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General Introduction

Adapted from:

Epigenetics in atherosclerosis and inflammation

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Introduction

Cardiovascular diseases (CVD) are the number one cause of death in the western society.[1,2] Atherosclerosis is the primary cause of coronary artery disease (CAD) and stroke and is regarded as a (chronic) inflammatory disease.[3,4] Atherosclerosis is characterized by asymmetrical focal thickenings of the arterial intima that are often referred to as atherosclerotic lesions or atheromas.[5] These thickenings are caused by the accumulation of lipids and inflammatory cells in the vessel wall.[5] Especially T cells and macrophages play an important role in pathogenesis of atherosclerosis.[6] Besides lipids and inflammatory cells, the lesion – depending on the stage of the disease – further consists of vascular endothelial cells (vECs) and vascular smooth muscle cells (vSMCs), and extracellular matrix (ECM).[7] Extreme thickening of the intima or even plaque rupture with subsequent thrombus formation may seriously hamper coronary blood flow, eventually leading to myocardial infarction.[8] Thus, atherosclerosis is a potential life-threatening condition and understanding the cause of atherosclerosis may contribute to the treatment and possibly prevention of this disease.

Atherosclerotic plaque formation is a dynamic multi-cellular process in which the activity of the different cell types involved is essentially determined by the regulation of different genes.[3–8] Understanding these processes is critical for our understanding of inflammatory responses and disease. Nowadays we are fully aware of the important involvement of epigenetic processes in the regulation of gene expression. For instance, not only is cytokine expression under epigenetic control, cytokines themselves induce (indirect) changes to the chromatin, providing an essential link between inflammation and epigenetic programming.[9] To understand the involvement of epigenetic process in atherosclerosis it is important to have a clear understanding of the disease process as well as an understanding of epigenetic phenomena. A brief introduction of both atherosclerosis as well as epigenetic gene regulation is provided below, before the potential involvement of epigenetic regulation in atherosclerosis is discussed.

Atherosclerosis

The first step in atherosclerosis is thought to be endothelial dysfunction,[10] possibly triggered by oxidized low-density lipoprotein (OxLDL).[5] Activation of the arterial endothelium results in the expression of cytokines and chemokines, enhancement in the permeability of the endothelial cell layer and an increased expression of adhesion molecules. Immune and inflammatory cells such as monocytes and

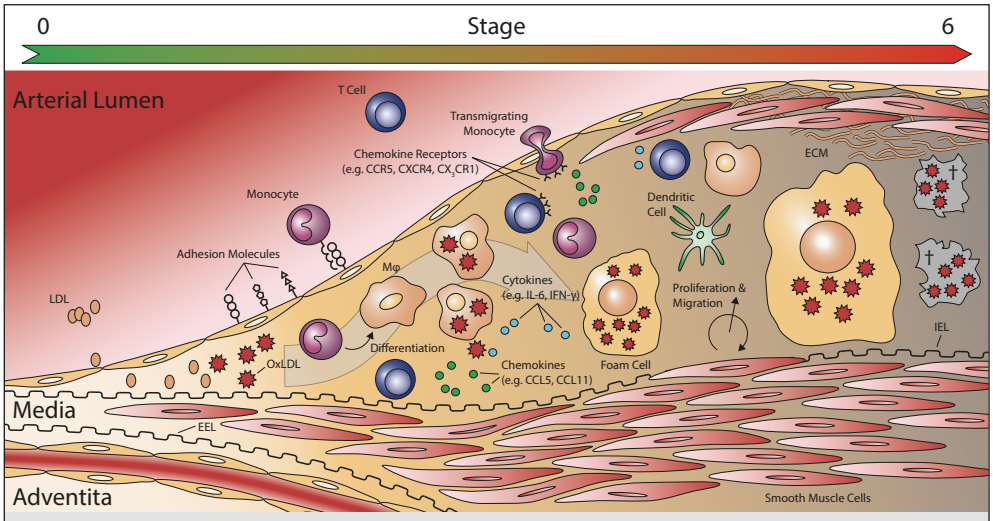

Figure 1–1. Schematic representation of plaque development. Oxidized low-density lipoprotein (OxLDL) is thought to trigger endothelial dysfunction. OxLDL is often associated with proteoglycans in the subendothelium. Activated endothelial cells start to express various adhesion molecules. Immune cells, mainly monocytes and T cells, are attracted by chemokines and migrate across the endothelium, using adhesion molecules. Monocytes then differentiate into macrophages (Mφ) and dendritic cells. These macrophages take up OxLDL and transform into 'foam cells'. This process of foam cell formation is indicated with the large grey arrow. T cells recruited to the plaque express several cytokines and chemokines. In response to the stimuli, vSMCs start to proliferate and migrate, and start to produce ECM. Abbreviations: ECM: extra cellular matrix; EEL: external elastic lamina; IEL: internal elastic lamina; LDL: low-density lipoprotein; Mφ: macrophages; OxLDL: oxidized low-density lipoprotein. Illustration inspired on Sherer *et al.*[11]

T lymphocytes are then attracted by chemokines (a process know as chemotaxis), followed by firm adhesion and transendothelial migration. These cells subsequently infiltrate the subendothelium of the vascular wall and form the main players in the formation of the 'fatty streak': the first identifiable lesion.[10] The process of plaque formation is shown schematically in figure 1–1.

Foam cells – cholesterol-engorged monocyte-derived macrophages – are the dominant type of immune cells found within the lesions. Macrophages take up the OxLDL present in the subendothelium, but are unable to digest it sufficiently, resulting in the formation of foam cells.[5] Although fatty streaks are not clinically significant, they are thought to be the precursors to more advanced lesions, although there is some dispute whether fatty streaks are truly precursors of advanced lesions.[7,12] If the causative agents for this endothelial dysfunction are not effectively removed, the inflammatory response can continue indefinitely. The

atherosclerosis-associated immune response is driven by monocyte-derived macrophages and specific subsets of T cells.[6,13] Monocytes and T lymphocytes migrate from the blood and proliferate within the lesion, resulting in the accumulation of inflammatory cells. If these cells become activated, they release cytokines, chemokines and growth factors,[10] contributing to the on-going inflammatory process. Moreover, immune activation eventually induces focal necrosis leading to the necrotic core found in advanced lesions.

In later stages of the disease other cell types (e.g. vSMCs) become involved. Vascular SMCs start to proliferate as a response to the various signalling molecules present in the lesion, thereby thickening the arterial wall. Eventually the lesion will evolve into a 'fibrolipid plaque', consisting of a lipid-rich necrotic core covered by a fibrous cap of vSMCs and a collagen-rich matrix.[3,14] The resulting thickening of the arterial wall caused by the plaque formation will partially be compensated by gradual dilation (remodelling). If the plaque volume becomes too large, this process cannot compensate enough, leading to decreased lumen size and hampered blood-flow.[15]

As stated above, the development of the atherosclerotic lesion is for the larger part determined by the release of various cytokines and chemokines. Pro-inflammatory cytokines such as tumour necrosis factor α (*TNF-α*) and interferon γ (IFNγ) released by activated T cells – recruited to the vascular cell wall – play an essential role in disease development. This critical role for pro-inflammatory cytokines in atherosclerosis development is illustrated in atherosclerotic mouse models. It has been shown that deletion of the genes coding for pro-inflammatory cytokines (e.g. IL-12, IFNγ, IFNγ receptor and TNF-α) resulted in reduced atherosclerosis development in these knockout mice.[16–18] Conversely, deletion of anti-inflammatory cytokine genes (e.g. IL-10 and TGF-β) resulted in increased atherosclerosis.[19,20] Different genetic variants of the *TNF-α* gene also showed to be important for the outcome of percutaneous coronary intervention (PCI, one of the treatments used for opening of occluded vessels) with respect to restenosis – one of the major limitations of the PCI technique.[21] Genetic variants of the *IL-10* gene have also proven to be important for the development of restenosis.[22]

The cross-talk between immune system and vascular wall results in upregulation of major histocompatibility complex (MHC) class II (MHC-II) molecules and of the MHC class Ib molecule, HLA-E.[3,23] Furthermore, it results in up regulation of chemokines such as CX3CL1 (fractalkine), CCL2 (MCP-1), CCL5 (RANTES) and their receptors.[3,24–27] All of these factors influence the gene expression profiles of cells

present within the lesion. These observations not only feed the current theory that atherosclerosis is an inflammatory disease – and not just a disease of lipid metabolism. For many of these inflammatory and immune modulating factors epigenetic components have been identified that are responsible for the transcriptional regulation of the genes encoding these factors. Thus epigenetic processes might prove to be important contributing factors to disease pathogenesis that should be taken into consideration.

Epigenetics explained

Although all cells in our body contain the same genetic material, each cell acts in a cell-type specific manner, as determined by its gene expression profile. Epigenetic mechanisms control gene expression without modifying the actual genetic code, whilst the altered gene expression patterns can be passed to the daughter cells upon cell division or even transgenerationally.[28–30]

In its natural state DNA is packaged into chromatin, a highly organized and dynamic protein-DNA complex which consists of DNA, histones and non-histone proteins.[31] Epigenetic mechanisms alter the accessibility of chromatin by modifying DNA and by modification or rearrangement of nucleosomes, which include post-translational modifications of histones.[32–34] Accessible chromatin allows gene-regulatory proteins (transcription factors) to interact with their cognate binding sites within the regulatory regions of genes, such as proximal promoters and enhancer/silencers. In this way, global gene activation and local control of gene-specific transcription is exerted by components of the epigenetic machinery. Moreover, environmental factors have an important role in the establishment of the epigenome.[35–38] Since epigenetic alterations can accumulate in time, environmental factors can have profound effects on the cellular repertoire of expressed genes.[28]

Already in 1975 DNA methylation was proposed to play a role in regulating gene transcription.[39,40] Generally speaking, DNA methylation is associated with low gene activity. Actively transcribed genes are usually maintained in an unmethylated state in their promoter regions.[41–43] DNA methylation involves methylation at the C5 position of cytosine residues in a CpG dinucleotide (i.e. cytosine followed by a guanine) context, exerted by DNA methyltransferases (DNMTs). DNMTs are capable of both methylation and demethylation – as has been postulated in two recent reports – thus making the modification reversible.[44,45] Notably, CpG dinucleotides are underrepresented in the genome of eukaryotes, but can be found in clusters – so-called 'CpG islands' – which in turn are mainly found in promoter regions. The

term 'CpG island' is defined as a region of at least 500 base pairs with a CG-content greater than 55%.[46]

For many years it was thought that functionally relevant DNA methylation occurs at the CpG islands within the promoter region. However, in a recent study by Irizarry *et al.* it was shown that most tissue-specific DNA methylation occurs in regions up to 2kb distant of the promoter region,[47] which were dubbed 'CpG island shores' by the authors. Furthermore they showed that cancer-specific methylation also occurs at conserved tissue-specific CpG island shores.

Besides methylation of DNA, post-translational modifications of N-terminal tails of histone proteins are key-components in the epigenetic regulation of genes. Over 60 distinct modifications are currently known – mostly in the histone tails – although some have been observed in the globular domain.[48,49] Modifications of histone tails include (amongst others) acetylation and methylation of lysine residues. Whereas acetylation of histone tails is correlated with gene activation,[50–52] the influence of histone methylation depends on the exact residue methylated and the number of added methyl groups.[53–55] Histone modifications and DNA methylation provide a close interplay with respect to gene regulation as both activities are functionally linked.[56]

Whereas lysine methylation and acetylation are the most studied modifications, there are many more histone modifications known. Arginine residues can also be methylated and acetylated, just as lysine residues. As is the case with lysine methylation, whether arginine methylation results in repression or activation of transcription depends on which arginine residue is methylated.[57] In addition, SUMOylation and ubiquitination of histones has also been observed.[49] SUMOylation appears to be associated with transcriptional repression,[58] whereas ubiquitination has been suggested to play a role in transcriptional activation and elongation.[59] However, the exact function of these modifications remains to be elucidated.

Epigenetic alterations are reversible

Interestingly, epigenetic modifications are reversible, which is illustrated by the counterbalancing actions of the various enzymes that are responsible for maintaining the epigenome. Lysine acetyltransferases (KATs, formerly known as histone acetyltransferases or HATs) are counteracted by histone deacetylases (HDACs) and Sirtuins (SIRT) in establishing histone acetylation modifications at lysine residues in the N-terminal tails. Lysine methyltransferases (KMTs, formerly known

as histone methyltransferases or HMTs), finally, are counteracted by the recently discovered lysine demethylases (KDMs, formerly known as histone demethylases or HDMs) in establishing histone methylation modifications (Figure 1–2). In this way

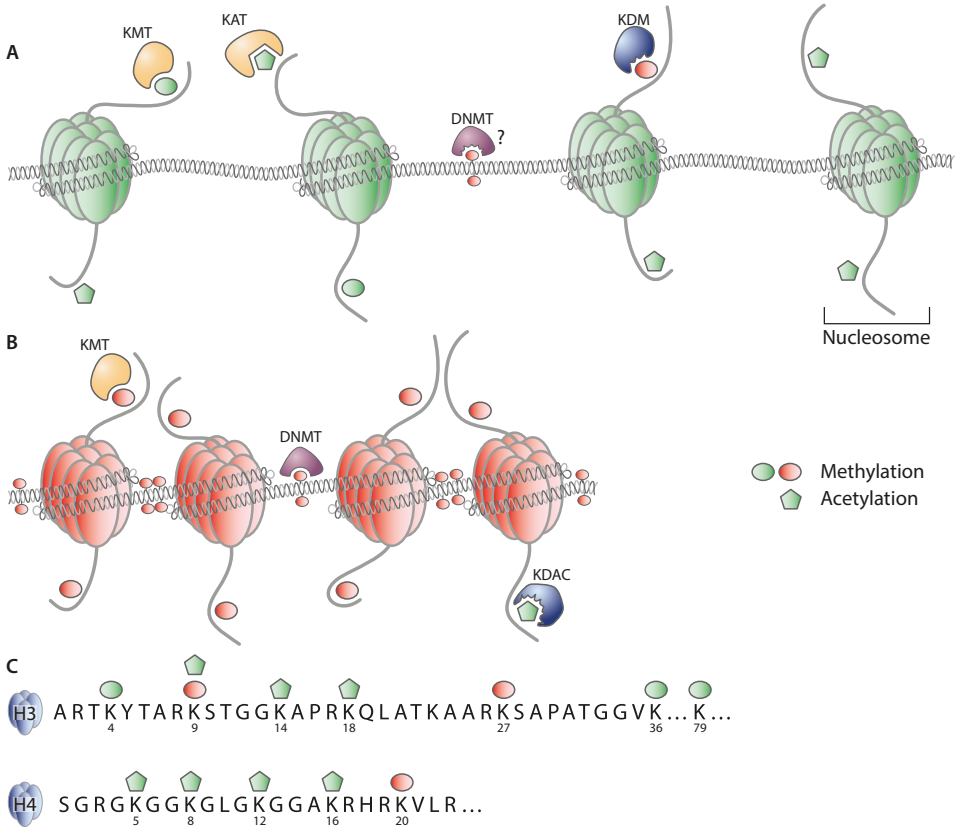

Figure 1–2. Schematic representation of chromatin. DNA is wrapped around the octamer core of histones to form a structure called the nucleosome. Transcriptionally active chromatin (euchromatin) is hallmarked by low frequency of DNA methylation. High levels of histone acetylation and histone methylation correlated with activation (MeK4H3, MeK36H3, MeK79H3) are detected in euchromatin. KATs are responsible for acetylation of histone tails, whereas KDMs remove methylation marks histones. DNMTs probably remove methylation marks from DNA, although this has not yet been proven definitively (A). Transcriptionally silent chromatin (heterochromatin) is hallmarked by high frequencies of repressive methylation markers (i.e. DNA methylation and MeK9H3, MeK27H3 and MeK20H4). Methylation of histone tails is catalysed by KMTs whereas DNA methylation is catalysed by DNMT. Acetylation markers, associated with activation, are removed by HDACs (B). Post-translational modifications of histone tails include (but are not limited to) acetylation (Ac) and methylation (Me), which can be associated with transcriptionally active (green) or silent (red) chromatin (C).

these enzymes promote a return to respectively repressive or active chromatin structure.[60–62]

Importantly, the reversible nature of these epigenetic modifications makes the chromatin-modifying enzymes interesting therapeutic targets[63–65] and a myriad of small molecule inhibitors (SMI) that can influence the enzymatic activity of these chromatin-modifying enzymes are currently being tested for their efficacy (see Mai *et al.*[66] and references therein). These inhibitors are mostly evaluated in the field of cancer research, in cell lines, in animal models as well as in clinical trials.[66] Notably suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) (HDAC inhibitors (HDACi)) are already FDA approved for cancer treatment and epilepsy respectively.[67–69] With respect to atherosclerosis, administration of curcumin (a KAT inhibitor) resulted in significantly lowered low-density lipoprotein (LDL) levels and raised high-density lipoprotein (HDL) levels in healthy volunteers.[70–72] It has also been reported that curcumin has an anti-proliferative effect on peripheral blood mononuclear cells (PBMCs) and vSMCs.[73]

Epigenetics and association with atherosclerosis

Epigenetics provides an attractive explanation on how diet, environment and lifestyle may contribute to disease. In principle, epigenetics explains how such external factors can impose aberrant gene expression patterns in an individual lifetime and even trans-generationally. One of the earliest studies linking DNA methylation to atherosclerosis showed that the extracellular superoxide dismutase (ec-SOD) gene was hypomethylated in atherosclerotic lesions in rabbits.[74] The importance of DNA methylation as a contributing factor to the pathogenesis of atherosclerosis is underscored by a study linking global DNA hypermethylation with predisposition to, and natural history of atherosclerosis.[75] In this study the correlation was found by comparing methylation sensitive restriction of peripheral blood leukocyte DNA. However, this approach evaluates only the inflammatory component of the disease process in PBMCs and not other components such as vECs and vSMCs. In particular, DNA hypermethylation was found to be significantly associated with both all-cause and cardiovascular mortality, even following the adjustment for age, CVD and diabetes mellitus. Hypermethylation was found in patients suffering from inflammation (high C-reactive protein (CRP) levels) and also in 13 patients who died from CVD. This paper suggests that global DNA hypermethylation is associated with inflammation and increased mortality in chronic kidney

disease. Importantly, hypermethylation was found to be the strongest independent risk factor for CVD mortality.

In the case of coronary artery disease (CAD) an epigenetic component associated with disease was also identified.[76] Sharma *et al.*, found that angiographically confirmed CAD patients showed a higher level of genomic DNA methylation – determined in peripheral blood lymphocytes – when compared to healthy controls.[76] Furthermore, a positive correlation was found between global DNA methylation and homocysteine levels. Homocysteine is known to be an independent risk factor for CAD.[77,78] Sharma and co-workers also tested the specific methylation status of the ApoE gene by bisulphite-sequencing. The ApoE promoter was previously shown to have a degree of methylation that varies with homocysteine levels.[79] However, no significant difference could be detected between patients and controls.[76] Both these studies underline the potential effect DNA methylation can have with respect to disease development and outcome. However, the precise genes targeted by DNA methylation and thus the precise mechanisms that induce DNMT activity remain to be elucidated.

Although DNA methylation is one of the most studied epigenetic phenomena, more and more research is performed on the specific contribution of histone modifications. Recently, a study was published directly linking epigenetic histone modifications and atherosclerosis. Hastings *et al.* showed that atherosclerosis-prone shear stress profiles globally decreased histone H4 acetylation and increased H4 acetylation at the c-*fos* promoter in smooth muscle cells (SMCs).[80] Perhaps more importantly, they showed that atheroprone hemodynamics resulted in differentially regulated phenotypes for endothelial cells (ECs) and SMCs. Shear stress (flow) was applied to ECs that were layered on top of SMCs in forces that correlate to atheroprone or atheroprotective-conditions. Atheroprone flow reduced the expression of eNOS, TIE2 and Krüppellike factor in ECs, and smooth muscle actin and myocardin in SMCs, whereas VCAM-1, IL-8 and MCP-1 were increased in both cell types. This correlated to a decrease in total H4 acetylation and serum response factor binding to the smooth muscle cell actin CArG promoter. Furthermore atheroprotective conditions induced a polygonal shape in EC whereas atheroprone conditions induced an elongated EC phenotype. In vSMCs atheroprotective conditions induced an elongated shape whereas atheroprone conditions showed a random SMC organization and cells aligned toward a perpendicular orientation relative to the direction of flow.[80]

Another linkage between atherosclerosis and histone modifications was found when the administration of the HDACi trichostatin A (TSA) in *LDR*-/- mice led to exacerbated neointimal lesions.[81] TSA increased CD36 mRNA, protein and cell surface expression levels, which were related to increased acetylated histone binding at the promoter region. CD36 recognizes OxLDL, thus the obtained results might be due to increased OxLDL uptake by macrophages. Also levels of TNF-α and VCAM-1 were increased in aortic plaques. It has also been noted that application of TSA resulted in inhibition of SMC migration.[82] Unfortunately, the exact mechanisms are not yet fully understood, but these studies clearly indicate a role for histone acetylation in disease pathogenesis.

Interestingly, some components relevant for the development of atherosclerosis, such as OxLDL, are able to modulate the activity of lysine deacetylases.[83] In cultured ECs it was shown that OxLDL reduced expression of HDAC1 and HDAC2. Global HDAC activities were partly restored by statins: pre-treatment of ECs for 24h with simvastatin or fluvastatin blocked the OxLDL-related modifications in H3 and H4. Addition of mevalonate could revert the effects observed after statin treatment. The researchers postulate that this, at least in part, may be due to inhibition of small GTP-binding Rho proteins. Furthermore, decreased expression of HDAC2 in ECs was shown in atherosclerotic plaques of human coronary arteries.[83] Taken together, these data show the importance of histone modifications in atherosclerosis.

A study performed by Barker *et al.* in 1989 sheds light on how long-term cellular memory can have an effect on the development of atherosclerosis. Nowadays it is generally accepted that long-term cellular memory is mediated via epigenetic phenomena. Barker *et al.* demonstrated that low birth weight is associated with a higher risk of cardiovascular events in adult live.[84] This led to the hypothesis that the environment the foetus is exposed to during pregnancy might partially determine cardiovascular risk. This so-called 'foetal origins hypothesis' states that adaptation to an unfavourable maternal environment is beneficial to the developing embryo *in utero*.[84] However, when the adult environment differs from the in utero environment, this may lead to an increased risk for cardiovascular disease. Studies in pregnant rats show that a protein-restricted diet results in reduced expression of Dnmt1, leading to hypomethylation of specific promoters.[85] These observations provide a strong indication that the in utero environment leads to epigenetic (re-) programming, which later in life might contribute to disease development.

Epigenetic regulation of cell activity

Cell differentiation (e.g. monocyte differentiation into macrophages) and cell-specific gene expression in most cases is controlled by epigenetic mechanisms. Below, the most important cellular factors that contribute to atherosclerotic plaque formation and how their phenotype is regulated by epigenetic processes will be discussed.

T cells

Several lines of investigation illustrate that T cells are important in the formation of (early) lesions.[86–89] Within the plaque, several subsets of T cells can be found. Mainly $CD4^+$ T cells are dominantly found in plaques, but there are some $CD8^+$ T cells found as well.[5] The T cells present in human plaques are predominantly of the Th1 phenotype.[89] Th1 cells produce IFN-γ – a macrophage activating cytokine – and are generally considered pro-atherogenic. Th2 cells are rarely detected in lesions.[90] They are generally considered anti-atherogenic, but may also contribute to aneurysm formation.[91] Regulatory T cells (Treg cells) control the balance between Th1 and Th2. Tregs are considered to be atheroprotective.[92]

All T cells derive from the same precursor: the naïve T-cell. Not surprisingly, differentiation of e.g. $CD4^+$ T cells into a Th1, Th2 or Treg phenotype is regulated by epigenetic processes.[93,94] The differentiation of naïve T cells into Th1 or Th2 is determined by IL-12 and IL-4 cytokines respectively. In response to these signals, transcription is initiated of lineage specific cytokine genes including *IFN-γ* and *IL-4*.[95] The *IFN-γ* and *IL-4* loci are maintained in a poised state in naïve T cells, meaning they show both repressive and activating epigenetic marks. In 1998 it was first proposed that epigenetic, chromatin-based structural changes, underlie the Th1/Th2 differentiation.[96] These epigenetic mechanisms – that are necessary to stably maintain gene expression patterns and eliminate the need for feedback loops – will be discussed below.

In Th1 cells expression of IFN-γ is preceded by remodelling of the *IFN-γ* locus.[97] Upon initial stimulation of naïve T cells, the lineage determining factors GATA3 and T-bet mediate many of the structural changes to the chromatin.[93] These factors will respectively render the *IFN-γ* or *IL-4* genes accessible to regulatory enzymes and other transcription factors.[98–100] On the level of DNA methylation, there are numerous findings supporting the epigenetic regulation of IFN-γ expression. Increased expression of IFN-γ was found in T cells from *Dnmt* knockout mice[101] and in T cells

treated with DNMT inhibitors (DNMTi).[102,103] In addition, expression of IFN-γ by NK and NKT cells is also controlled by epigenetic processes.[97] The ability of NK and NKT cells to rapidly produce substantial amounts of IFN-γ implies that the *IFN-γ* locus is accessible to transcription factors, meaning that the chromatin at the *IFN-γ* locus is maintained in an 'open' state. Indeed the *IFN-γ* locus in NK cells was found to be hyperacetylated and in a poised state.[104,105]

In the differentiation to Th2 cells, IL-4 expression is preceded by remodelling of the *IL-4* locus, similarly to the *IFN-γ* locus remodelling in Th1 cells. In naïve T cells, the *IL-4* locus is 'poised', allowing rapid, early transcription. The IL-4 promoter region exhibits a low basal level of H3 acetylation and DNA hypomethylation, but also shows H3K27Me3 (i.e. triple methylation of lysine 27 on histone H3) modifications. When naïve T cells are stimulated under Th1 conditions, transcription activating chromatin marks at the *IL-4* locus are replaced with repressive marks, whereas the contrary happens under Th2 stimulating conditions (e.g. at the *IFN-γ* locus*)*. These processes have been excellently reviewed by Ansel *et al.*[106]

The Foxp3 transcription factor is considered the master switch for Treg. The promoter of this transcription factor showed difference in methylation levels between Tregs and non-Treg $CD4^+$ cells.[107] Furthermore, this study also showed difference in activating histone marks (H3Ac, H4Ac and H3K4Me3) in *FOXP3* promoter chromatin. Epigenetic regulation of T cell subtypes has also been shown *in vivo*. Mice, which were treated with the HDACi TSA showed an increase in $Foxp3^+$ $CD4^+$ Treg cells – both proportionally as well as in absolute numbers – in the lymphoid tissues.[94] Besides influencing T cell populations, the differentiation of monocytes to dendritic cells (DCs) can also be modulated by HDACi.[108]

Monocytes

Monocytes are critical players in the formation of atherosclerotic plaques. Monocytes migrate from the vessel lumen into the arterial wall, where they differentiate into either DCs or macrophages (Figure 1–1). These macrophages later transform into foam-cells, one of the major components of the atherosclerotic plaque. It has been shown by two independent researches that interfering with HDAC function during differentiation resulted in the formation of function-impaired DCs. Notably, treatment with the HDACi's butyrate and MS275 resulted in lowered expression of CD1. CD1 expression is a marker for mature DCs, but notably it is also important in the presentation of lipid antigens and thus may play an important role in the development of the atherosclerotic plaque.[108,109]

Endothelial cells

Epigenetic mechanisms are also directly involved in the transcriptional regulation of immune genes in the vascular wall. For instance, in cultured human ECs it was found that administration of the HDACi TSA inhibited TNF-α induced monocyte adhesion. This was the result of downregulation of the *VCAM-1* gene, however *ICAM-1* and E-selectin – two other cytokines inducible genes – where not affected.[110] This suggests that the HDACi modulated TNF-α–mediated signalling specifically targets the *VCAM-1* promoter, instead of general inhibition of the NF-κB pathway. This notion is interesting, since NF-κB is a target for chromatin modifying enzymes as well (see "Non-histone Targets" on page 31). Similar results have been presented for the induction of tissue factor in human ECs. The human tissue factor (TF) promoter contains an NF-κB–related binding site: TF-κB. HDACi administration inhibited agonist induced (TNF-α, IL-1ß or LPS) TF activity in ECs by blocking activation of TF-κB (TF activity was reduced 90% in HUVEC; 50 % reduced in PBMC, *in vitro*). TSA nearly abolishes TF-κB binding, without affecting NF-κB binding (as determined by electrophoretic mobility shift assays (EMSAs), chromatin immunoprecipitation (ChIP) and luciferase promoter reporter assays). These results were achieved using a variety of structurally distinct HDACi's.[111]

Work performed in the group of Stefanie Dimmeler showed an important role for HDACs in regulating several endothelial specific genes. Using HDACi's, it was shown that Endothelial Nitric Oxide Synthetase (eNOS) mRNA and protein levels decreased after HDACi administration.[112] Furthermore the 'master-switch' for some typical endothelial expressed genes (*HOXA9*, which regulates eNOS, VEGF receptor 2 and VE-cadherin expression) was also downregulated in its expression by HDACi administration.[113]

Smooth Muscle Cells

The data reported in literature regarding epigenetic regulation in SMCs provides an explanation how epigenetics may control lineage specificity. Nearly all SMC-restricted protein genes contain the CArG box DNA sequence within their promoter and this sequence is also required for SMC gene transcription *in vivo*. Genes important for SMC phenotypic switching (e.g. genes important for migration, proliferation and ECM production) also contain CArG boxes within their promoters.[114] CArG boxes serve as a binding site for Serum Response Factor (SRF), a transcription factor.[115] However SRF expression is not limited to SMCs and serves many functions in different cell types including cell migration and cell-cell adhesion.[116]

Multiple pieces of evidence suggest that SRF binding to CArG boxes in SMCs is regulated at the level of the chromatin structure. First of all, the x-ray crystal structure of SRF showed that SRF requires several contacts within the minor groove of DNA. The minor groove would be obscured if DNA was wrapped around the nucleosome structure. Also from thermodynamic considerations, binding of SRF to the CArG box would be greatly inhibited if the CArG box was bound to nucleosomes (see McDonald *et al.*[117] and references therein).

Secondly, using EMSAs it was shown that SRF can bind to synthetic DNA with the same affinity as to DNA obtained from lysates of both SMC and non-SMC cell cultures. Conversely, by using ChIP assays, it was shown that SRF binds to promoter regions in SMCs much more effectively then to promoter regions in non-SMCs. This suggests that in SMCs the chromatin is in such a state that it is accessible to SRF.[118–121] This observation is supported by micrococcal nuclease digestion experiments, which showed that the CArG box of gene promoters in SMCs is more accessible to digestion then in non-SMCs.[122]

Finally, it was shown that histone H3 and H4 acetylation was increased in SMCs when compared to numerous different cell-types, including cultured endothelial stem cells. In addition, non-SMCs where enriched for the repressive chromatin marks H3K27Me3 and H4K20Me2 at SMC-specific promoter regions (see McDonald *et al.*[117] and references therein).

Evidence from diabetic mouse models suggests an important role for epigenetic regulation in the inflammatory phenotype of vSMCs. The recently discovered KDM, lysine-specific demethylase (LSD-1 or KDM1, a H3K27Me2 demethylase), was shown to play a role in the pro-inflammatory phenotype of vSMCs in diabetic mice.[123] In this mouse model levels of H3K4Me2 (a histone modification correlated with expression) were increased at the MCP-1 and IL-6 promoter. Upon stimulation with TNF-α there was recruitment of RNA polymerase II to the promoter and increased expression of MCP-1 and IL-6. Expression of LSD-1 was shown to be decreased in vSMCs. Knockdown of LSD-1 with siRNA increased inflammatory gene expression whereas over expression decreased inflammatory gene expression in vSMCs of these mice.

It may very well be possible that similar mechanisms apply in the case of atherosclerosis. As discussed in a previous section, vSMCs isolated from rat thoracic aorta, which were treated with the HDACi TSA, displayed an inhibition of vSMC proliferation.[82] It was subsequently shown that expression of p21WAF1 was induced

by this treatment. However, HDACi treatment had no effect on SMCs isolated from p21WAF1 knockout mice.

Together, these findings illustrate how epigenetic mechanisms play an important role in the processes that control differentiation and activation of hematopoietic cells, but also of vECs and vSMCs.[117,124]

Chemokines, their receptors and other genes involved in inflammation

Expression of immune response genes in vECs and vSMCs is a major determinant in atherosclerosis onset and development because it contributes to the vascular-immune crosstalk. The vascular wall modulates inflammation by the expression of numerous cytokines and chemokines. For many of these genes epigenetic components, influencing expression, have been identified. Below, some of the best-studied examples will be discussed in detail.

eNOS

As stated in the previous sections, many inflammatory processes are recognized by an epigenetic component. One of the best-studied cases of epigenetic regulation in the vascular wall involves the transcription of eNOS, an enzyme which is regarded as endothelial specific. Its catalytic product, Nitric Oxide (NO), was first identified as a vasodilator,[125] although recent evidence also suggest a role in inflammation. NO has been attributed roles in leukocyte adhesion and vSMC proliferation.[126] Furthermore, dysfunctional eNOS is believed to be implicated in atherosclerosis development.[127]

The human eNOS promoter does not contain a canonical TATA box, nor does it contain a proximal CpG island. Expression of eNOS is restricted to ECs *in vivo*. Various assays in both expressing and non-expressing cell types showed that a majority of the non-expressing cell types demonstrated transgene promoter activity.[128–130] In eNOS expressing ECs, eNOS promoter DNA was only slightly methylated or unmethylated, whereas non-expressing cells showed a high amount of DNA methylation. Treatment of non-expressing cell types with 5-azacytidine (a DNMTi) led to an increase in eNOS mRNA levels.[130] Later experiments showed the involvement of histone modifications in the expression of eNOS as well. ChIP showed enrichment of H3Ac, H4Ac and H3K4Me(2/3) in chromatin of the eNOS promoter in eNOS expressing cells. Treatment of non-expressing cells with the HDACi TSA

– effectively increasing histone acetylation – also led to an increase in eNOS mRNA (See Fish *et al.*[126] and references therein).

iNOS

Not only is eNOS regulated by epigenetic mechanisms, of interest is the observation that also Inducible Nitric Oxide Synthase (iNOS) is actively regulated by the epigenetic mechanisms.[131] This gene is expressed in macrophages, but also in ECs and vSMCs, under inflammatory conditions. It has been suggested that NO – derived from macrophages expressing iNOS – can result in apoptosis of vSMCs, promoting plaque instability.[132] A study performed by Mellott *et al.* showed that cytokine induction of iNOS resulted in a change of chromatin structure at the iNOS promoter.[9] Later work showed that the *NOS2A* gene, encoding iNOS, had high levels of DNA methylation and of the H3K9Me2 and H3K9Me3 repressive marks in non-expressing cells. Cell lines capable of iNOS induction had lower levels of CpG-methylation and histone 3 lysine 9 methylation at the *NOS2A* promoter. Treatment with a DNMTi resulted in an increase of iNOS mRNA.[131]

CCR5

CCR5 is important for plaque formation as it attracts T cells and mononuclear cells. CCR5 knock-out mice show less neointima formation and an increase in production of the anti-inflammatory IL-10 molecule by SMCs.[133,134] One of the ligands for the CCR5 receptor, CCL5 or RANTES, has also been shown to be involved in unstable angina pectoris (UAP).[135] UAP is generally the result of erosion or rupture of an atherosclerotic plaque. CCR5 is epigenetically regulated in T cells. In naïve T cells, lacking CCR5 expression, DNA of the CCR5 receptor is hypermethylated and the acetylation-level of histone-tails is strongly reduced in CCR5 promoter chromatin. When T cells are activated and express CCR5, DNA-methylation is significantly lowered combined with an increase in histone acetylation modifications. These results are extensively discussed in **chapter 3**.

The observations above indicate that epigenetic mechanisms play a key role also in regulation of (immune) genes in cells that participate in atherosclerosis and vascular remodelling, and in the crosstalk of immune cells and vascular wall components.

CCL11 (eotaxin)

In human atherosclerotic plaques, the chemokine CCL11 (also known as eotaxin) was shown by immunohistochemistry to be expressed.[136] In healthy tissue only negligible amounts of CCL11 were found. Polymorphisms within the CCL11 gene are also associated with the development of restenosis after PCI.[137] CCL11, an eosinophil chemoattractant, has been suggested to play a role in atherosclerosis development, although the precise relation remains to be elucidated.[136,138,139] At the same time, CCL11 is expressed by SMCs, while macrophages in the human atherosclerotic plaque express the CCL11 receptor, CCR3.[136] CCL11 transcription is induced by inflammatory TNF-α signalling and is mediated through NF-κB. TNF-α induces acetylation of histone H4 in the CCL11 promoter. This results in an open chromatin structure, promoting subsequent binding of the NF-κB subunit p65 to the CCL11 promoter (as shown by ChIP).[140] Notably, glucocorticoids repress CCL11 transcription through selective inhibition of histone H4 acetylation.[140] Although only histone H4 acetylation and no other epigenetic markers were investigated, this research provides a very strong indication for epigenetic regulation of the CCL11 gene.[140]

Epigenetics in (Vascular) Inflammation

KDM6B

De Santa *et al.* recently showed that inflammation and Polycomb-mediated gene silencing are linked.[141] Polycomb Group (PcG) proteins are important for the maintenance of a repressive chromatin that is stable over many cell divisions. As such, PcG proteins play a critical role in differentiation processes and maintenance of cellular identity. Gene silencing is mediated by the Polycomb Repressive Complex 2 (PRC2) through the H3K27Me3 chromatin mark, which is subsequently read by the PRC1 maintenance complex (reviewed by Kohler *et al.*[142]).

The JmjC-domain protein Jmjd3 (KDM6B, a H3K27 demethylase) is expressed in macrophages upon stimulation with bacterial products and pro-inflammatory cytokines.[141] Jmjd3 binds to Polycomb Group (PcG) target genes, regulates H3K27Me3 levels and therewith their transcriptional activity.[143] For the first time, an inducible enzyme was shown to erase epigenetic modifications. Later work showed that 70% of lipopolysaccharide-inducible genes in macrophages are Jmjd3 targets.[144] This provides an essential link between inflammation and reprogramming of the

epigenome and might prove to be of vital importance in chronic inflammation and autoimmune diseases, including atherosclerosis, in the near future.

Oestrogen receptor

Oestrogen receptors (ERs) are present in the coronary arterial wall on both SMCs and ECs.[145–147] ERs may play an important role in protection against atherosclerosis.[146] Deficiencies in oestrogen receptor alpha (ER-α) lead to accelerated atherosclerosis in men, furthermore ER-α mediates the loss of expression of some cytokine induced cell-adhesion molecules (see Miller *et al.*[148] and references therein). ER-α was shown to have a varying degree of methylation throughout the cardiovascular system.[149] In the same study it was shown that an age related increase of methylation occurred in the right atrium. Furthermore, higher degrees of methylation were found in coronary atheromas when compared to macroscopically normal tissues.

Similarly, the oestrogen receptor beta (ER-ß) also displays a correlation in methylation of its promoter and atherosclerosis.[150] Contrary to ER-α expression, expression of ER-ß correlates with atherosclerosis independent of age.[151] By comparing plaque vs. non-plaque tissue from the same vessels, it was recently shown that the ER-ß promoter has higher methylation levels in atherosclerotic lesions.[150] In this study, increased expression of ER-ß was also observed – *in vitro* – in ECs and SMCs after administration of a DNMTi (5-aza-2′-deoxycytidine) and a HDACi (TSA). These experiments provide supporting evidence for epigenetic regulation of the ER-ß receptor.

COX-2

Another example for the linkage between epigenetics and cardiovascular disease related inflammation is transcription of the cyclooxygenase-2 *(COX-2)* gene. TNF-α – as well as several other cytokines – can induce expression of COX-2, a protein associated with atherosclerosis development.[152] Cytokines (such as TNF-α and IFN-γ) can also induce (indirect) changes to the chromatin, allowing effector-genes to be expressed upon stimulation.[9] For COX-2 it was demonstrated that mRNA expression and protein synthesis can be repressed *in vitro*, in cell lines by the HDACi's sodium butyrate (NaBu) and TSA revealing a significant role for epigenetic components in Cox-2 expression.[153] These experiments were performed in a colon cancer cell line, and thus need to be confirmed in cells relevant for vascular

biology and atherosclerosis (e.g. vECs, vSMCs or monocytes) to extrapolate these results to atherosclerosis.

Transcriptional regulation of MHC molecules – the role of CIITA

Constitutive expression of MHC-II surface molecules is restricted to professional antigen presenting cells (APC) of the immune system. On all other cell types, their expressions can be induced in an environment rich in inflammatory cytokines – such as the atherosclerotic plaque – or upon activation such as in human T cells.[154] Therefore, under inflammatory, conditions MHC-II molecule expression can be induced on vECs and vSMCs, which normally are not expressing MHC-II.[155,156]

The 'master regulator' of MHC-II expression is the co-activator CIITA (Class II Transactivator)[157] Within the context of atherosclerosis, CIITA is of importance, since it has been shown to be involved in transcriptional regulation of collagen type 1.[158,159] Collagen 1 is one of the main components of extracellular matrix and is essential in cap formation and plaque stabilisation.[10]

Transcriptional regulation of *MHC2TA*, the gene encoding CIITA, is mediated through the activity of four independent promoter units (CIITA-PI through CIITA-PIV, Figure 1–3A).[160] These promoter units are employed in a cell type- and activation-specific manner. In aortic SMCs CIITA-PIII and –PIV are expressed after IFN-γ stimulation and CIITA is needed for IFN-γ–mediated repression of collagen type 1 genes (*COL1A1* and *COL1A2*).[158,159] Furthermore, collagen transcription is also repressed by the RFX family proteins. RFX1 only weakly interacts with the unmethylated collagen promoter, but binds with higher affinity when the promoter is in a methylated state.[159]

In MHC-II transcription, CIITA exerts its transactivating function through protein-protein interactions with a multi-protein complex, which is comprised of RFX, CREB/ATF and NFY (Figure 1–3B).[161–163] Together with CIITA, this complex acts as an enhanceosome driving transactivation of these genes.[161–163] In the MHC-enhanceosome, CIITA acts as a platform for recruitment of chromatin-modifying activities, which include KATs, HDACs and an arginine methyltransferase.[164–166] Interactions of these chromatin-modifying enzymes and CIITA result in higher-order structural changes within the chromatin, effectively activating or silencing MHC-II gene transcription.[165,167] A number of studies now have shown that epigenetic processes not only control MHC-II transcription, but also contribute to the activating/silencing of *MHC2TA* transcription. This is illustrated by the observation that in DC

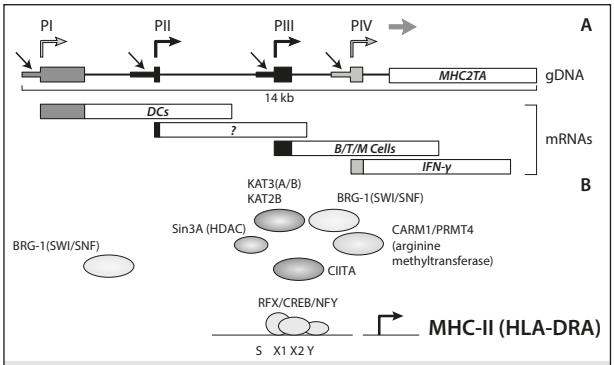

Figure 1–3. (A) Organization of the MHC2TA multipromoter region. Each promoter (indicated with black arrows) transcribes an unique first exon (shaded boxes). Transcription start sites are indicated with coloured, angled arrows. Promoter PI is mainly used for constitutive CIITA expression in dendritic cells, whereas PIII is mainly used in B cells, activated T cells and macrophages. Promoter PIV is mainly used for IFN-γ induced CIITA expression in non-bone marrow-derived cells. The function of PII is still poorly understood. (B) Factor assembly on the MHC-II promoter. Shown is the regulatory SXY module, to which the MHC enhanceosome binds.

maturation deacetylation occurs at the *MHC2TA* locus coinciding with transcriptional inactivation.[168] During DC differentiation histone acetylation of the type I promoter is increased. This increase was blocked by IL-10, inhibiting *MHC2TA* transcription.[169]

Non-histone Targets

Acetyltransferases/deacetylases and methyltransferases/demethylases discussed in the previous sections also target non-histone proteins which could lead to alterations in function of the targeted proteins or the generation of novel epitopes which now can evoke an adaptive (auto-) immune response.[170] Especially since NF-κB – playing crucial roles in inflammation – is showing up as one of the non-histone substrates. Both NF-κB and p53 are acetylated by KAT3A (CBP) and KAT3B (p300),[171–174] whereas p53 can also be acetylated by KAT2B (PCAF).[175,176]

As is the case with most genes, transcription of pro-inflammatory cytokine genes such as IFN-γ and TNF-α is positively regulated by transcription factors. However, these cytokines can also induce changes to the epigenome. Thus not only are cytokines under epigenetic control, cytokines themselves induce (indirect) changes to the chromatin, allowing effector-genes to be expressed upon stimulation. Activation of the IFN-γ pathway results in activation of e.g. NF-κB. NF-κB is found in the cytoplasm of unstimulated cells, together with its inhibitor, IκB, that prevents NF-κB from entering the nucleus.[177] Upon stimulation with inducers – e.g. reactive oxygen species (ROS) and lipid peroxidase products – IκB is rapidly degraded via the ubiquitin-proteasome pathway.[178,179] Hereupon, NF-κB is transported to the nucleus, where it can activate the transcription of various targets with

κB-elements in their promoter.[180,181] This process is known to be activated by KATs and repressed by HDACs (See Glozak *et al.*[170] and references therein). The p65 subunit of NF-κB is acetylated by KAT3A/B (P300/CBP) at lysines 218, 221 and 310.[182] The acetylated form of p65 shows weak affinity for IκB. HDAC3 in turn deacetylates p65, promoting association with IκBα and thereby nuclear export of NF-κB.[171] In addition to HDAC3, HDAC1 has been shown *in vitro* and *in vivo* to deacetylate p53.[183]

Besides to acetylation, methylation of non-histone proteins has also been observed. For example, methylation occurs at lysine 831 of the vascular endothelial growth factor receptor 1 (VEGFR-1), which is di-methylated by Smyd3 (a H3K4 methylase).[184] The biological function of methylation at lysine 831 is yet to be discovered. These examples perfectly illustrate how epigenetic and inflammatory processes are interwoven.

Thesis Outline

As discussed above, epigenetic control is one of the most fundamental regulatory systems within the cell. Not surprisingly, it fulfils essential roles in the regulation of inflammatory and immune responses involved in atherosclerosis pathology (summarized in Table 1–1). Precise determination of histone modifications at specific genes might prove its value in understanding gene expression profiles in vascular disease. Especially since small molecules can inhibit the function of histone modifying enzymes, altering the expression of genes. Therefore, modulation of epigenetic gene regulation might prove to be the therapeutic intervention of the future, especially in complex multi-factorial diseases such as atherosclerosis, multiple sclerosis and rheumatoid arthritis.

Table 1–1: Overview of genes regulated by epigenetic processes involved in atherosclerosis development. Genes for which evidence of epigenetic regulation is solely based on administration of inhibitors of histone modifying enzymes are not shown.

Gene	Type of Regulation	Reference
ec-sod	DNA Methylation	74
c-fos	Histone Code	80
CD36	Histone Code	81
IFN-γ	DNA Methylation & Histone Code	97–99
IL-4	DNA Methylation & Histone Code	106
Foxp3	DNA Methylation & Histone Code	94,107
eNOS	DNA Methylation & Histone Code	126,130
iNOS	DNA Methylation & Histone Code	9,131
CCL11	Histone code	140
CCR5	DNA Methylation & Histone Code	185
oestrogen receptor α	DNA Methylation	149
oestrogen receptor ß	DNA Methylation & Histone Code	150
CIITA	DNA methylation & Histone Code	164–169

In atherosclerosis much information has been gathered over the years regarding the individual components involved in plaque formation as discussed above. Systematic investigation however, of epigenetic phenomena in atherosclerosis has so far only been conducted in mouse models.[186,187] In **chapter 2** epigenetic modifications in human plaques are examined. The plaques investigated are cross-sectional for disease pathology. Subsequently, epigenetic regulation of monocytes is investigated in **chapter 3**. The transcriptional patterns of the genes encoding epigenetic modifying enzymes where studied during the differentiation of monocytes in macrophages and DCs. In **chapter 4** interventions by small molecule inhibitors is discussed by studying the transcriptional regulation of the CCR5 gene. **Chapter 5** illustrates the interplay between genetic and epigenetic code and discusses a SNP found in the upstream promoter region of the epigenetic modifying enzyme PCAF. Finally, in **chapter 6** the findings presented in this thesis will be summarized and discussed.

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