and focal hypermethylation [124].

4.5. DNA methylation in endothelial dysfunction

Endothelial cells show altered gene expression at regions of disturbed flow, at hotspots of atherosclerosis initiation and progression [125]. Disturbed flow was shown to promote endothelial inflammation through a DNMT1-dependent promoter hypermethylation [126]. Disturbed flow was also shown to increase promoter CpG hypermethylation of KLF4 in a DNMT3A-dependent manner in swine aortas [127]. In *ApoE*$^{-/-}$ mice, autophagy flux was impaired together with TET2 protein downregulation during atherosclerotic lesion progression [128]. Furthermore, TET2 was downregulated under low shear stress conditions *in vitro*, but TET2 overexpression increased eNOS, reduced endothelin-1 expression and autophagy under low shear stress conditions *in vitro*. This study suggests a role for TET2 in maintaining endothelial function under low shear conditions [129].

LDL - induced proinflammatory and prothrombotic endothelial phenotype was shown to be mediated in part via DNMT1 mediated promoter hypermethylation of KLF2 leading to down regulation of its target genes thrombomodulin and eNOS *in vitro* [130]. Lentiviral mediated TET2 overexpression promoted endothelial autophagy and downregulated inflammatory factors such as VCAM1, ICAM1, MCP1 and IL-1β in lesions of high fat fed *ApoE*$^{-/-}$ mice. TET2 overexpression decreased the methylation levels of Beclin 1 promoter leading to increased endothelial cell autophagy and decreased inflammatory factors in endothelial cells exposed to oxidized LDL [128]. TET2 overexpression improved endothelial function in response to oxidized LDL by increasing 5hmc levels and promoter demethylation of cystathionine -γ-lyase (CSE). This was associated with increased H2S availability, inhibition of NF-kB and decreased ICAM-1 and VCAM-1 [131]. Age-dependent losses in DNA methylation lead to genome instability [16] and senescence [132] in human endothelial cells.

4.6. DNA methylation in lesions

Genome-wide DNA methylation studies [57,88,91] have identified thousands of differentially methylated sites in human atherosclerotic lesions compared to disease-free segments. Zaina et al., 2014 identified an epigenetic signature of 1858 atherosclerosis-specific methylated CpGs and 91% of those appeared hypermethylated in the diseased vessel [88]. Interestingly, a follow-up study revealed 1631 CpG loci in atherosclerotic plaques that were increasingly methylated as the lesions mature [90]. Yamada et al. identified 2609 differentially methylated CpG loci in Japanese population and 85% of the detected loci (2272) were hypermethylated [91]. Aavik et al. reported predominant hypomethylation of promoter sites (3997 sites = 84%) in atherosclerotic lesions [57], but the ultimate reason for such contrasting results remains unclear.

The top lists of the most differentially methylated sites identified in atherosclerotic lesions demonstrate little overlap, which makes the results from different DNA methylation studies difficult to analyze. Difficulties in DNA methylation pattern analyses are in part caused by the unknown cellular composition of the sampled tissues and the stochasticity of metabolic processes that have shaped the local vascular microenvironment.

Maintenance of a healthy epigenome depends on cellular metabolic intermediates (e.g. SAM, alpha-ketoglutarate) and imbalance in metabolites instructs the epigenetic machinery to reshape the epigenome. For example, DNA methylase DNMT1 protein is destabilized by methylation and targeted to proteolysis, which may significantly amplify small-scale alterations in SAM concentration [133]. Alternatively, mitochondrial dysfunction can reduce alpha-ketoglutarate production, which may lead to a drop in DNA demethylation by TET enzymes [134]. Novel single-cell based analytical tools may help to differentiate the atherosclerotic tissue DNA methylation pattern into distinct cell-types specific epigenetic signatures.

4.7. DNA methylation regulated by ncRNA

MiRNAs show a tendency of upregulation (e.g. miR21, miR34a) in atherogenesis is leading to suppression of genes involved in endothelial regeneration and leading to clonal hematopoiesis [135–137], but the mechanisms responsible for miRNA upregulation upon chronic inflammation and ageing are mainly unknown. Mir-34a ties DNA methylation together with specific histone modifications and mir-34a expression is inhibited by DNA hypermethylation at its promoter [138]. Furthermore, mir-34a is upregulated in senescent endothelium and it downregulates SIRT1, which remodels chromatin by direct deacetylation of histones and recruits nuclear enzymes for histone and DNA methylation [139].

Little is known about the role of ncRNAs in chronic inflammation and atherosclerosis, but some of them (e.g. ANRIL, NKILA, MALAT1, Meg3, HOTTIP) most likely play a substantial role [140]. ANRIL has been shown to activate NFkB signaling [141], when NKILA does the opposite and carries a great potential to reduce inflammation [142]. MALAT1 and Meg3 demonstrate similar opposing effects in endothelial cells. Inhibition of MALAT1 expression suppresses endothelial cell proliferation [143], but Meg3 downregulation allows endothelial cells to proliferate and sprout [61].

Tens of thousands of different circular RNA molecules have been identified, and about 10% of genes can produce nuclease resistant circular RNAs [144]. CircRNAs carry a great potential in gene regulation (e.g. miRNA sponging) and their link to DNA methylation needs to be determined, but there is evidence that on the contrary to linear ANRIL the circular ANRIL may have atheroprotective effects [145]. RNA molecules bound to DNMT1 [146] prevent methylation of specific genes and the original finding by Di Ruscio et al. has opened up new avenues in inhibiting DNMT1 allosterically [147].

Collectively, these findings suggest a critical role of the regulators of DNA methylation in linking DNA methylation to atherogenesis by promoting clonal hematopoiesis as well as modulating vascular smooth muscle phenotype, endothelial function, and macrophage function in response to a multitude of stressors including low shear stress, hyperhomocysteinemia, ox-LDL, hyperlipidemia and hyperglycemia (Fig. 3).

DNA methylation in HSC leads to clonal hematopoiesis and preferential skewing towards myeloid lineage leading to increased inflammatory monocytes in circulation. Upon entering plaques these monocytes differentiate to inflammatory macrophages. Additionally, local alteration in cell specific DNA methylation can lead to endothelial dysfunction, smooth muscle dedifferentiation and macrophage activation – key contributors of atherosclerosis initiation and progression.

5. Clinical perspectives

Atherosclerosis- and age-related changes in DNA methylation represent a complex pattern of general losses (hypomethylation) and focal gains (hypermethylation) of 5 mC marks. It is not straightforward to extract clinically useful information from DNA methylation studies because of the interdependence of DNA sequence polymorphisms, methylation patterns and gene expression levels. Atherosclerosis-specific patterns of DNA methylomes develop in conjunction of two opposing processes. Namely, upon ageing methylomes tend to diversify (= epigenetic shift) as shown in the case of twins [148] and unique individual methylomes obtain common features (= epigenetic clock) that allow to determine the persons true biological age [13]. DNA methylation

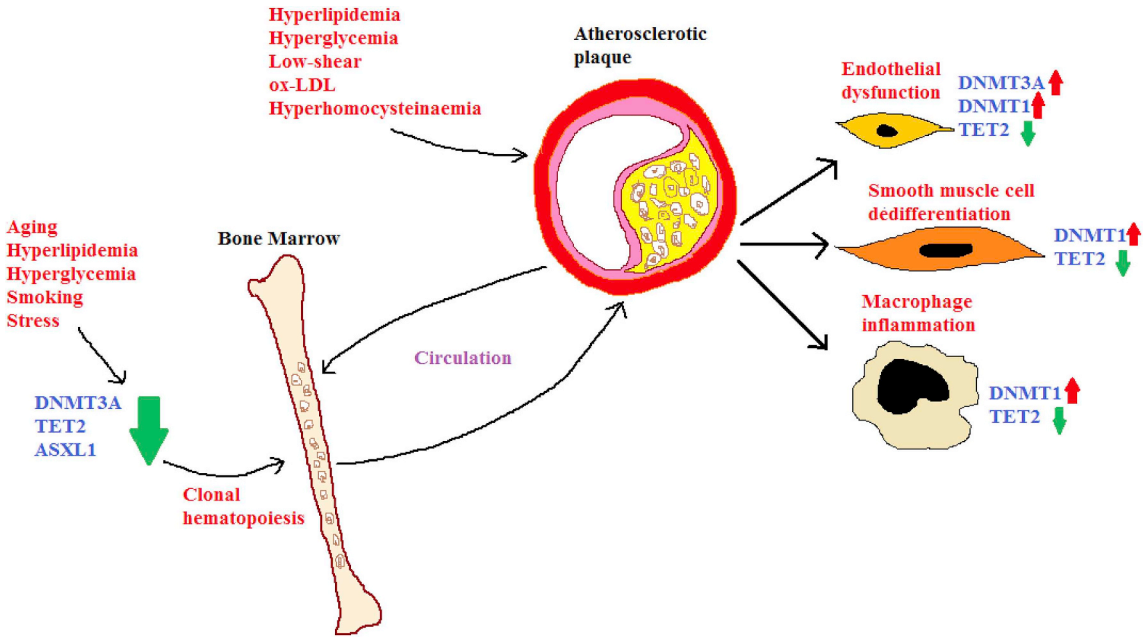

Fig. 3. Bone marrow and lesion specific alterations in DNA methylation are combined in atherogenesis.

changes and epigenetic modifications in general are reversible and this makes them attractive targets for interventions.

Potential pharmacoepigenetic interventions include 1) stimulation of mitochondrial biogenesis in dysfunctional endothelium, 2) prevention of cellular senescence by down-regulating NFkB expression, 3) removal of senescent cells from atherosclerotic lesions to promote angiogenesis and regeneration of ischemic tissue, 4) refreshing stem cell pool to promote angiogenesis, 5) restoring polyclonality among hematopoietic stem cells by resetting the epigenetic clock, 6) restoration of the ability of stem cells to differentiate into endothelial cells and smooth muscle cells etc. In addition to pharmacoepigenetics it is appropriate to mention that even the sickest patients benefit from physical activity which induces changes in DNA methylation both in gene and tissue specific manner [149].

Despite of the limited understanding of DNA methylation events, there are some promising examples of pharmacoepigenetics from preclinical studies as well as from clinical trials (see Table 2). For example, in humans, the BET (Bromodomain and Extra-Terminal proteins, histone modification readers) inhibitor apabetalone (RVX-208) trial for atherosclerosis has reached Phase 3 and preliminary data indicate that those receiving apabetalone had fewer cardiovascular events, especially in patients with diabetes [150]. Apabetalone stimulates ApoAI expression, increases HDL and decreases CRP in patients [150].

DNA methyltransferase inhibitor RG108 relieved replicative senescence in human BM cells by downregulating senescence-related genes [124]. Rejuvenation of stem cells deserves special attention to be paid as genome-wide studies in aged stem cells have revealed a decrease in DNA methylation at the promoter of genes associated with self-renewal, whereas promoters of genes regulating differentiation were hypermethylated [151,152]. Manipulating the equilibrium of DNA methylation and demethylation processes is a very delicate issue and DNA methylation inhibitors have been demonstrated to cause autoimmunity disease like state and procainamide may cause lupus erythematosus in 25–30% of patients [153]. There are commonly used drugs that have epigenetic effects: for example, vasodilator hydralazine is a weak inhibitor of DNA methylation used to reactivate tumor suppressor gene expression in combination with valproate and rapamycin has the potential to eliminate senescent cells [154].

ABT263 (specific inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL) selectively clears senescent cells and rejuvenates aged bone marrow in mice [155]. Clearance of p16Ink4-positive senescent cells extended the median lifespan of both male and female mice [156]. ABT263 carries a great potential to treat chronic inflammatory conditions in CVD.

Several drugs targeting epigenetic regulators (Table 2) are in preclinical or clinical development for various cancers, but hold also a potential to be useful in treating CVD as has been demonstrated for decitabine. Decitabine (5-aza-2′-deoxycytidine) was shown to reactivate ESR1 and ESR2 expression in cancer cells in 1995 but only in 2014 it was demonstrated that decitabine can restore anti-inflammatory phenotype of arterial endothelial cells in a mouse model of atherosclerosis [126].

6. Conclusions

Novel molecular level information has broadened our understanding of CVD pathology and has offered new therapeutic approaches for treatments. However, despite of the new mechanistic information suggesting novel treatments, it should be remembered that the priority of active preventive measures appears higher than ever.

Epigenetically inherited DNA methylation marks are established as a result of complex developmental processes. Intricacy in DNA methylation represents only a fraction of the complexity inherent to the regulation of eukaryotic gene expression. The complexity of gene regulation in eukaryotes allows organisms to adapt their behavior to the rapid changes in the environment. We are just starting to realize the true complexity of gene regulation and this necessitates a need to define achievable goals. In the context of atherogenesis, for example, it will be of utmost importance to identify factors defining the state of dynamic equilibrium of two opposing biochemical activities (i.e. methylation of DNA by DNMTs and demethylation by deaminases and TETs) both needed for the homeostasis of contractile smooth muscle cells in conjunction with healthy endothelium and functional bone marrow.

Table 2
Potential therapeutic avenues to prevent atherosclerosis development based on epigenetic effects.

I. Reversal of epigenetic modifications

Modulator	Mechanism	Target	Tissue/cell	*In vitro/In vivo*	Reference
5-Aza-dC	DNMT1 inhibition	Myocardin, TET2	Atherosclerotic lesions, SMCs	*In vitro and in vivo*	[121] Zhuang 2017
5-Aza-dC	DNMT1 inhibition	Beclin 1 and LC3	Macrophages	*In vitro*	[157] Li 2015
5-Aza-dC	DNMT1 inhibition	ABCA1	Atherosclerotic lesions, macrophages	*In vitro and in vivo*	[158] Lv 2016
5-Aza-dC	DNMT1 inhibition	CSE	Macrophages	*In vitro*	[119] Li 2015
RVX-208	BET inhibitor	Increased apoA-I, HDL-C	Serum	Humans	[150] Nicholls 2018
DECITABINE	DNMT inhibition	Estrogen receptor	MDA-MB-231 tumor cell line	*In vitro*	[159] Ferguson 1995
Statins	HDAC activation	IL8 and MCP-1	HUVECs	*In vitro*	[160] Dje 2009

II. Elimination of HSC clonality

Modulator	Mechanism	Target	Tissue/cell	*In vitro/In vivo*	Reference
Vitamin C	Co-factor of $Fe2^{+}$ and α-KG-dependent dioxygenases	TET2	HSPC	*In vivo/In vitro*	[161] Cimmino 2017
Ascorbate	TET2 activation	TET2	HSPC	*In vivo*	[162] Agathocleous 2017
Rapamycin	MTOR inhibition	p16(Ink4a), p19(Arf), and p21(Cip1) inhibition	HSC	*In vivo*	[163] Chen 2009

III. Elimination of senescent cells

Modulator	Mechanism	Target	Tissue/cell	*In vitro/In vivo*	Reference
RG108	DNMT inhibition	TERT, bFGF, VEGF, and ANG activation	hBM-MSCs	*In vitro*	[124] Oh 2015
Navitoclax (ABT263)	BCL-2 and BCL-xL inhibition	BCL-2 and BCL-xL	HSC	*In vivo*	[155] Chang 2016
AP20187	Clearance of p16(Ink4a)-positive cells	p16(Ink4a)	Heart, kidney, fat	*In vivo*	[156] Baker 2016
Navitoclax (ABT263)	BCL-2 and BCL-xL inhibition	Mmp3, Tnfα, and Vcam1	Aorta	*In vivo*	[164] Childs 2016
Dasatinib + Quercetin	Nitric oxide bioavailability	Various	Aorta	*In vivo*	[165] Roos 2016
Rapamycin	MTOR inhibition	IL6, IL1A, NF-kB inhibition	HCA2 fibroblasts	*In vitro*	[154] Laberge 2015
Rapamycin	Autophagy activation	NRF2 activation, STAT3 inhibition	Fibroblasts, fat and lung	*In vitro/In vivo*	[166] Wang 2017

IV. Prevent/slow down vascular ageing

Modulator	Mechanism	Target	Tissue/cell	*In vitro/In vivo*	Reference
Exercise	Nitric oxide bioavailability	eNOS, NOX2	Aorta	*In vivo*	[167] Guizoni 2016
Calorie restriction	Triglycerides and factor VIIc reduction	HDL-Cholesterol induction	Serum	Human	[168] Lefevre 2009
Spermidine	Autophagy activation	LC3II, p62 activation	Aorta, VSMC, Macrophages	*In vitro/In vivo*	[169] Michiels 2016
NMN	SIRT1 activation	SIRT1, mnSOD	Artery	*In vivo*	[170] de picciotto 2016
NMN	SIRT1 activation	SIRT1, H2S	Capillary endothelial cells	*In vivo*	[171] Das 2018
Thieno[2,3-c]isoquinolin-5-one	Poly (ADP-ribose) polymerase inhibition	MCP1, ICAM1, TNF-α	Thoracic aorta	*In vivo*	[172] Hans 2009
Quercetin	SIRT1 activation	NOX2 and NOX4 inhibition	oxLDL treated HUVECs	*In vitro*	[173] Hung 2015
Quercetin	VCAM1, E-Selectin, NF-kB inhibition	IL-1R, Ccl8, IKK, and STAT3	$ApoE^{-/-}$ mice, HUVECs	*In vitro/In vivo*	[174] Kleemann 2011
Rapamycin	p-AMPK, PTEN, p27kip activation	p19 inhibition	Aorta	*In vivo*	[175] Lesniewski 2017
Statins	Enhanced DNA damage repair	ATM and H2AX phosphorylation	Human VSMCs, rabbits atherosclerotic aorta	*In vitro/In vivo*	[176] Mahmoudi 2008

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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