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Dissertação orientada por:
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The studies presented in this thesis were performed within the Metabolic & Genetics (Met&Gen) research group, at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Rita Castro, Ph.D, and Isabel Rivera, Ph.D. This work was supported by grant PEstOE/SAU/UI403 by FCT.

Resumo

As doenças cardiovasculares são a principal causa de morte nos países desenvolvidos. Assim sendo, é importante estudar os mecanismos moleculares causadores destas patologias, descobrindo novos mecanismos moleculares subjacentes e factores de risco, bem como novas formas de prevenção e terapias mais eficazes.

A desregulação do metabolismo da homocisteína, que leva ao aumento dos seus níveis circulantes no plasma (designada por hiperhomocisteinémia), está associada às doenças cardiovasculares. No entanto, os mecanismos inerentes à toxicidade vascular da homocisteína permanecem ainda por elucidar. Recentemente foi colocada a hipótese de que a acumulação do precursor da homocisteína, a S-adenosil-homocisteína (SAH), que ocorre em situações de hiperhomocisteinémia, poder conduzir à hipometilação celular, explicando assim a toxicidade vascular associada à hiperhomocisteinémia. A SAH funciona como inibidor da actividade enzimática da maioria das metiltransferases celulares que usam S-adenosilmetionina (SAM), o precursor metabólico da SAH, como dador de grupos metilo para as reacções de transmetilação que actuam sobre diversos compostos como DNA e proteínas, incluindo as histonas. A metilação do DNA e das histonas constituem mecanismos epigenéticos que regulam a expressão genética.

A Enhancer of Zeste Homolog 2 (EZH2) estabelece a trimetilação da lisina 27 da histona H3 (H3K27me3), uma marca epigenética envolvida na repressão de genes, através do aumento da condensação da cromatina. De entre os genes cuja transcrição é reprimida no endotélio, incluem-se aqueles que têm um papel na promoção da aterosclerose. A EZH2 é uma histona metiltransferase dependente da SAM e, consequentemente, a acumulação de SAH poderá afectar negativamente o funcionamento da EZH2. Assim sendo, se os níveis intracelulares de SAH aumentarem, o conteúdo de H3K27me3 celular poderá diminuir, com a consequente activação de genes pró-aterogénicos, o que contribuirá para o estabelecimento da aterosclerose e da patologia cardiovascular. Esta possibilidade constitui a hipótese de trabalho investigada no presente trabalho.

Para confirmar esta hipótese avaliámos se a acumulação intracelular de SAH levaria à diminuição de H3K27me3, quer *in vitro,* em células endoteliais humanas, quer *in vivo* usando células mononucleares do sangue periférico obtidas voluntariamente de doentes cardiovasculares e de controlos. Os nossos resultados indicam que, *in vitro,* a acumulação intracelular de SAH diminui a marca H3K27me3. Nas células endoteliais, a quantificação dos níveis intracelulares de SAM e SAH revelou uma acentuada diminuição da sua razão, confirmando uma capacidade de metilação celular diminuída, quando comparada com células controlo. Relativamente aos estudos *in vivo,* os resultados obtidos demonstraram que o conteúdo de H3K27me3 não diferiu de forma significativa nos doentes cardiovasculares e a na população controlo, apesar da razão SAM/SAH intracelular estar diminuída nos doentes cardiovasculares em relação aos controlos.

Embora os resultados *in vitro* corroborem a nossa hipótese, fomos incapazes de evidenciar *in vivo*, um relação inversa entre o ambiente de hipometilação celular e o conteúdo da marca epigenética dependente da EZH2 em células mononucleares do sangue periférico de doentes cardiovasculares. Assim, serão necessários estudos futuros, numa população-alvo mais alargada, para confirmar os resultados obtidos *in vivo*. Idealmente, estes estudos futuros deveriam incidir em doentes cardiovasculares que não estejam submetidos a nenhum tipo de medicação. Isto porque os resultados da avaliação de mediadores da aterosclerose, como o colesterol por exemplo, foram nitidamente influenciados pela terapia a que os doentes cardiovasculares estavam sujeitos aquando da colheita de sangue. Por outro lado, estes resultados foram, ainda assim, interessantes, comprovando a eficácia da medicação aconselhada aos doentes cardiovasculares, uma vez que estes revelaram, no geral, níveis de lípidos circulantes significativamente mais baixos do que os indivíduos controlo.

Adicionalmente, investigações futuras deverão alargar o estudo a outro tipo de células, como por exemplo células do tecido vascular. Com efeito, o conteúdo da marca epigenética H3K27me3 das células estudadas *in* vivo poderá não representar o conteúdo das células endoteliais. Só assim poderemos avaliar inequivocamente se a marca H3K27me3 está relacionada com o aparecimento e/ou desenvolvimento de um fenótipo endotelial pró-aterogénico. Deste modo poderemos também clarificar se a desregulação da actividade da EZH2 induzida pela SAH contribui efectivamente para o aparecimento da aterosclerose e doença cardiovascular.

Palavras-chave: doença cardiovascular, Enhancer of Zeste Homolog 2, epigenética, hipometilação, homocisteína.

Este texto não obedece às normas do Novo Acordo Ortográfico.

Abstract

Cardiovascular diseases (CVD) are the main cause of death in developed countries. Thus, it is important to study the mechanism that causes disease progression, uncover new risk factors, and new ways of effective prevention and therapeutic approaches.

Increased levels of circulating homocysteine are associated with cardiovascular disease. However, the mechanisms underlying this association remain elusive. Cellular hypomethylation caused by accumulations of the homocysteine precursor, S-adenosylhomocysteine (SAH), may explain homocysteine's toxicity. SAH inhibits the enzymatic activity of most cellular methyltransferases, which use S-adenosylhomocisteine (SAM), SAH's precursor, as a methyl group donor, acting upon several important compounds like DNA and proteins, including histones. Methylation of DNA and histones are epigenetic mechanisms that regulate gene expression and whose deregulation may cause diseases.

Enhancer of Zeste Homolog 2 (EZH2) is a histone methyltransferase that establishes trimethylation of lysine 27 on histone H3 (H3K27me3), an epigenetic mark associated with repression of pro-atherogenic genes in endothelial cells. EZH2 activity depends on SAM, therefore SAH accumulation may negatively affect EZH2 methyltransferase activity diminishing H3K27me3 content, and leading to the expression these pro-atherogenic genes, and to CVD. This possible mechanism constitutes the basis of our work.

To study this hypothesis, we evaluated whether SAH accumulation leads to H3K27me3 decrease either *in vitro,* in human endothelial cells, or *in vivo,* in peripheral mononuclear blood cells (PMBC) of CVD patients. Our *in vitro* results demonstrate that H3K27me3 content decreases if SAH accumulates in the cells. Yet, our *in vivo* results show no significant difference in the H3K27me3 content in PBMC from CVD patients and controls. Although our *in vitro* results corroborate our hypothesis, additional studies with a larger cohort of patients and with other cellular material, namely vascular tissue, are necessary in order to ascertain whether SAH-induced deregulation of EZH2, and consequent H3K27me3 endothelial content, contributes to atherosclerosis and CVD.

Key words: cardiovascular disease, DNA hypomethylation, Enhancer of Zeste Homolog 2, epigenetics, homocysteine.

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Este texto não obedece às normas do Novo Acordo Ortográfico.

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Abbreviations

Adenosine-2,3-dialdehyde	**ADA**
Angiotensin Converting Enzyme	**ACE**
Apoliprotein A-I	**apoA-I**
Apoliprotein B	**apoB**
Cardiovascular Diseases	**CVD**
C-reactive Protein	**CRP**
Cytosine preceding Guanine	**CpG**
Diethylpyrocarbonate	**DEPC**
DNA methyltransferases	**DNMT**
Dr. Joaquim Chaves Laboratories	**JCLab**
Endothelial Cells	**EC**
Endothelial Dysfunction	**ED**
Enhancer of Zeste Homolog *2*	**EZH2**
High-density Lipoprotein	**HDL**
High-density Lipoprotein cholesterol	**HDL-c**
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Núcleo de Prestação de Serviços de Bioquímica, FFULisboa	**NPSB**
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Soluble Vascular Cell Adhesion Molecule-1	**sVCAM-1**
Total Homocysteine	**tHcy**
Trimethylation of histone H3 at lysine 27	**H3K27me3**
Vascular Cell Adhesion Molecule-1	**VCAM-1**
Vascular Smooth Muscle Cells	**VSMC**

I – INTRODUCTION

1. Cardiovascular Disease

Cardiovascular diseases (CVD) are a group of diseases resulting from dysfunctional settings of arteries, veins, and heart[1]. Within this context, CVD morbidity and mortality are usually caused by ischemic heart disease, stroke, and congestive heart failure[2], and the amount of deaths rises with age[3].

CVD is considered the leading cause of death in developed countries[4], causing 17.1 million deaths every year[5], and this number is expected to grow up to 23.6 million by the year of 2030[6]. In Portugal, the percentage of deaths attributed to CVD by gender is higher in women than in men, as illustrated in Figure 1, where the percentage of death due CVD in 2013 is shown.

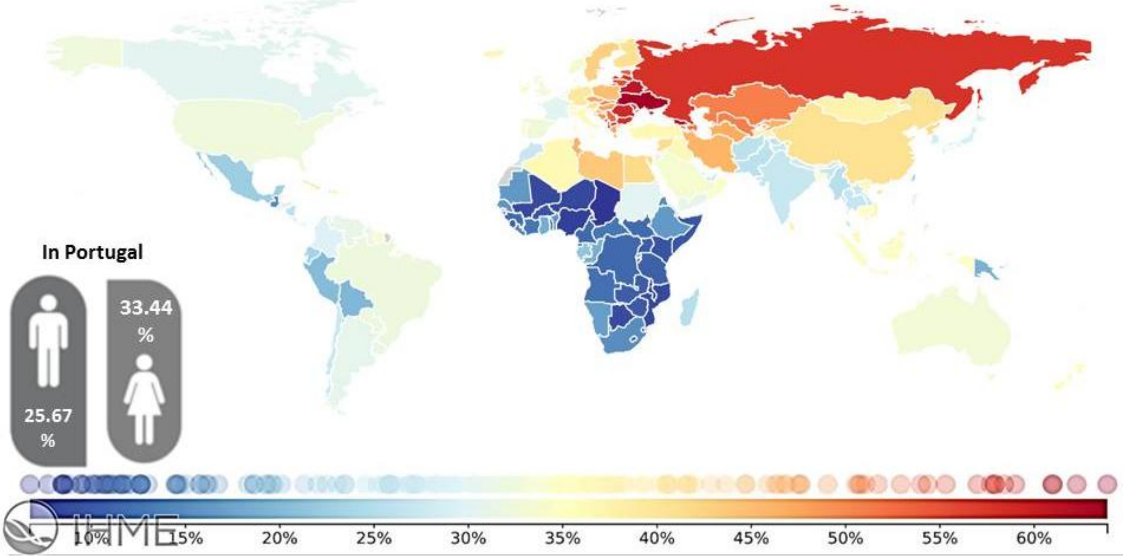

Figure 1. Percentage of deaths due to CVD in relation to total deaths in 2013.
Results worldwide correspond to CVD death percentage in relation to total deaths at a national level. The color bar scheme indicates the percentage of CVD death rate, from the lowest, represented in dark blue, to the highest, represented in red. Results of Portugal correspond to CVD death percentage in relation to total deaths per gender.
Abbreviation: CVD, cardiovascular disease.
Data source collected from references[7,8].

In approximately ten per cent of cases CVD are an heritable condition[9], but there are numerous non-heritable risk factors contributing to CVD, such as hypertension, smoking, diabetes, bacterial infections, inappropriate vitamin consumption, abnormal blood cholesterol, high blood pressure, lack of exercise, obesity or overweight, aging, and high plasma levels of homocysteine (Hcy)[1,6,10–15]. Air pollution has also shown to increase the risk for CVD morbidity and mortality, even though it depends on the individuals' ethnicity[16].

CVD develops after a prolonged asymptomatic phase[17] and negative cardiovascular events may occur in the absence of notorious risk factors[18], therefore, it is important to find new ways to diagnose and prevent these group of diseases.

The cardiovascular systems is composed by the heart, connected to a network of arteries, veins, and capillaries[19]. Different cell types constitute the vessel wall, such as smooth muscle cells, and endothelial cells (EC)[20]. The endothelium is composed by a monolayer of EC, coating the entire vascular system[21]. In normal conditions, the endothelium is in a quiescent state, with a turnover rate from months to years. It conducts the blood ensuring the nutritional support for organs, and regulates vascular tone, platelet activity, and blood vessel formation and growth. The endothelium also plays a role on innate and adaptive immunity, by regulating leukocyte adhesion and hemostasis[22–25]. It also limits clot formation, by maintaining blood's fluidity, and functions as a barrier between blood and surrounding tissues[24].

1.1. Endothelium dysfunction, atherosclerosis, and CVD

The homeostasis of the endothelium may be disturbed by metabolites, hormones[23] and by microbiological, immunological or mechanical insults, resulting in vascular injury[26]. Upon injury, the endothelium will undertake major changes[27], causing the reshaping of the vessel wall, both in size and composition[28]. In this process, the endothelial barrier permeability is disrupted[29], leading to arterial stiffness, and to endothelium dysfunction (ED)[19]. ED is characterized by vasoconstriction, vascular inflammation, upregulation of cytokines and adhesion molecules, and decreased bioavailability of nitric oxide (NO)[30,31]. NO is an important molecule with vasodilatory properties and cardioprotective effects[31]. The reduction of NO bioavailability occurs due to NO synthase disruption[32], as NO is synthesized from ʟ-arginine *via* endothelial NO synthase (eNOS), which has anti-atherogenic functions[33]. ED is linked with eNOS dysfunction[34].

ED is the initial stage of atherosclerosis, which is the leading cause of CVD[24,35]. Atherosclerosis is a multifactorial disease that progresses with age, usually begins in youth and continues asymptomatic until a later point in life[36,37]. It develops in the intima of middle and large size arteries, mainly in vessel bifurcations[38] and inner curvatures[39], mostly in areas of disturbed blood flow[40]. Alterations like vascular remodeling, calcification, oxidative stress, cell death, and in lipoprotein metabolism occur during atherosclerosis development[36]. Ages over 45 years for men and 55 years for women are a risk factor[41]. Aged vessels are more susceptible to develop vascular injuries and impaired angiogenesis, therefore they can easily develop atherosclerosis[19]. Until today, there is no way to reverse atherosclerosis by medical therapeutics[42].

The accumulation of low-density lipoprotein (LDL) in the subendothelial matrix is an initial step of atherosclerosis[43]. LDL can diffuse passively through EC junctions, and its interaction with apoliprotein B (apoB) seems to be important for its retention in the vessel wall[43], and consequent progression of atherosclerosis. ApoB is the principal apolipoprotein of LDL and the largest isoform present in the body[35], allowing the internalization of LDL particles by the cells, so they can absorb cholesterol[44]. LDL and other apoB-containing lipoproteins can accumulate in the intima[43]. LDL can undertake modifications like oxidization. Oxidized LDL (ox-LDL) can activate EC, monocytes and macrophages[38].

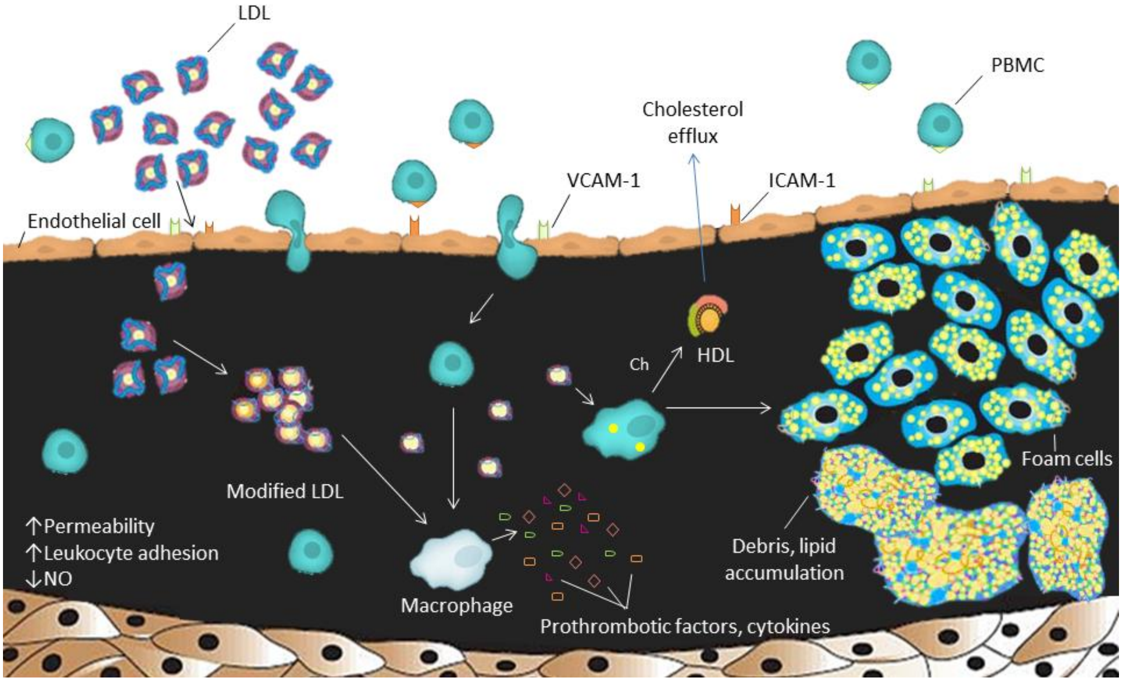

Figure 2. Schematic representation of the atherosclerotic process.
The structure of a normal artery is represented with the endothelial cell layer at the top, followed by the intima (exaggerated in this figure), and smooth muscle cells at the bottom. Changes in the blood flow affect EC, influencing their permeability and gene expression, leading to a decreased NO bioavailability. The retention of LDL and other lipoproteins are a key initiator event. LDL undergoes oxidative modification, which are, inhibited by HDL. Oxidized LDL stimulates the production of adhesion molecules (like ICAM-1 and VCAM-1) by EC, resulting in PBMC recruitment into the vessel wall. Oxidized LDL forms aggregates that are recognized by macrophages (derived from monocytes), that forms foam cells. When they die, foam cells contribute to a growing mass of debris and lipids that accumulate over time.
Abbreviations: LDL, low-density lipoprotein, NO, nitric oxide; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PBMC, peripheral blood mononuclear cells; Ch, cholesterol; HDL, high-density lipoprotein; EC, endothelial cells.
Adapted from references[43,45].

The upregulation of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), will favor the adhesion of peripheral blood mononuclear cells (PMBC - namely lymphocyte and monocytes) to the endothelium[31], as shown in Figure 2. Moreover, the dysfunctional EC phenotype will increase the expression of pro-inflammatory cytokines, promoting the entrance and retention of cholesterol containing LDL in the artery wall and modifications of LDL structure can increase the expression of adhesion molecules in EC[46–48]. LDL is composed by phospholipids and free cholesterol, and acts as a cholesterol transporter[44].

These accumulations can occur over time[42], as lipoprotein retention seems to be a self-perpetuating process[35], maintained by the dysfunctional EC phenotype[46]. This process results in EC activation, and acts as a chemoattractant for monocytes[38], and can also be an indicator of oxidative stress[18].

Atherosclerosis is characterized by high plasma concentrations of cholesterol[49], that leads to the formation and growth of the atherosclerotic plaque[41]. These plaques are formed by accumulation of fibrous materials, cell debris, minerals, and lipids[50]; infiltration of immune cells leads to the establishment of a collagen fibrous cap[45]. Proliferation and migration of vascular smooth muscle cells (VSMC) can occur, as well as accumulation of collagen and proteoglycans, resulting in blood vessel obstruction[28].

In opposition to LDL, high-density lipoprotein (HDL) has a protective function against atherosclerosis. HDL is responsible for the cholesterol efflux from macrophages, through the action of apolipoprotein A-I (apoA-I), transporting the cholesterol back to the liver[51]. From there, HDL go to the intestine where apoA-I is liberated from lipids, so it can be attached to new HDL particles, or it is degraded in the kidneys[52]. HDL may have a protective role upon ED, by enhancing NO release, preventing apoptosis, and reducing oxidative damage[29,51]. Nevertheless, HDL seems to function as a chameleon-like lipoprotein, having anti-inflammatory functions in the absence of an acute phase response or systemic inflammation, and promoting inflammation in the reverse case scenario[53].

With the progression of atherosclerosis, deposits of C-reactive protein (CRP), an innate immune response protein, can also be found in the intima[54,55]. CRP may affect the expression of endothelial cell adhesion molecules, chemokine production and contributes for EC’s apoptosis[21]. CRP levels may depend on the individuals genetic variation[56].

Besides CRP, fibrinogen is also associated with atherosclerosis development[15]. High levels of plasma fibrinogen are found in patients with acute thrombosis, and acute coronary syndrome[57]. Fibrinogen is a complex hexamer composed by α, β and γ chains[58], and it is the major plasma coagulation factor[59]. The isoform γ’ is associated with atherothrombotic events[60].

As the plaques grow, EC proliferate[43] and plaques can extend beyond into the arterial lumen[61], being able to form a necrotic core[45]. The lesions will grow continuously until a critical point, leading to the narrowing of the lumen (stenosis). At some point, the dilation of the artery can no longer compensate stenosis[49], macrophages will become apoptotic[42] and will destabilize the atherosclerotic plaque[62]. The plaques can grow enough to block the blood flow, leading to vein occlusion and eventually resulting in adverse vascular events as myocardial infarction (MI) or stroke[43]. Alternatively, the degradation of the fibrous cap may lead to plaque rupture[43,63], releasing prothrombotic factors, promoting thrombosis[38,41]. The beginning and development of CVD complications depend on the interplay between inflammation response and oxidative stress[64]. If the inflammatory response continues, the artery wall will thicken and harden[41], and the number of macrophages and lymphocytes will increase, leading to the release of cytokines, chemokines, growth factors, and hydrolytic enzymes. Over time, this could lead to focal necrosis[49].

Elevated shear stress can lead to platelet aggregation and further plaque rupture. On the other hand, a low shear stress permits the formation and development of atherosclerotic plaques[10]. Activated EC will also interact with leukocyte through the same adhesion molecules and through pro-inflammatory cytokines, like IL-6 and -8[29]. Monocytes produce IL-1β, a powerful cytokine, and high levels of IL-1β seems to contribute for atherosclerosis development[65].

2. Homocysteine Metabolism

Homocysteine (Hcy) is formed during the metabolism of methionine (Met)[66], an essential amino acid found in foods with high protein content[1].

Met is transformed in S-adenosylmethionine (SAM), a reaction catalyzed by methionine adenosyltransferase (MAT, EC 2.5.1.6), which has two isoenzymes: one distributed along all tissues and another present in the liver[67]. SAM is the universal methyl donor in the cell, and specific methyltransferases transfer SAM's methyl group to various molecules like DNA, RNA, proteins or lipids[68,69]. After the transfer of the methyl group, S-adenosylhomocysteine (SAH) is formed. SAH is a strong competitive inhibitor of the catalytic action of most SAM-dependent methyltransferases[67,70]. As such, high levels of SAH may decrease the cellular methylation status[71]. SAM/SAH ratio is used as an indicator of cellular methylation potential, and decreased SAM/SAH values indicate that the cell is hypomethylated[72].

SAH is further hydrolyzed into Hcy and adenosine by SAH hydrolase (SAHH, EC 3.2.2.9) through a reversible reaction that favors SAH synthesis. However, in physiological conditions, Hcy is quickly metabolized, favoring SAH hydrolysis[66,67,72].

Following its production, Hcy can be catabolized by the transsulphuration pathway, in which Hcy and serine are condensed into cystathionine, by the action of cystationine-β-synthase (CBS, EC 4.2.1.22), that uses pyridoxal phosphate (PLP), the active form of vitamin

B_6, as co-factor[67]. Cystathionine is converted into cysteine which will be further oxidized into urinary sulphates that are eliminated from the body[73], as illustrated in Figure 3. Interestingly, the transsulphuration pathway is absent in vascular and myocardial cells due to the lack of CBS, and for this reason the cardiovascular system is highly susceptible to Hcy accumulations and resulting toxicity[32].

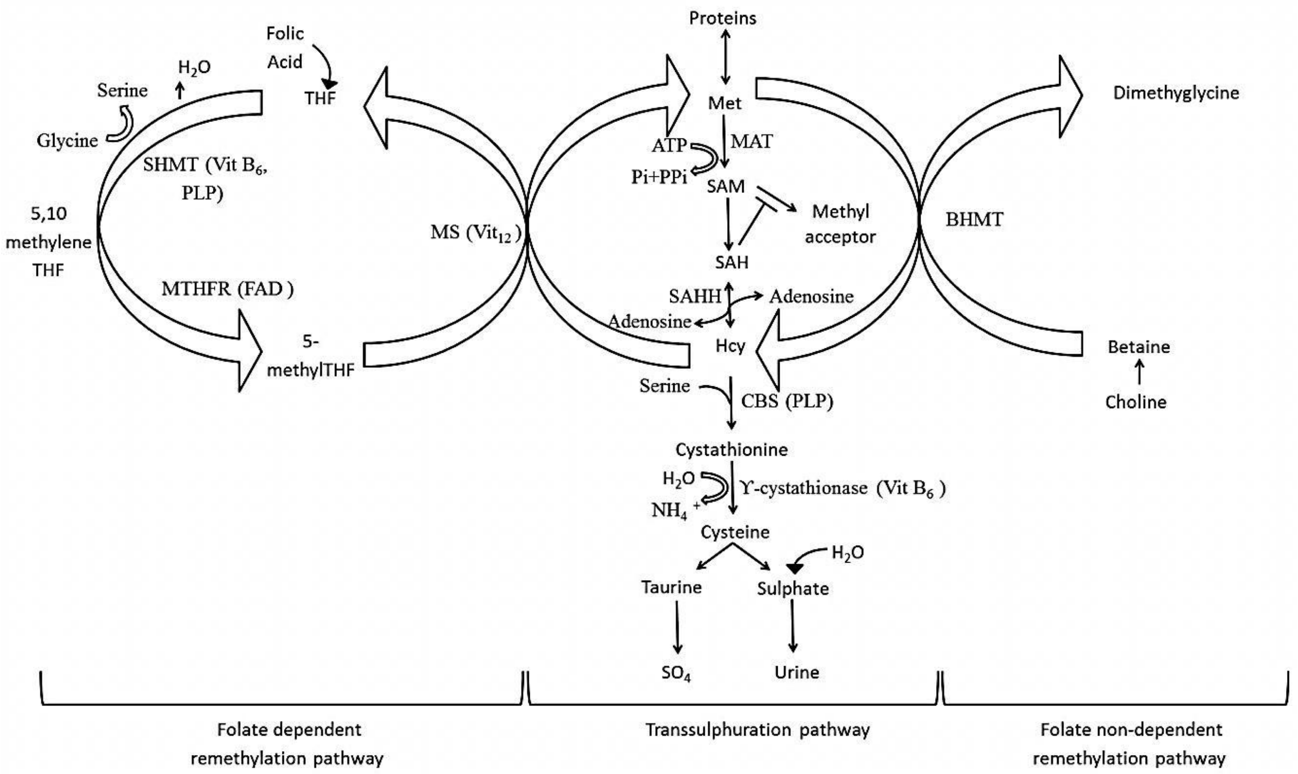

Figure 3: Homocysteine metabolic pathway.
Abbreviations: Met, methionine: MAT, ATP-ı-methionine S-adenosyltranferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; Hcy, homocysteine; THF, tetrahydrofolate; MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; CBS, cystathionine β-synthase; PLP, pyridoxal phosphate; Vit, vitamin.
Adapted from reference[67].

Met consumption only provides a portion of the body's needs and its synthesis requires a lot of energy; so, the organism depends on optimal Hcy recycling[1]. Hcy can be remethylated into Met through two ways: a folate-dependent pathway or a folate-independent pathway[68]. In the folate-dependent pathway, methionine synthase (MS, EC 1.16.1.8) is responsible for the conversion of Hcy into Met[74]. 5-Methyl tetrahydrofolate (5-methylTHF) supplies the methyl group used by MS for Hcy remethylation[66]. MS requires vitamin B_{12} as a co-factor and produces also tetrahydrofolate (THF)[75]. THF is further reduced of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), and then converted into 5-methylTHF, by the action of methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20)[75]. Mutated MTHFR can lead to Hcy accumulation[76].

The betaine-homocysteine pathway is a folate-independent remethylation cycle, in which Met is produced from Hcy by a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) that uses betaine as a methyl donor group. Betaine is then converted into dimethylglycine[67,68].

Adequate levels of B vitamins are mandatory to ensure a regular metabolism of Hcy[77]. Accordingly, deficiencies in B vitamins can lead to an increase Hcy circulating levels[74]. On the other hand, treatments with folic acid can lower the levels of accumulated Hcy[78].

Women tend to have lower Hcy circulating levels than men, probably due to the influence of sex hormones[1]. Also, Hcy levels increase progressively with age[79].

2.1. Homocysteine and link to disease

High Hcy plasma levels are associated with atherosclerosis[69], and Hcy is considered to be an independent cardiovascular risk factor[70]. Meta-analyses have shown that increases of 3 - 5 µmol/L of Hcy in the plasma can enhance 10 % - 27 % the risk for thromboembolism, myocardial infarction, or stroke[80].

The increase of plasma Hcy levels (hyperhomocysteinemia or HHcy) have been linked to impaired endothelial vasodilation[81], platelets aggregation, vascular inflammation[32], and ED[82]. A large cohort study, performed in Italy, demonstrated that high plasma levels of Hcy were linked to coronary atherosclerosis[32]. Subjects presenting HHcy also exhibited high levels of circulating SAH in the plasma and alterations in DNA methylation pattern in the lymphocytes[67].

Several mechanisms have been proposed to explain the vascular toxicity of Hcy. Various studies showed that increased levels of plasma Hcy are important for the initiation and progression of vascular disease, including ED, inhibition of methylation due to SAH accumulation, protein modification at a post-translational level, decreased NO levels, and increased oxidant stress[83].

The beneficial effects of folate supplementation as an Hcy-lowering therapy aiming to diminish CVD risk are still controversial. Oral administration of folic acid, either alone or in combination with vitamin B_{12} has shown to lower Hcy levels[84], and folic acid supplementation in HHcy subjects can ameliorate endothelial-dependent dilatation[32]. Also, vitamin B ingestion may lead to carotid plaque decay, even in patients with normal Hcy levels[85]. Other studies also confirmed the benefits of folate. For example, a Canadian cohort study revealed that low folate levels in the serum were linked to a higher coronary heart disease. Also, the US Physician Health Study showed that low folate levels were linked to a higher probability of acute myocardial infarction[86].

However, the NORVIT trial showed that treatments with folic acid and with vitamin B_6, although effective in diminishing plasma Hcy levels, did not lowered CVD risk[78]. Other studies also confirmed these negative results. For example, the SEARCH study, a large Hcy-lowering trial could not show the advantage of vitamin B consumption on reducing the CVD risk[84]. Another example is the VISP study, which did not show benefits of high-dose vitamin therapy on the outcome of stroke, coronary heart disease or death[85].

These controversial studies lead to an impasse in the scientific field of Hcy research. In fact, several authors claimed that it would be expected that treatments aiming to lower Hcy levels should reduce the associated cardiovascular risk[47]. Nonetheless, current views hold that several factors may explain these contradictory results. For example, most studies had a less than 5 years period of follow-up, an insufficient period of time to allow accurate conclusions about CVD risk decline, when atherosclerosis is a chronic condition that takes years to develop[87]. Importantly, SAH, and not Hcy, is now considered the main culprit for vascular toxicity associated to HHcy. In accordance, a study revealed that vitamin B supplementation, which can reduce Hcy plasma levels, did not decreased SAH levels[82].

2.2. Homocysteine and cell hypomethylation

Currently SAH, the Hcy precursor, which accumulates in the setting of HHcy, is considered a more accurate measure of CVD risk than Hcy[82,88]. As discussed before, under normal conditions, the reaction catalyzed by SAHH favors Hcy rather than SAH synthesis[67]. Nevertheless, if Hcy accumulates (due to genetic or nutritional causes), SAH will increase as well[82]. As referred above, SAH inhibits almost all SAM-dependent methyltransferases[70]. In fact, this inhibitory effect of excess SAH may explain the vascular toxicity attributed to HHcy. Thus, excess SAH may promote hypomethylation of DNA, proteins, and other small molecules. Several observations support this possibility. For example, in cultured cells, elevated SAH levels leads to gene-specific DNA hypomethylation[80]. Additionally, increased levels of SAH (and low SAM/SAH ratios) were observed in plasma and in erythrocytes from HHcy subjects with occlusive vascular disease[74]. Furthermore, the Met&Gen Group have demonstrated that intracellular accumulation of SAH results in DNA and protein hypomethylation. Moreover, a SAH-induced hypomethylating environment promotes ED and a pro-atherogenic phenotype of EC[89].

The methylation status of DNA and histone-proteins constitute epigenetic marks that modulate gene expression. As such, these observations support the hypothesis that SAH-induced epigenetic deregulation may underlie HHcy-related CVD.

3. Epigenetics

Epigenetic changes are heritable changes in gene expression that do not involve any alteration in the underlying DNA sequence[90] and that may be mediated through complex genome-environment interactions[91]. The environment can induce stable modification in the cell function that persist until adulthood, leading to a panoply of phenotypes, including disease risk[92]. Epigenetic changes affect gene function by altering the packing of chromatin (chromosomal DNA associated with histone proteins) and, consequently, the genome stability and the accessibility of regulatory proteins[69,71,93]. Epigenetic changes can also result in inappropriate gene silencing or expression[17,94].

Numerous types of human diseases, including CVD, have been linked to epigenetic alterations[28]. Environmental factors, like diet, can trigger epigenetic modifications associated with CVD, as exemplified by the Dutch Hunger Winter case, where it was observed that food restriction during pregnancy had long-lasting consequences for adult health, including increased risk for coronary heart disease[69]. Besides diet, other habits like smoking, exercise, and endocrine exposures may lead to transgenerational nongenomic modifications[92].

There are various types of epigenetic modifications and these include histone and DNA modifications[95], which are linked to Hcy metabolism.

3.1. DNA methylation

DNA methylation has an important role in various biological processes including development, inactivation of the X chromosome in females, imprinting, and suppression of DNA sequences that are considered as parasitic[96]. Methylation in repetitive gene sequences, like satellite DNA, are a necessary step for normal gene regulation, as it enables spatial positioning of the chromosomes[97]. DNA methylation is a powerful way to silence gene expression[93] and can also influence transcriptional elongation process, alternative splicing[98], and cellular growth, differentiation, survival and senescence[99].

In mammalian genomes, methylation deposition normally occurs in 5’ cytosine (C) that precedes guanine (G), or CpG dinucleotides. High density of CpG dinucleotides form the so-called CpG islands[100], the majority located in gene promoters, including housekeeping genes, tissue-specific genes and developmental regulatory genes[96]. DNA methylation generally leads to increased chromatin condensation and inhibition of normal DNA-protein binding, which leads to gene silencing[97,100].

DNA methyltransferases (DNMT) are SAM-dependent enzymes that establish and maintain DNA methylation patterns. Mammals possess different types of DNMT: DNMT1, and DNMT3A and DNTM3B. DNMT1 functions at a post DNA-replication level, maintaining the methylation pattern[100] as it copies the methylation pattern during mitosis[92], although it can also have *de novo* methylation capacities[40]. DNMT1 activity seems to be lower in EC exposed to Hcy[71], and in advanced stages of atherosclerosis, *DNMT1* expression decreases significantly, causing a global reduction of DNA methylation[101]. Also, *DNMT1* expression can be induced by disturbed blood flow[102].

DNMT3A and DNMT3B are associated with *de novo* methylation processes[93,100] which can occur in 3 - 5 % of adult somatic cells during mitosis[17]. In peripheral blood mononuclear cells (PBMC), low DNMT3A/B levels are associated with high SAH levels. Nevertheless, this relation between increased Hcy levels and decreased DNMT-mediated hypomethylation is not widely accepted[103].

It has been observed that *in vivo* inhibition of DNMT led to low levels of atherosclerotic plaque development[102]. In fact, alterations in the DNA methylation capacity leading to hypomethylation have been reported in CpG islands of patients with heart failure[69]. Also, DNA methylation was found to play an important role in atherosclerosis development[72], as DNA hypomethylation was reported in VSMC of advanced atherosclerotic plaques in human patients[17]. Also, *DNMT* expression seems to respond to disturbed blood flow, which in turn, regulates endothelial gene expression and function, which could lead to atherosclerosis[46].

Experiments with animals models demonstrated that DNA methylation has great implications in the development of atherosclerosis, as mice without *DNMT* genes showed DNA hypomethylation in their lymphocytes and an increase in inflammatory mediators[17].

DNA methylation patterns can be modified by various factors: nutrition, chromatin accessibility, HHcy state, oxidative stress, inflammation, aging, or SAM and SAH availability[93]. SAH has the ability to bind to DNMT with more affinity than SAM does, thus inhibiting their activity and subsequent DNA methylation reactions. This explains why patients with HHcy can show alterations in DNA methylation pattern of their PBMC[72]. Alteration of the DNA methylation pattern induced by SAH results in hypomethylation and enhances DNA's sensitivity to SAH toxicity[82].

Furthermore, hyper-mutability of distinct cardiac genes can be attributed to disturbed DNA methylation patterns[104]. Atherosclerotic patients showed DNA strands breaks and chromosomal damage in VSMCs and other circulating cells[105]. In coronary arteries of patients with advance atherosclerotic plaques, hypomethylation of genes involved in inflammation and immune responses was reported[106].

New sequencing chemistries have the advantage to reveal direct DNA methylation measures[107]. Recently, the epigenome-wide survey of human candidate genes that partake in atherogenesis revealed, in atherosclerosis patients, a genome-wide increase in DNA methylation levels, contrary to expectations[108,109]. Large genome sequencing studies also reported an increase of global DNA methylation levels as atherosclerosis evolves[9]. Although, genome-wide and age-related studies showed that DNA hypomethylation was predominant[9]. Differences in the methylation pattern in different studies can be explained by the response of different cell types upon atherogenesis[106].

Lastly, DNA methylation can enable histone modifications but on the other hand, histone methylation can facilitate DNA methylation[101], as methylated intragenic regions have a strong effect on histone modifications[98]. Histone modification can act as docking sites, recruiting chromatin-modifying enzymes, regulating specific transcriptional gene's states[110].

3.2. Histone methylation

Histones are nuclear proteins that form globular octamers of H2A, H2B, H3 and H4 assemblies, around which 146 base pairs of DNA segments are wrapped. These assemblies form the nucleosomes, which are the basic units of chromatin[111–113]. Nucleosome structure changes according to histone charges, which affect the chromatin structure by interfering with the contact between histones and DNA and between different histones[114,115]. Nucleosome disposition can block the access of transcription factors and polymerases[111], thereby blocking gene transcription[93,116].

Changes of histone charges usually occur in amino acid residues present in their tails[117]. Histones are susceptible to different modifications, including methylation[115].

The effects of histone methylation on transcription depend on the specific residues that are methylated. For example, methylation of lysines 4, 36 and 79 in histone H3 are mainly associated with active transcription[110], while methylation at lysines 9 and 27 are associated with gene repression. Furthermore, the methylation status of a single lysine residue can be determinant for gene expression[112]. In fact, lysines can be mono-, di-, or trimethylated[115], these marks are associated with both active and inactive chromatin, depending on the methylation degree and the position within the nucleosome and gene[112]. Trimethylated histone H3 lysine 4 (H3K4me3) is a mark of active promoters[110], while di- and trimethylated histone H3 lysine 9 (H3K9me2/3) residues are strongly associated with transcriptional repression[118].

Additionally, one of the most studied histone modifications is the epigenetic mark H3K27me3 (trimethylation of histone H3 at lysine 27), known for heritable gene silencing expression[110,114]. DNA hypomethylation and global alterations in histone tail can lead to an increase of H3K27me3 levels[119].

Histone methyltransferases (HMT) are enzymes with the ability to methylate histones and depend on SAM as the methyl donor compound. Although there are other HMT[100], we will focus on the one responsible for the catalytic activity towards lysine residues[112]: the Enhancer of Zeste Homolog 2 (EZH2).

3.2.1 Enhancer of Zeste Homolog 2

Polycomb group proteins (PcG) are widely expressed[120] and represent an evolutionary conserved multiprotein family that include the polycomb repressor complex 2 (PCR2)[121]. PcG are epigenetic regulators of transcription, leading to repression of gene expression[97]. EZH2 is a SET-domain (Su(var)3-9; E(z); Trithorax)[122,123] HMT, that represents the catalytic core of PCR2[99,124], which epigenetically silences gene expression[125]. Also, EZH2 relies on SAM as a methyl-donor compound[99]. EZH2 can associate with DNMT as they both might function with a similar mechanism for silencing gene expression. The interaction between EZH2 and DNMT seems to facilitate the binding to promoters of genes that are targets of EZH2[126].

PCR2 is known to respond to high nucleosome density, leading to a higher level of chromatin condensation, and therefore, gene repression[127]. In fact, H3K27me3 in repressed genes will recruit PCR2 to copy the methylation pattern, during DNA replication. This way, the methylation pattern will be conserved in both DNA strands even after cell division, assuring the cell's identity as it controls the gene expression profile[121].

H3K27me3-enriched repressive chromatin structure is crucial for stem cells to maintain their quiescent and undifferentiated status[128–130]. In a wide variety of cancer cells EZH2 is overexpressed[97,131]. In contrast to studies in cancer cells, little is known concerning EZH2 role in differentiated cells. Recent studies revealed that EZH2 epigenetically regulates EC proliferation, migration, and communication, and angiogenesis[125,132]. Additionally, EZH2 target genes consist of several mediators implicated in ED, including pro-inflammatory cytokines that induce endothelial cell expression of adhesion molecules[125]. The same phenotype that Met&Gen Group reports had attributed to excess SAH[89]. As such, these observations raise the possibility that deregulation of epigenetic control mediated by EZH2 may contribute to ED and CVD[125]. In support, a reduction in global histone H3K27me3 in atherosclerotic plaques was just reported[133].

In addition, the Met&Gen Group recently reported that, in cultured human endothelial cells, SAH decreases *EZH2* expression and reduces the content of the epigenetic mark H3K27me3. Furthermore, *EZH2* knockdown recapitulated the effects of excess SAH on endothelial activation, i.e., it induced up-regulation of adhesion molecules and cytokines.

These findings suggest that suppression of EZH2 activity by excess SAH may contribute to the vascular toxicity of HHcy[89]. This constitutes our working hypothesis that is illustrated in Figure 4.

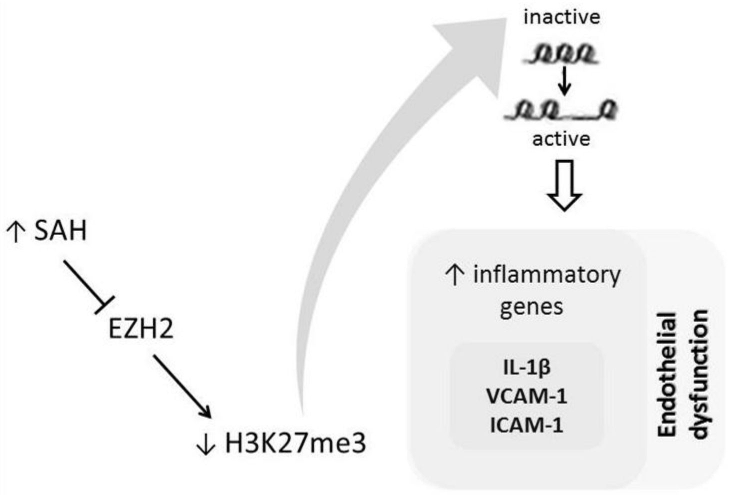

Figure 4: Potential mechanism(s) by which SAH causes endothelial activation.
SAH can inhibit EZH2 activity leading to a decrease in repressive histone mark H3K27me3, which suppresses the expression of various genes, including *IL-1β*. Exposure to excess of SAH activates the transcription of cytokines (such as IL-1β) and adhesion molecules (such as ICAM-1 and VCAM-1), contributing to endothelial cell activation, which leads to endothelial dysfunction.
Abbreviations: SAH, S-adenosylhomocysteine; EZH2, Enhancer of zeste homolog 2, H3K27me3, trimethylation of lysine 27 in histone H3; IL-1β, interleukin 1-β; VCAM-1, vascular adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1.
Adapted from reference[89].

II - OBJECTIVES

We postulate that hypomethylating stress induced by high levels of SAH will suppress histone H3 specific methylation, therefore contributing to a pro-atherogenic endothelial phenotype. We believe that a decreased content of the repressive epigenetic mark H3K27me3 induced by excess SAH will promote atherogenesis and CVD. Recently, Loscalzo and colleagues[134] validated the use of peripheral blood cells in the study of the pathophysiology of atherosclerosis-related diseases. Thus, and considering our working hypothesis, the objectives of this work are:

- to confirm, *in vitro*, the ability of SAH to decrease the H3K27me3 mark
- to determine the content of H3K27me3 mark in PMBC of healthy individuals and CVD patients
- to evaluate whether H3K27me3 mark is associated with plasma and PMBC markers of hypomethylation and atherosclerosis, and whether they correlate with CVD

III – METHODS AND MATERIALS

A. *In vitro* studies: incubation procedure in endothelial cells

Human umbilical endothelium cells (HUVEC)[135] are a cellular model widely used to study the atherosclerosis process[136], since their phenotypic changes are similar to those of the adult vascular endothelium[137]. To test the ability of SAH to diminish the endothelial H3K27me3 epigenetic content we performed *in vitro* studies in HUVEC.

A.1. Cell culture and treatments

HUVEC from pooled donors (Lonza) in passage 2 were cultured in T_{75} flasks with filter cap (Orange Scientific, Belgium), at 37 °C with 5 % CO_2, in a Hera Cell 150 incubator (Thermo Scientific by Thermo Fisher Scientic, Waltham, MA, USA). Cells were grown in EGM™-2 Medium containing SingleQuot™ Kit supplements, including antibiotic and antimitotic (Lonza) until 70 - 80 % confluency and were further harvested with a 1:3 split. In this process, the medium was removed, cells were washed twice with DPBS (Lonza) without calcium and magnesium, and 0.5 % trypsin – EDTA (Gibco™ by ThermoFisher Scientific, Waltham, MA, USA) diluted in DPBS (1:10) was added. After 2 min at 37 ^{o}C, the cells were collected and grown in fresh culture medium at 37 ^{o}C, which was replaced 12 h after tripsinization, and further, every 48 h.

Both DPBS and EGM™-2 Medium were filtered before usage, with the help of a stericup® and steritop™ vacuum-driven filtration TM PLUS system with 0.22 µm connected to a chemical duty pump, 220 V/50 Hz WP6122050 pore (Merck Millipore, Darmstadt, Germany).

Experiments were performed between passages five and eight and with cells 70 to 80 % confluent. Adenosine-2,3-dialdehyde (ADA), a SAHH inhibitor, was used to increase the intracellular levels of SAH[135]. Treatments with ADA were performed for 24 or 48 h at 20 µM. A sterile solution of ADA was used, which was obtained using a 5 mL Terumo® sterile syringe (Terumo Corporation, Japan) and a sterile syringe-driven 0.22 µm filter (Millex® from Merck Millipore, Darmstadt, Germany). Cells incubated in unsupplemented medium were used as control.

All cell manipulations were executed in a Holten Laminar flow hood HVR 2640 (Thermo Scientific).

A.2. Sample preparation

After the incubation period, the culture medium was removed and the cells were washed twice with DPBS (Gibco™ by ThermoFisher Scientific). Cell detachment was executed in 500 µL of DPBS using a 23 cm cell scrapper (Orange Scientific, Belgium); a process repeated two more times. All manipulations were performed on ice, outside the flow chamber. The cells were collected into a 2 mL sterile microtube (Eppendorf, Hamburg, Germany) and centrifuged at 4000 rpm for 2 min in a Hermle Z233M-2 centrifuge (Labnet International, Inc, Edison, USA). Part of the pelleted cells was immediately deproteinized by adding 100 μL of 10 % perchloric acid (v/v) and stored at -80 ^{o}C in an ultra-low temperature freezer (Panasonic Biomedical Sales, Japan) until SAH and SAM analysis. The remaining cells were also stored at -80 ^{o}C until further Western blot analysis.

A.3. SAM and SAH analysis

Deproteinized cell extracts were sent to the Metabool Laboratory at VU Medisch Centrum, in Amsterdam, The Netherlands, to measure SAM and SAH content by tandem mass spectrometry, as previously described[138].

A.4. Western blot analysis

A.4.1. Histone extraction

Histone can be extracted following nuclei isolation[139]. With this purpose, the cells were lysed by hypotonic lysis buffer (HLB: 10 mM Tris-HCl pH 8.8; 1 mM KCl; 1.5 mM $MgCl_{2;}$ 1 mM diothiothreitol; 25x complete protease inhibitor; in diethylpyrocarbonate (DEPC) treated water) followed by 30 min incubation on ice in a Rocker 25 agitator (Labnet International, Inc). Protein extraction is usually executed at 4 ^{o}C, as they can easily denaturate once released from cells[140].

After the incubation with HLB, the microtubes containing the cell lysates were centrifuge at 10000 g for 10 min at 4 ^{o}C. To the obtained pellets, 50 μL of 0.25 M H_2SO_4 were added and lefted incubating on ice in a Rocker 25 agitator for at least 2 h. At this point, most of nuclear proteins had precipitated[139]. Then 200 μL of 100 % ethanol was added and a centrifugation at 1000 g for 10 min at 4 ^{o}C was performed. The obtained pellet (histones) was washed 3 times, each with 500 μL of cold PBS (Sigma-Aldrich, St. Louis, Missouri, USA) followed by centrifugation at 1000 g at 4 ^{o}C for 10 min. Lastly histones were resuspended in 100 μL of 0.1 % PBS-Triton and stored at -20 ^{o}C until quantification.

A.4.2. Histone quantification

Before subsequent analyses, samples were sonicated for 50 cycles, with 100 % amplitude using a UP100H Ultrasonic Processor (Hielscher Ultrasound Technology, Teltow, Germany).

Histones were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) according the manufacturer’s instructions. This method is based on the reduction of Cu^{2+} to Cu^{1+} by proteins followed by colorimetric detection of Cu^{1+} by

BCA[141]. BCA readings were executed using a microplate reader ASYS Expert PLUS (LaboControle, Oeiras, Portugal).

A.4.3. Western blotting

Histone samples were diluted in loading buffer were denatured at 95 °C for 7.5 min in the thermocycler Swift™ Maxi® (ESCO®, Singapore). Subsequently, histone samples were loaded in a 20 % (w/v) SDS/polyacrylamide gel (Nzytech Company, Lisbon, Portugal) in a Mini-Protean® Tetra Vertical Electrophoresis Cell (Bio-Rad, California, USA), and ran under reducing conditions, according to the method described by Laemmli[142]. Electrophoresis conditions were maintained at 15 mA, per gel, connected to a PowerPac™ Basic (Bio-Rad).

Histones were then electrotransferred onto a 0.45 µm pore membrane (Amersham Hybond™-P PVDF; GE Healthcare, Buckinghamshire, UK), which was stirred in 100 % methanol for about 20 seconds prior to use.

The transference process was undertaken using a Mini Trans-Blot® Cell Kit (Bio-Rad), with cooled transfer buffer circulating at 600 rpm in a Heidolph MR 3001 agitator (Heidolph, Schwabach, Germany), at 124 mA for 45 min, and connected to a PowerPac™ Basic (Bio-Rad). The transfer buffer consisted in 25 mM Tris-HCl pH 8.2 - 8.7, 192 mM glycine and 20 % (v/v) methanol.

Further, membranes were incubated in a 5 % blocking solution (1 % (w/v) milk fatty acids-free diluted in TBS (14.2 % NaCl (w/v), 10 % Tris-HCl 1 M pH 7.5, (v/v)) with 0.1 % Tween® 20 (v/v) (Sigma-Aldrich, St. Louis, USA), for 2 h in a Mini LabRoller™ Dual Format Rotator at RT (room temperature). TBS with 0.1 % Tween® 20 (v/v) was used to wash the membranes. Then, the membranes were incubated overnight at 4 °C with anti-rabbit Histone H3K27me3 antibody (pAb) (1:500, Active Motif, Carlsbad, California, USA) using a Mini LabRoller™ Dual Format Rotator.

After another washing with TBS with 0.1 % (v/v) Tween® 20, the membrane was incubated with anti-rabbit immunoglobulin G, HRP-linked antibody (1:2000, Cell Signaling Technology®, Danvers, MA, USA) for 1 h 45 min at RT in a Mini LabRoller™ Dual Format Rotator followed by washings (4 times) with TBS with 0.1 % Tween® 20 (v/v), in a Rocker 25 agitator at 100 rpm, for 5 min each. Immunocomplexes were revealed using the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) in 1:1 concentration, using the Chemidoc™ XRS+ System (Bio-Rad).

The membranes were stripped off their antibodies in a Rocker 25 agitator at 100 rpm. Each step takes 5 min, involving a washing step with water, incubation with the stripping solution (25 % acetic acid (v/v), 0.5 % Tween® 20 (v/v), 0.05 % sodium dodecyl sulfate (w/v), 0.75 % glycine (w/v)), two series of washings with water, and one last washing with TBS with 0.1% Tween® 20 (v/v).

The membrane was blocked again and washed, as previously described above and was further incubated with anti-mouse Histone H3t antibody (mAb) (1:20000, Active Motif) overnight at 4 °C. After proper washing and incubation with peroxidase-conjugated anti-mouse secondary antibody IgG+IgM (H+L) (1:4000, Jackson ImmunoResearch, West Grove, PA, USA), immunocomplexes were detected as previously described.

All antibodies specified in this protocol are described in Table 1.

Table 1. List of primary and secondary antibodies used in immunoblot assays.

Antibody	Brand	Reference	Dilution
Histone H3K27me3 (Host: rabbit)	Active Motif	39157	1:500
Histone H3t (Host: mouse)	Active Motif	39763	1:20000
Anti-Rabbit IgG, HRP-linked Antibody	Cell Signaling Technology®	7074	1:2000
Anti-Mouse: affinipure goat anti-mouse IgG+IgM antibody	Jackson ImmunoResearch	115-035-044	1:4000

A.4.4. Image and Statistical analysis

All images were captured using Quantity One® 4.6 software for Windows (Bio-Rad), exposing the membrane in the Chemidoc XRS+ System, using the chemiluminescent option for incubations with Histone H3t antibody, and the chemiluminescent high-sensitive option for incubations with Histone H3K27me3 antibody. Membrane expositions where stopped when the signal reached saturation point.

Images where processed and analyzed by using ImageJ 1.48v software from National Institutes of Health, (USA); converted into 8-bit format. The percentage of H3K27me3 was normalized to H3t percentage. One way ANOVA followed by post hoc Bonferroni's multiple comparison test was performed in GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, "www.graphpad.com".

B. *In vivo* studies: characterization of CVD patients and healthy individuals

To determine whether EZH2 suppression and decrease of H3K27me3 content were associated with markers of hypomethylation and CVD, we studied plasma and PBMC from CVD patients and controls.

B.1. Participants and sample collection

We studied 14 atherosclerotic vascular patients [mean age 56.08 ± SD 10.56 years] and 15 healthy controls [mean age 52.63 ± SD 6 years]. The patients group was constituted by 4 women and 10 men; the control group was constituted by 8 women and 7 men. Cases were recruited by Prof. Doutor Fausto Pinto’s team (Centro Hospitalar Lisboa Norte, Lisboa, Portugal) amongst patients who had been admitted to Hospital de Santa Maria, Lisbon, Portugal with a diagnosis of acute coronary syndrome; samples were collected at least 6 months after the occurrence. Controls were selected among staff of the Faculty of Pharmacy from the Universidade de Lisboa whose lifestyle details (i.e., alcohol consumption, medication, smoking, physical exercise, and personal, and family histories) were established by use of standardized questionnaires and protocols. The criteria for inclusion in the control group were: normal hematology and liver/renal function tests and no history of vascular pathology. Exclusion criteria for both groups were metabolic, hepatic, or renal pathology; cancer; alcohol or drug abuse. All patients, but none of the controls, were under therapy with the following drugs: non-steroidal anti-inflammatory + antiplatelet + angiotensin converting enzyme (ACE) inhibitors + statins. Written informed consent was obtained from all participants, and the study was approved by Local Ethical Committees.

B.2. Preparation of biological samples

A total of 50 mL of EDTA-blood samples were collected after a fast of 12 h; plasma and PBMCs were isolated no more than 4 h after the blood collection[66]. We used 10 mL of blood for plasma separation by centrifugation at 1800 rpm at 8 ^{o}C for 10 min, using an Eppendorf 5810R centrifuge (Eppendorf). From the obtained plasma, 750 µL were immediately prepared to preserve SAH and SAM by adding 750 µL of 10 % perchloric acid (v/v). The remaining plasma was further used to perform the biochemical analysis detailed in section B.3. All preparations were stored at −80 ^{o}C until use. From the remaining blood, PBMC were immediately isolated using density gradient centrifugation (section B.2.1). The obtained cells were further used to measure intracellular SAM/SAH levels (section A.3) and to isolate histones and RNA (section B.2.2) for the studies detailed in B.3 and B.4, respectively.

B.2.1. PBMC isolation

PBMC were isolated from the blood samples by density gradient centrifugation[143], using LEUCOSEP™ tubes (Greiner Bio-One GmbH, Kremsmünster, Austria), that were put on ice and filled up to the filter with Ficoll-Paque™ PLUS (GE Healthcare, Buckinghamshire, UK). After homogenizing carefully, the EDTA-blood was layered on the LEUCOSEP™ tubes and centrifuged at 2700 rpm for 20 min, with no brake. Differential migration during centrifugation resulted in the formation of layers, containing different cell types[143], as illustrated in Figure 5.

The bottom layer contains red blood cells and granulocytes, and the layer above contains the Ficoll-Paque™ PLUS solution. Because of their lower density, PBMC were found at the interface between the plasma and the Ficoll-Paque™ PLUS solution[143].

The PBMC were recovered with a plastic sterile Pasteur pipette (FL Medical, Torreglia, Italy) and were divided into two 15 mL Cellstar® tubes (Greiner Bio-One GmbH). The tubes are centrifuged at 4000 rpm for 7 min at 8 °C. The supernatant was discarded and 3 mL of Hanks Solution (Sigma-Aldrich) was added to the PBMC pellets. The tubes were centrifuge again, in the same conditions as previously described, and the supernatant was discarded. All centrifugations up to this point were executed in an Eppendorf 5810R centrifuge (Eppendorf).

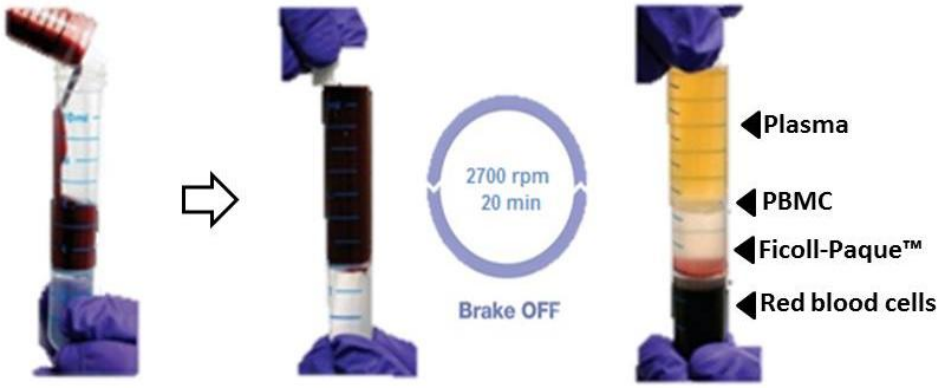

Figure 5. Isolation of leukocytes from EDTA-blood using Ficoll-Paque™ density gradient centrifugation.
Abbreviations: PBMC, peripheral blood mononuclear cells.
Adapted from reference[144].

The PBMCs' pellets were washed twice with 750 µL of Hanks Solution, and then transferred into a 2 mL sterile microtube (Eppendorf). After a centrifugation at 12000 rpm for 20 seconds, the supernatant discarded and 750 µL of cold DEPC-treated water were added to ressuspend the pellet, and another 750 µl of Hanks solution were added to the microtubes, which were centrifuged again in the same conditions. These last two centrifugations were executed in a Hermle Z233M-2 centrifuge (Labnet International, Inc).

100 µL of cold PBS were added to one of the microtubes, which was immediately frozen at -80 °C until further SAM/SAH determinations (after immediate deproteinization with 200 µL of 10 % perchloric acid (v/v)). To the other microtube, 1000 µL of RNAlater® solution (Ambion, ThermoFisher Scientific, Waltham, USA) was added, and followed by an overnight incubation at 4 °C. The microtube was stored at -80 °C, until further histone and RNA extractions, as detailed in section B.2.2.1 and B.2.2.2.

B.2.2. Isolation of histones and RNA

A methodological approach illustrated in Figure 6 was optimized in order to allow the isolation of histones and RNA from the same PBMC sample conserved in RNAlater® solution (Ambion) or in PBS.

For PBMC conserved in RNAlater® solution, 500 µL of cold PBS (Sigma-Aldrich) were added, followed by a centrifugation at 2500 g for 10 min in an Eppendorf 5810R centrifuge (Eppendorf). After discarding the supernatant, 1000 µL of HLB were added to the PBMC, which were incubated for 30 min on ice in a Rocker 25 agitator (Labnet International, Inc). Afterwards, the samples were centrifuged at 10000 g for 10 min at 4 ^oC. The obtained pellet was further used for histone extraction by adding 50 µL of 0.25 M H_2SO_4, whereas the supernatant was transferred to another 2 mL sterile microtube (Eppendorf), and kept on ice, until RNA extraction.

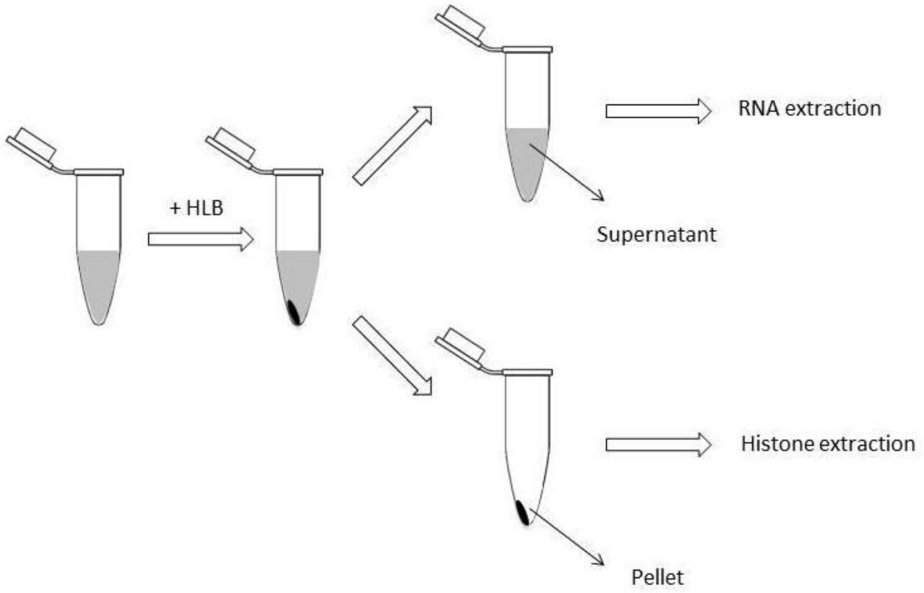

Figure 6. Simplified scheme of RNA and histone extractions executed from the same sample of PBMC. Abbreviations: HLB, hypotonic lysis buffer.

B.2.2.1. RNA extraction

To the supernatant obtained in B.2.2., 1 mL of Isol-RNA lysis Reagent (5 PRIME, Hilden, Germany) was added. After 5 min at RT, 200 µL of chloroform was added; the microtubes were agitated and incubated at RT for 2 min. During this incubation period, a separation into phases occurred: RNA remain in the aqueous phase, proteins stay down in the organic phase, in between stands DNA[145]. The tubes were then centrifuged at 4 ^oC for 15 min at 12000 rpm and the obtained supernatant, approximately 500 – 700 µL, was transferred to a new 2 mL sterile microtube (Eppendorf). An equal volume of 100 % isopropanol was added for RNA precipitation and after 10 min at RT, the tubes were centrifuged for 10 min at 4 ^oC at 12000 rpm and the supernatant was discarded. RNA pellet was washed with 1 mL 75 %

ethanol in DEPC-treated water (v/v), followed by 5 min centrifugation at 4 ^{o}C at 12000 rpm. Lastly, the supernatant was discarded and the tubes were left to dry at RT, in an upside-down position. Then 50 – 100 µL of DEPC-treated water, and after 10 min incubation at RT, the tubes were stored at -80 ^{o}C.

B.2.2.2. Histone extraction

After RNA extraction, we proceed to histone extraction by adding 200 µL of 100 % ethanol and following the protocol as described in section A.4.1.

B.3. Biochemical analysis

Part of the plasma (1 mL) obtained in B.2. was sent to the laboratory of NPSB (Núcleo de Prestação de Serviços de Bioquímica, FFULisboa) for determination of the levels of total cholesterol, HDL-c, LDL-c, triglycerides, and tHcy (total homocysteine). The remaining plasma was sent to Dr. Joaquim Chaves Laboratories (JCLab, Algés) to determine the concentrations of the following parameters: fibrinogen, CRP, apoB-100, apoA-I, IL-1β, ICAM-1, and VCAM-1. Details about the methodology used at NPSB and at JCLab are shown in Table 2.

SAH and SAM were measured in the deproteinized PBMC extracts obtained in B.2. using the same procedures described in section A.3.

B.3.1. Statistical analysis

Unpaired Student’s T-test with Welch’s correction was performed to compare differences between control and patients groups, with the accepted value of $P<0.05$ as significant, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, “www.graphpad.com”.

B.4. Western blot analysis and statistical analysis

The histones obtained in B.2. were used for Western blot studies to analyze H3K27me3 and histone H3 contents, as described in section A.4.3.

Unpaired Student’s T-test with Welch’s correction was performed to compare differences between control and patients groups, with the accepted value of $P<0.05$ as significant, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, “www.graphpad.com”.

Table 2: Resume table of the parameters analyzed in the blood.

Parameter	Method	Volume of plasma	Location
TC	Enzymatic endpoint[146] from Randox CH 200	10 µL	NPSB
HDL-c	Precipation method[52] from Randox CH 203	10 µL	NPSB
LDL-c	Calculated based on the levels of total cholesterol, HDL-c, and triglycerides[147].	-	NPSB
Triglycerides	GPO-PAP[148] from Randox TR210	10 µL	NPSB
tHcy	High-performance liquid chromatography (HPLC)[149]	100 µL	NPSB
Fibrinogen	Fibrinogen bindarid™ radial immunodiffusion kit (The Binding Site Group, Birmingham, United Kingdom)[59]	10 µL	JCLab
CRP	Latex-enhanced immunoturbimetric assay in Siemens Advia® 1650 chemistry analyzer (Siemens Healthcare)[150]	10 µL	JCLab
apoB-100	Immunoturbidimetry enhanced by polyethylene glycol (PEG) in Siemens Advia® 1650 chemistry analyzer (Siemens Healthcare)[151]	10 µL	JCLab
apoA-I	Immunoturbidimetry enhanced by polyethylene glycol (PEG) in Siemens Advia® 1650 chemistry analyzer (Siemens Healthcare)[151]	10 µL	JCLab
IL-1β	Enzyme Amplified Sensitivity Immunoassay[152] (DIAsource IL-1β-EASIA from DIAsource ImmunoAssays S.A.)	200 µL	JCLab
ICAM-1	Enzyme immunoassay for the quantitative determination of soluble VCAM-1[153] (sVCAM-1 ELISA -Enzyme from ILB international)	30 µL	JCLab
VCAM-1	Enzyme immunoassay for the quantitative determination of soluble ICAM-1[153] (sICAM-1 ELISA -Enzyme from ILB international)	30 µL	JCLab

Abbreviations: NPSB, núcleo de prestação de serviços de bioquímica, FFULisboa; JCLab, Dr. Joaquim Chaves Laboratories; TC, total cholesterol,; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; tHcy, total homocysteine; CRP C-reactive protein; apoB-100, apoliprotein B-100; apoA-I, apoliprotein A-I; IL-1β, interleukin-1 β; ICAM-1, intercelular adhesion molecule 1; VCAM-1, vascular adhesion molecule 1.

B.5. RT-qPCR

RNA is an unstable molecule and its half-life is very short so it is important to ascertain a good RNA quality and integrity beyond extraction, which depends on the quality of cell lysis[140]. RNA integrity was verified by 1 % SeaKem® LE agarose (Lonza) gel electrophoresis (data not shown), and its quality and quantity were evaluated on NanoDrop ND-100 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) using NanoDrop 1000 Operating Software (version 3.7.1 compatible with Windows). Moreover, possible contamination with genomic DNA was eliminated by treatment with deoxyribonuclease I, DNase I recombinant, RNAse-free 10U/µL (Roche, Basel, Switzerland). Briefly, 1 µg of total

RNA were made up to 7 µL with DEPC-treated water and 0.25 U of DNase I was added in the laminar flow chamber. The mixture was incubated for 15 min at 25 ^{o}C in the thermocycler. The reaction was stopped by adding 1 µL 80 mM EDTA and further incubated for 10 min at 75^{o}C.

The resulting DNase I-treated RNA samples were reverse transcribed using the Super Script® III First-Strand Synthesis Super Mix (Invitrogen Life Technologies by ThermoFisher Scientific, Waltham, USA), adding 10 µL of reaction buffer and 2 µL of enzyme mix to the 8 µL sample and incubated for 10 min at 25 ^{o}C, 30 min at 50 ^{o}C, and 5 min at 85 ^{o}C. Then added 1 µL of RNAse H was added and the tubes were incubated at 37 ^{o}C for 20 min.

Afterwards, the samples were put on ice and were quantified on NanoDrop ND-1000 spectrophotometer (NanoDrop Products), using NanoDrop 1000 Operating Software (version 3.7.1 compatible with Windows), to assure that the amount of DNA template was similar amongst different samples.

All incubations were performed in the thermocycler Swift™ Maxi® (ESCO®).

First-strand cDNA (250 ng) was used as template for qPCR using a CFX96™ Real-Time System C1000 Touch™ thermocycler (Bio-Rad), programed by Bio-Rad CFX Manager 3.0 for Windows with the following cycling conditions: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, and 40 cycles 95 ^{o}C for 15 s followed by 60 ^{o}C for 1 min.

Gene expression analyses were performed using the TaqMan® Gene Expression Assays (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA) IDs: Hs00544833_m1 for *EZH2* and Hs015555410_m1 for *IL-1β* and as endogenous control the TaqMan$^{®}$ β-actin Control Reagents (4352935 - 1104025). In every reaction the TaqMan$^{®}$ Gene Expression Master Mix was used following enclosed instructions. The plaques were centrifuged at 500 g for 3 min at 4 ^{o}C in Eppendorf 5810 R V6 centrifuge (Eppendorf) before the amplification.

Each sample was assayed in triplicate and results represent a minimum of three independent experiments. Relative expression of target mRNA was calculated from the target *Ct* values and β-actin mRNA *Ct* values, using the comparative C_T method ($\Delta\Delta\ C_T$ Method).

B.5.1. Statistical analysis

Unpaired Student's T-test was performed to compare differences between the control and patients groups, with the accepted value of $P<0.05$ as significant, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, "www.graphpad.com".

IV – RESULTS AND DISCUSSION

A. *In vitro* studies: incubation procedure in endothelial cells

A.1. Influence of ADA in H3K27me3 on HUVEC cells

HUVEC are vulnerable to high SAH levels, which can affect the cellular methylation pattern[135]. We wanted to investigate if hypomethylation, induced by SAH accumulation, influenced the content of the histone epigenetic mark, H3K27me3. To test this, we incubated HUVEC in the presence and absence of ADA, as this compound inhibits SAH hydrolysis, thus increasing intracellular SAH levels[154]. Plus, SAH hardly crosses the cell membrane, so SAH build-up due to ADA treatment is quite effective[33].

To confirm the efficacy of ADA to trigger cellular hypomethylation, we quantified SAH and SAM levels, as well as SAM/SAH ratio, an indicator of the cellular methylation status[67]. Actually, we observed a dramatic increase of intracellular SAH levels in HUVEC treated with 20 µM ADA after 24 h, when compared to control cells (1797 *vs* 142.5 pmol/mL, respectively), whereas SAM levels only increased from 396.4 to 709.5 pmol/mL, respectively. However, after 48 h of incubation, SAH levels decreased to 416.5 pmol/mL and to 126.9 pmol/mL in treated and in control cells, respectively, whereas SAM levels decreased to 105.9 and 259.1 pmol/mL, respectively. Accordingly, as illustrated in Figure 7, at 24 h the SAM/SAH ratio of cultured cells incubated in the presence of 20 µM ADA revealed a 7-fold reduction when compared with control cells, thus proving the impaired methylation status of the cellular environment.

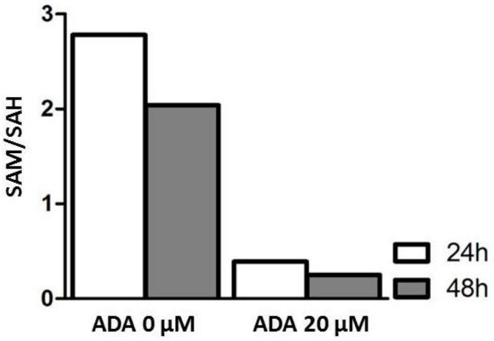

Figure 7. Intracellular SAM/SAH ratio in HUVEC incubated with ADA 0 µM and 20 µM for 24 h and 48 h. Abbreviations: ADA, adenosine-2,3-dialdehyde; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HUVEC, human umbilical vein endothelial cells.

These results clearly showed that under physiological conditions, SAM and SAH levels are strictly regulated, showing a narrow variation. After treatment with ADA, SAH levels increase dramatically whereas SAM levels' variation is much more restricted, suggesting a tight regulation.

We then quantified H3K27me3 content in control and ADA-treated HUVEC and our results showed that, after 24 h incubation with 20 μM ADA, a significant reduction of relative H3K27me3 levels occurred, as illustrated in Figure 8.

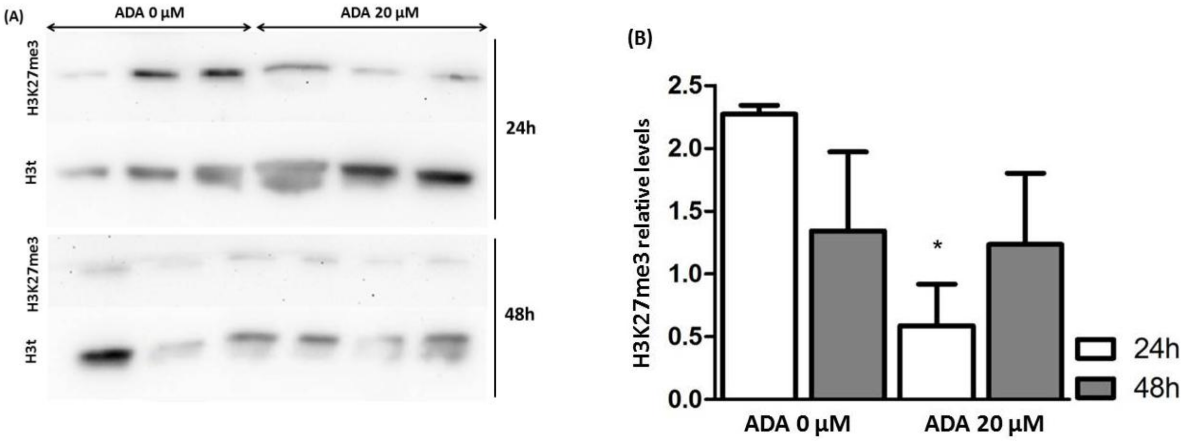

Figure 8. Effect of excess of SAH on H3K27me3 mark in HUVEC.
(A) Western blot images of H3K27me3 (17 kDa) and H3t (17 kDa) content in HUVEC, upon incubation with 0 μM or 20 μM ADA for 24 h and 48 h. (B) Results correspond to mean ± SD (*n*=3) upon H3K27me3 content normalization against H3t. Significant difference was inferred at $^{*}p<0.05$ *vs* 0 μM ADA at 24 h using one way ANOVA followed by post hoc Bonferroni's multiple comparison test.
Abbreviations: ADA, adenosine-2,3-dialdehyde; H3K27me3, trimethylation of histone H3 at lysine 27; H3t, histone H3 total; SAH, S-adenosylhomocysteine; HUVEC, human umbilical vein endothelial cells.

However, in HUVEC incubated with 20 μM ADA, after 48 h the H3K27me3 relative levels are similar to the ones observed in control cells. A plausible explanation for this observation is the decay in ADA efficiency along the incubation time, and that enzymatic SAHH activity can be recovered. Effectively, ADA inhibiting activity may not survive beyond 24 h because of its two labile aldehyde groups[155]. In addition, as we previously referred, we observed a dramatic impairment of SAH levels at 48 h, and these lower levels of SAH do not seem to be sufficient to inhibit the activity of the methyltransferase responsible for the trimethylation of the H3K27, thus explaining the increase of the H3K27me3 levels, which reached values similar to the control cells. An increase in SAH, independent from SAM decrease, is a more powerful predictor of cellular methylation decrease[156]. Nevertheless, this experience proved that, *in vitro* and after 24 h incubation, an increase of SAH levels does alter the cellular methylation status, leading to a decrease of the epigenetic mark H3K27me3.

In line with these observations, our group previously described that elevated SAH levels induces H3K27me3 hypomethylation in human coronary artery endothelial cells (HCAEC)[89]. The same study also showed that intracellular SAH accumulation can also lead to cytokine production, like IL-1β, through NF-κB pathway activation[89], which is a primed inflammatory pathway in atherosclerosis[157]. This pathway has an important role in initiation and development of atherosclerosis and can lead to the induction of pro-apoptotic genes, depending on the cell type[158]. Hcy has a pathogenic effect through oxidative stress increase, which could result in apoptosis[154], *via* NF-κB activation[159].

Another study with cultured EC demonstrated that high levels of Hcy, which leads to increased intracellular SAH levels, result in cell growth arrest through gene hypomethylation[160].

Another interesting study with HUVEC showed that intracellular SAH accumulation, triggered by 3-deazaadenosine (DZA), another SAH hydrolase inhibitor, led to inhibition of cellular growth and proliferation, morphological alterations, and apoptosis. Furthermore, it also resulted in global DNA hypomethylation[161]. Our group also observed cell growth decreased upon ADA treatment in HCAEC[89]. This suggests that intracellular SAH accumulations may also cause other damages to EC, besides the alterations to the cell methylation pattern. Our group also observed NO decreased bioavailability in cultured human EC under excess of SAH[89]. NO is a vital compound for normal endothelial functions[33], and whose decreased bioavailability is a hallmark of ED.

B. *In vivo* studies: characterization of CVD patients and healthy individuals

B.1. Quantification of Hcy levels and SAM/SAH ratio

In the past decades, many studies have linked high levels of plasma Hcy to CVD risk[87,162,163]. Hcy levels differ between gender; men tend to have higher Hcy levels than women, although levels rise in women when they reach menopause[66]. These differences in gender are explained by men's larger muscle mass, and by the hormonal estrogen therapy in women[164], which has shown to affect CVD risk[165]. Additionally, the metabolism of Hcy is more efficient in women than in men[66].

Our results revealed that plasma tHcy concentration (mean ± SD) was higher in CVD patients than in controls (11.09 ± 2.3 μM and 8.41 ± 2.80 μM, respectively), though not reaching statistical significance. Furthermore, as shown in Figure 9, we stratified our results according to gender. Control men presented higher plasma tHcy levels than control women (9.47 ± 2.96 μM and 7.35 ± 2.20 μM, respectively), though the difference did not reach statistical significance. Amongst CVD patients, no difference in tHcy levels was observed between men and women (11.14 ± 2.46 μM and 10.95 ± 2.20 μM, respectively). Nevertheless and interestingly, CVD women presented statistically significant higher tHcy plasma levels than control women (10.95 ± 2.15 and 7.35 ± 2.20, respectively), whereas such difference was absent between CVD and control men (11.14 ± 2.46 and 9.47 ± 2.96, respectively). On average, all groups have normal tHcy levels, which range from 5 to 15 μM (in a fasting state)[1].

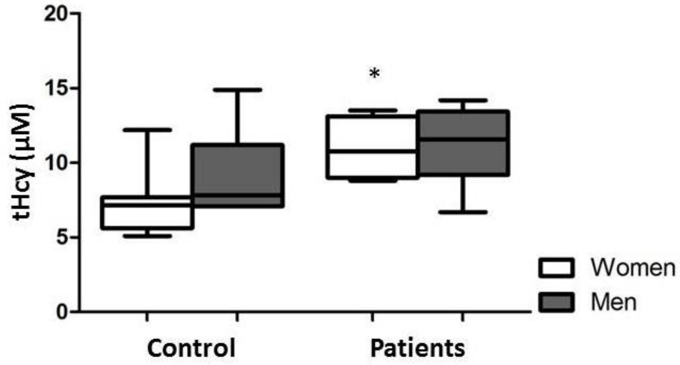

Figure 9. Evaluation of tHcy levels by gender in the plasma.
Results correspond to mean ± SD. Significant difference is inferred at *p<0.05 *vs* control using unpaired Student's T test with Welch's correction.
Abbreviations: tHcy, total homocysteine.

Results from the Hordaland Homocysteine Study showed that smoking and large caffeine consumption are associated with high levels of Hcy[1]. In fact, smoking can influence folate absorption, contributing to the elevation of Hcy levels due to impaired remethylation[166]. Since 64 % of the CVD population has smoking history, compared with 44 % of the controls, smoking might have influenced our results.

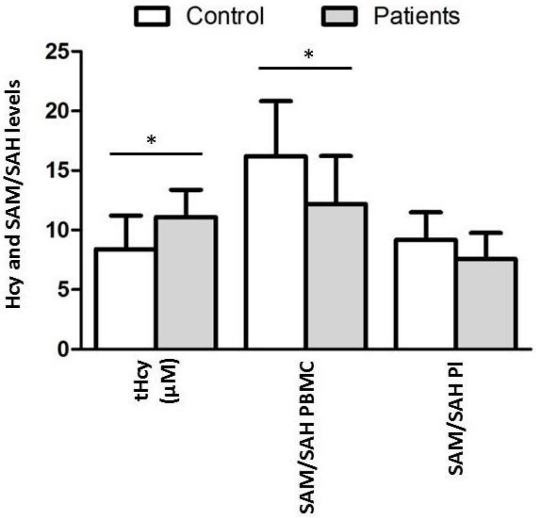

Figure 10. Evaluation of tHcy, SAM/SAH ratio in PBMC and in plasma in the studied population.
Results correspond to mean ± SD. Significant difference is inferred at *p<0.05 *vs* control using unpaired Student's T test with Welch's correction.
Abbreviations: tHcy, total homocysteine; SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine; PBMC, peripheral blood mononuclear cells; Pl, plasma.

SAH plasma levels have been suggested to be a better CVD risk indicator than Hcy[88], thus we also measured SAM and SAH levels as well as SAM/SAH ratio in both PBMC and in plasma of the studied populations. Our results, displayed in Figure 10, showed that the PBMC SAM/SAH ratios were significantly lower in CVD patients than in controls (12.21 ± 4 and 16.2 ± 4.63, respectively). However, the correspondent difference in plasma SAM/SAH ratios did not reached statistical significance (7.56 ± 2.2 and 9.17 ± 2.31, respectively), although it was previously shown that an increase of plasma tHcy was associated with an increase of plasma SAH levels[156]. As aforementioned, SAH is the precursor of Hcy that accumulates when Hcy accumulates[167]. We reason that evaluating SAM/SAH levels in PBMC is a more reliable measure of the extent of atherosclerosis, as these cells directly participate in endothelial inflammatory response.

We have also investigated the relation between circulating tHcy and SAM/SAH ratio either in PBMC or in plasma of CVD patients and controls, but we found the absence of statistical significance. Our results demonstrated a wide range of PBMC SAM/SAH values in both studied groups (Figure 11A) whereas the correspondent plasma values were much less dispersed (Figure 11B). Nevertheless, as can be observed in Figure 11A, where we correlated tHcy levels with PBMC SAM/SAH ratio, the controls values are spotted in the upper-left quadrant, corresponding to the lower levels of tHcy and the higher SAM/SAH ratios. Conversely, CVD patient values presented a tendency to higher tHcy levels and lower SAM/SAH ratios. Concerning the correlation between tHcy levels and plasma SAM/SAH ratios, displayed in Figure 11B, the values of both studied groups are condensed in the lower quadrants, revealing lower SAM/SAH ratios and thus confirming the absence of the statistically significant differences previously described between CVD patients and controls (Figure 11B).

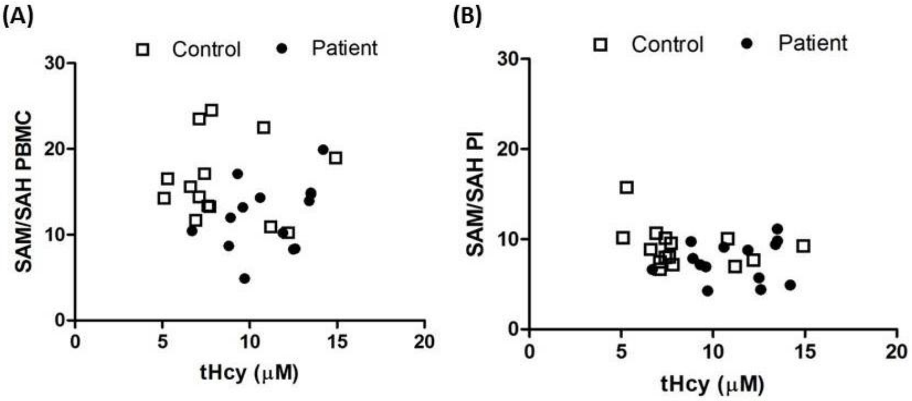

Figure 11. Plot of individual values of plasma tHcy *vs* SAM/SAH ratio.
(A) Distribution of individual values of tHcy *vs* and SAM/SAH ratio in the plasma. (B) Distribution of individual values of tHcy *vs* SAM/SAH ratio in the PBMC.
Abbreviations: tHcy, total homocysteine; SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine; PBMC, peripheral blood mononuclear cells; Pl, plasma.

Interestingly, when CVD patient and control values were taken together, we could observe a weak inverse correlation (*r* = - 0.20) although non-significant, between circulating tHcy levels and plasma SAM/SAH ratios, since we were measuring components in the same fluid. Furthermore, tHcy levels did not correlate with PBMC SAM/SAH ratios. We hypothesize that a similar trend would be observed if the correlation was achieved with intracellular tHcy levels.

Finally, we correlated the SAM/SAH ratios in PBMC and plasma and, though no statistically significant correlation was detected (*r* = 0.29), our results showed that an increase of SAM/SAH values in the plasma was accompanied by an elevation of the SAM/SAH ratio in the PBMC, as illustrated in Figure 12.

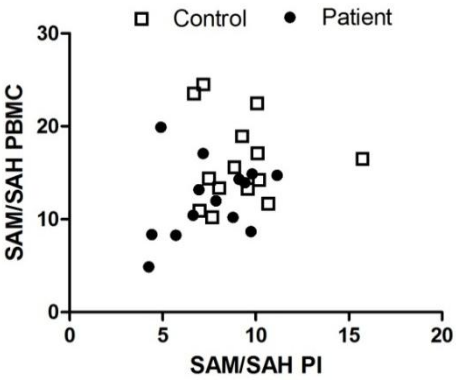

Figure 12. Plot of individual values of SAM/SAH ratios in the plasma *vs* SAM/SAH ratio in the PBMC.

Abbreviations: tHcy, total homocysteine; SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine; PBMC, peripheral blood mononuclear cells; Pl, plasma.

We also compared SAH levels in the plasma and PBMC in both studied groups, and the results revealed CVD patients have higher levels in the plasma and in PBMC than controls (Figure 13). Nevertheless, these results must be analyzed carefully since the absolute SAH levels were not normalized to total protein values. Interestingly, SAH values are higher in plasma than in PBMC, reaching a statistical significance in controls, but not in patients (data not shown). These observations are in concordance with the SAM/SAH ratios that we calculated above, since low SAM/SAH ratios correspond to higher levels of SAH, in the CVD patients group compared with controls, both in PBMC (12.21 ± 4 and 16.20 ± 4.63, respectively), and in plasma (7.56 ± 2.20 and 9.17 ± 2.31, respectively).

Our results confirmed previous reports stating that circulating tHcy level is a good indicator of CVD risk. However, and concerning the methylation index (SAM/SAH ratios) the present results clearly suggested that intracellular measurements are a more reliable parameter than plasma levels.

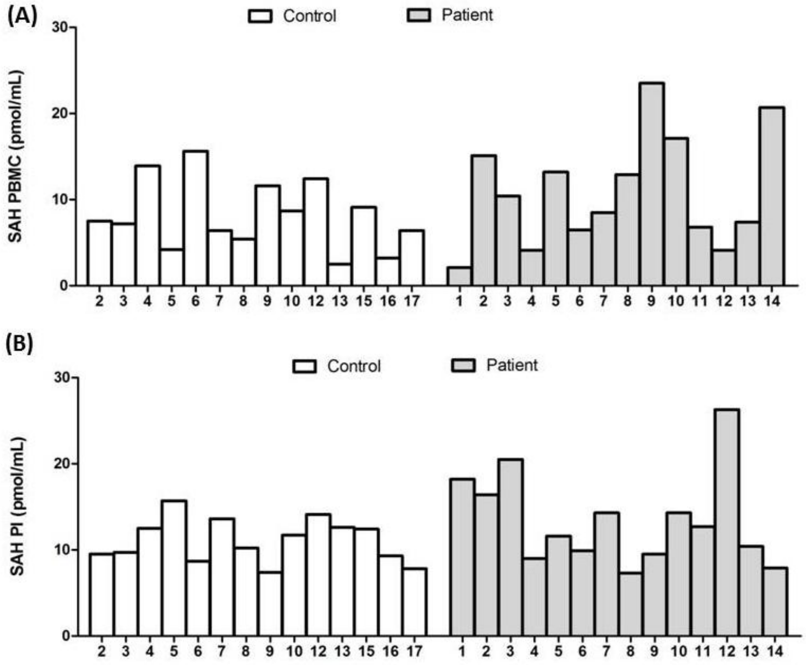

Figure 13. Evaluation of SAH levels in the studied population.
Abbreviations: SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine; PBMC, peripheral blood mononuclear cells; Pl, plasma.

B.2. Evaluation of cardiovascular risk parameters in the population

B.2.1. Evaluation of pro-atherogenic indicators in circulating plasma

Atherosclerosis is an inflammatory disease that begins with ED and progresses through lipid accumulations in the vascular wall; therefore, we used the levels of several circulating lipids to characterize our population, namely: triglycerides, total cholesterol, HDL, LDL, apo-AI, and apoB-100. Our results are shown in Figure 14.

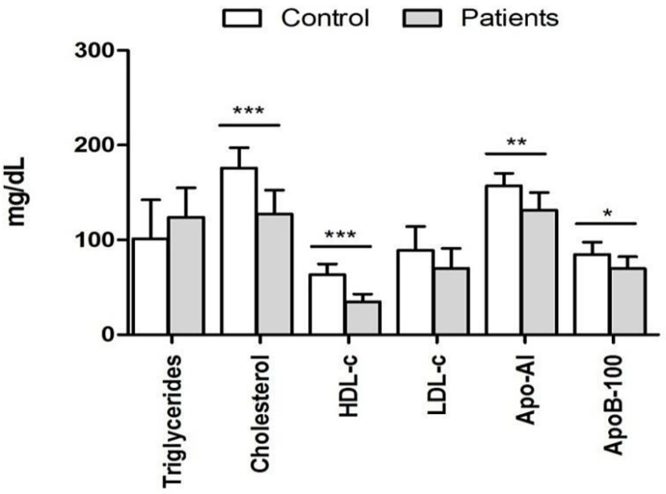

Figure 14. Levels of plasma circulating lipids in the studied population.
Results correspond to mean ± SD. Significant difference is inferred at *p<0.05;**p<0.01;***p<0.001 *vs* control using unpaired Student's T test with Welch's correction.
Abbreviations: HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; apo-AI, apolipoprotein AI; apoB-100, apolipoprotein B-100; CVD, cardiovascular disease.

- Triglycerides

In our study, the triglyceride plasma values were 124.03 ± 30.98 mg/dL for CVD patients, and 101.26 ± 40.97 mg/dL for controls; we did not find any statistical difference between both groups.

Various studies have demonstrated that high triglyceride levels are associated with high rates of CV events and deaths[168]. Other authors claim that triglyceride measurement does not give information about immediate CVD risk[169]. Accordingly, a recent study has shown that elevated levels of triglycerides are associated only with increased long-term mortality of coronary heart disease patients[170]. Nevertheless, triglycerides do not accumulate in the atherosclerotic plaque, as they are degraded in various cells in the body[171].

- Total cholesterol

Our results showed that total cholesterol plasma levels are significantly lower in the patient group (127.52 ± 24.86 mg/dL) than in controls (175.89 ± 21.53 mg/dL).

Cholesterol builds in the vessel's wall and participates in the atherosclerosis progression[43], and it is considered a CVD risk factor[172]. Also, high levels of circulating Hcy can induce cholesterol accumulation[50], and since tHcy levels are higher in patients it would be expected that cholesterol levels would have the same tendency. However, all CVD patients were subjected to cholesterol lowering therapy, like statins, which may explain these unexpected findings, as it will be discussed further below.

The cholesterol can be carried by HDL and LDL, labeled as HDL-cholesterol (HDL-c) and LDL-cholesterol (LDL-c)[173]. LDL-c is a measure of total cholesterol that exists in LDL, whereas HDL-c is a measure of total cholesterol that exists in HDL[174].

- LDL-c and HDL-c

CVD patients displayed decreased LDL-c plasma levels (70.11 ± 20.95 mg/dL) when compared to the control group (89.04 ± 20.95 mg/dL). Moreover, HDL-c levels are also significantly lower in CVD patients (34.64 ± 8.29 mg/dL) than in controls (63.22 ± 11.46 mg/dL).

Subjects with high CVD risk usually present high plasma levels of LDL-c[175]. In fact, high LDL-c levels are significantly linked to atherosclerosis[44] as they can promote inflammation[65]. Endothelial membrane permeability for LDL is enhanced in case of an atherosclerotic lesion and, as a consequence, LDL-c enters into the vascular intima. Modified LDL, like ox-LDL, can be easily phagocytosed by macrophages resulting in their accumulation in vascular cells, and causing local changes to the arterial wall[176,177]. These include increased adherence ability of monocytes[18] and the establishment of an inflammatory phenotype, a process that facilitates apoptosis through NF-κB pathway activation, thus perpetuating the pathological lesion[178].

As opposed to LDL, HDL has a protective vascular role by removing the excess of cholesterol from peripheral tissues[43], diminishing the levels of adhesion molecules on EC, enhancing the synthesis of NO, and preventing LDL oxidation[51].

Lipid lowering drugs are the first line of attack towards atherosclerosis[179]. Statin treatment, due to its cholesterol and LDL-c lowering effects, is a normal therapeutic approach for atherosclerosis and individuals with high CVD risk[175]. Statins function by inhibiting the enzyme HMG-CoA reductase that partakes in the cholesterol synthesis pathway. As a result of this drug intake, there is decreased intrahepatic cholesterol levels and an up-regulation of LDL receptors in hepatic cells, leading to increase LDL uptake from the circulation[174]. Pleiotropic effects of statins include anti-thrombotic and antioxidant effects on the vessels[180]. Interestingly, high HDL-c levels are claimed as a good indicator of cardiovascular risk in statin-treated patients that have LDL-c in low levels[168].

In addition to statins, the antiplatelet effect of aspirin (acetylsalicylic acid) is also used in the prevention of acute myocardial infarction. Aspirin inhibits platelet activation and aggregation, reduces thrombosis and inhibits the progression of atherosclerosis, by inhibiting LDL-c oxidation[181] and reducing LDL circulating levels[177].

Aspirin has antithrombotic effects through the inhibition of cyclooxygenase inhibition and prostangladin reduction, ultimately resulting on platelet aggregation inhibition, without affecting EC functions[182]. Aspirin has been shown to have cytoprotective functions that are unrelated with its antiplatelet activity, such as improving endothelium-dependent arterial relaxation by inducing the release of NO from the vascular endothelium[183], and reducing apoA levels in human hepatocytes by suppressing apoA gene transcription[184]. Clopidogrel, a drug that also inhibits platelet aggregation through a diverse mechanism is often associated with aspirin[185], to avoid atherosclerosis progression.

Atorvastatin also improves vascular NO bioavailability[186]. It leads to structural and metabolic modifications in HDL, enhancing HDL anti-atherogenic functions[187]. Besides HDL modulation, atorvastatin can also lead to LDL-c decrease[179].

ACE inhibitors, a therapy also given to the studied patients, can reduce the expression of LOX-1, the human receptor of ox-LDL, thus slowing down the atherosclerosis development. ACE or kininase II is an endothelial enzyme, which has a role in the balance of vasodilatation and constriction[188].

Our results showed that CVD patients display decreased LDL levels compared with controls, an unexpected finding that may well be a consequence of the medication of the CVD patients. In fact, all patients were submitted to statins and aspirin therapy. The significant lower HDL-c levels in the CVD patient group, when compared to controls, confirmed that HDL-c levels are a good indicator of cardiovascular risk in statin-treated patients that have LDL-c in low levels.

Interestingly, smoking has been linked with a decrease in HDL levels[31], which might contribute to our results, as some patients have a smoking history.

- apoA-I and apoB-100

Concerning apoA-I and apoB-100 levels, we observed that the plasma concentration of these molecules is lower in the patient group than in controls (131.50 ± 18.50 mg/dL *vs* 157.07 ± 13.14 mg/dL for apoA-I and 69.79 ± 12.61 mg/dL *vs* 84.60 ± 12.90 mg/dL). This observation corroborates our results from HDL-c and LDL-c measurements, since apoA-I is the major component of HDL, and apoB-100 is the major component of LDL[18,51]. Smoking seems to decrease apoA-I (and HDL-c) levels[189]. Furthermore, as we stated previously, aspirin reduces mRNA apoA levels in human hepatocytes[184], which could have influenced the patients' apoA-I levels.

It was reported that Hcy levels correlate inversely with HDL-c and apoA-I levels in case of HHcy[93], since Hcy can influence independently apoA-I levels[189]. Our results seem to follow that tendency as well.

B.2.2. Evaluation of CVD risk in the population

The CVD risk is the probability of developing the disease and it is calculated in accordance to physical manifestations, family history, and routine blood analysis[190]. These include the calculation of different ratios of lipids. It was reported that about 50 % of population with abnormalities in the lipoprotein metabolism are in risk to develop a CVD[191].

In our study we used total cholesterol/HDL-c and LDL-c/HDL-c ratios as indicators of cardiovascular risk[192,193]. Normal values for total cholesterol/HDL-c value are under 5 in men and under 4.5 in women, and for LDL-c/HDL-c ratio are under 3.5 in men and under 3 in women[191].

Our results, illustrated in Figure 15A, confirmed that amongst controls, most individuals are CVD risk free, for the exception of subjects 11, 14, and, 18. For presenting CVD risk, these individuals were excluded from our initial control group. Interestingly subject number 9 has really low values in both lipid ratios, comparing with the rest of the control individuals. These results could be due to the simvastatin therapy taken at the time. Simvastatin can lead to a decrease in LDL-c levels[168]. Control number 15, who also displayed low LDL-c levels, did not state any medication in the questionnaires.

Concerning CVD patients, and surprisingly, most individuals are not inserted in the CVD risk category, with the exception for individuals 1, 5, 9, and, 10 (Figure 15B).

We argue that this unexpected finding is explained by the lipid lowering medication to which CVD patients were subjected to alter total cholesterol/HDL-c and LDL/HDL-c ratios[173].

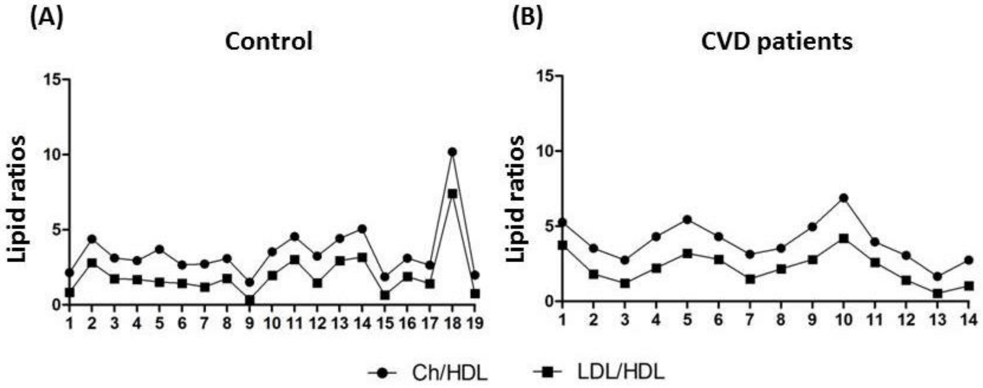

Figure 15. CVD risk analysis in the studied population.
(A) CVD risk distribution along the control group given by total Ch/HDL and LDL/HDL ratios. (B) CVD risk distribution along the CVD patient group given by total Ch/HDL and LDL/HDL ratios.
Abbreviations: Ch, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; CVD, cardiovascular disease.

We also compared the lipid ratios in the patient group *vs* controls but they were not statistically different (data not shown), showing the inefficacy of these blood lipids ratios as tools to identify high-CVD risk people for secondary prevention.

Another estimate of the CVD risk, besides the referred total cholesterol/HDL-c and LDL/HDL ratios, is the calculation of the apoB-100/apoA-I ratio[191]. Thus, we have also calculated this ratio (data not shown) but none of the studied individuals could be inserted in the CVD risk category. This is not surprising, considering that LDL/HDL-c and total cholesterol/HDL-c ratios have a greater predictive value and are used for a long time, including in the Framingham Heart Study, as in opposition of apoB-100/apoA-I ratio[173,191].

B.2.3. Quantification of pro-inflammatory indicators

As referred above, the atherosclerosis is an inflammatory disease of the vascular wall. Therefore, IL-1β, CRP, and fibrinogen were chosen as inflammatory indicators and were evaluated in CVD patients and in controls. Our results are shown in Figure 16.

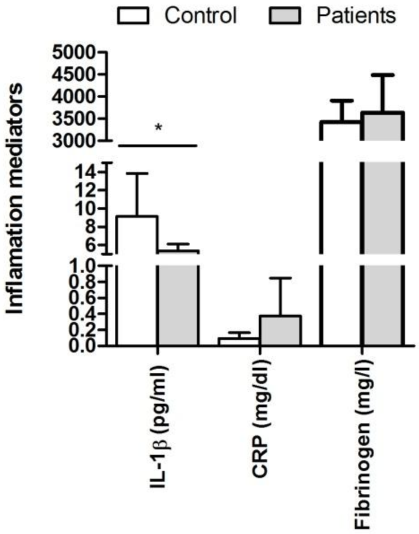

Figure 16. Inflammatory parameters in the studied population.

Results correspond to mean ± SD. Significant difference is inferred at $^*p<0.05$ *vs* control using unpaired Student's T test with Welch's correction.

Abbreviations: CVD, cardiovascular disease; IL-1β, interleukin-1β; CRP, C-reactive protein.

- IL-1β

IL-1β is an important pro-inflammatory cytokine, which partakes in the establishment and progression of atherosclerotic lesions. It has been reported that atherosclerosis patients show higher levels of IL-1β than healthy individuals[194]. Interestingly, Met&Gen Group observed that SAH accumulation in human EC activates NF-κB pathway, triggering IL-1β expression[89]. Nevertheless, our results showed that plasma IL-1β levels were lower in CVD patients (5.36 ± 0.73 pg/mL) than in controls (9.13 ± 4.73 pg/mL) although a high standard deviation of values was observed in the control group. However, we reason that, concerning the CVD patients, the anti-inflammatory therapy, like statins and high doses of aspirin, does inhibit cytokines release[179,186], explaining this observation. Also, accumulated cholesterol may induce IL-1β expression[195], and thus we speculate that the low cholesterol levels in CVD patients also contributed to the low levels of IL-1β.

- CRP

CRP is an acute-phase plasma protein produced by the liver in response to cytokine production during the inflammation response[196,197]. Our results showed the absence of any statistical variation of CRP levels amongst the studied population (0.37 ± 0.48 mg/dL *vs* 0.09 ± 0.07 mg/dL in patients and controls, respectively). We argue that this observation, may also be explained by the patients' therapy, specifically by the pleiotropic effects of statins, which reduces CRP circulating levels[195,198]. In addition, the anti-inflammatory therapy also targets cytokines like IL-1β[186].

CRP participates in all stages of atherosclerosis development[54]. Elevation of plasma CRP levels occurs due to a systemic response to the vascular inflammation[199]. A recent study showed a positive association between CRP and tHcy plasma levels[200]. Nevertheless, it still remains ascertained if inhibition of inflammation response *per se* will lead to a decrease in vascular events rates[194]. In fact, in a mouse model with CRP deficiency or reduction, it was not observed any reduction of the number/size of atherosclerotic lesions[201].

- Fibrinogen

We have also evaluated fibrinogen plasma levels since fibrinogen, an acute-phase inflammatory reactant, increases during the inflammatory response of the vascular wall[202]. Accordingly, high fibrinogen levels have been associated with an increased CVD risk[203]. Nevertheless, in our study, the levels of fibrinogen were similar in CVD patients and controls (3636.43 ± 850.20 mg/L and 3422.50 ± 485.31 mg/L, respectively). Once again, we argue that the patients' medication may explain these results. In fact, either the anti-inflammatory therapy (like atorvastatin) or the anti-coagulant medication can decrease the fibrinogen levels in the plasma, since fibrinogen is a coagulant factor[202,204].

B.2.3. Evaluation of circulating pro-atherogenic mediators

Adhesion of circulating leukocytes to the EC is an important step in the inflammatory process of atherosclerosis and is mediated by the expression of adhesion molecules in EC[205]. ICAM-1 and VCAM-1 are important adhesion molecules whose interaction with leucocytes is required for their firm adhesion to the endothelium[34]. Membrane-bound forms of these adhesion molecules are difficult to measure *in vivo*, but the soluble forms can be detected in the plasma and are used to evaluate the inflammatory response of the damaged endothelium and its levels of dysfunction[206]. Thus, in our study, we measured soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1) plasma levels. The results were 544.39 ± 59.50 ng/mL in controls and 1000.91 ± 272.20 ng/mL in patients for sVCAM-1, and 507.62 ± 57.28 ng/mL in controls and 687.20 ± 191.49 ng/mL in patients for sICAM-1. As illustrated in Figure 17, sVCAM-1 plasma levels were significantly higher in the patients group when compared to controls, but sICAM-1 plasma levels did not change amongst the studied groups.

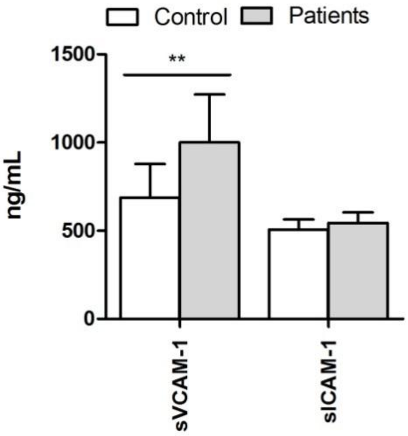

Figure 17. Evaluation of endothelial dysfunction indicators in the studied population.
Results correspond to mean ± SD. Significant difference is inferred at $^{**}p<0.01$ *vs* control using unpaired Student's T test with Welch's correction.
Abbreviations: CVD, cardiovascular disease; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1.

ICAM-1 is constitutively expressed in the vascular wall and its expression is upregulated upon a vascular inflammatory response, namely through NF-κB pathway[207]. Healthy blood vessels do not express VCAM-1[34,205]. In a human study, VCAM-1 levels were found to be associated with heavy lymphocyte and macrophages infiltration into the intima[205]. In another interesting study, Baccarelli and colleagues, found a link between hypomethylation of repetitive LINE-1 (Long Interspersed Nucleotide Elements) sequence and high VCAM-1 levels in the blood cells of healthy elderly individuals. Repetitive elements, like LINE-1, had been used as a measure of global DNA methylation. The authors hypothesized that these results could suggest that this link between hypomethylation and VCAM-1 levels may represent an early event in CVD[208]. Furthermore, higher levels of VCAM-1 were found in the human serum of atherosclerotic patients[101]. Additionally, in a study using mice with VCAM-1 deficiency also suggested that VCAM-1 has a major role in the early stages of atherosclerosis, when compared to ICAM-1[209].

Recently, it was found that a high Met intake was linked to higher levels of ICAM-1 and VCAM-1[210], and since tHcy levels are higher in patients, that could also influence the levels of these adhesion molecules. Nonetheless, an increase of these adhesion molecules is enough to increase the binding of leukocytes to EC[167] to perpetuate the inflammatory response.

The equilibrium between pro-atherogenic cytokines and anti-atherogenic cytokines is crucial to determine the atherosclerosis development. In fact, pro-atherogenic cytokine tumor necrosis factor-α (TNF-α) can lead to the upregulation of VCAM-1, increasing the interaction between leukocytes and EC in humans. On the other hand T-regulatory (Treg) cells mediate the secretion of IL-10, and anti-inflammatory and atheroprotective cytokine, which leads to down-regulation of ICAM-1 and TNF-α expression in EC[186]. This interaction could explain why ICAM-1 levels did not change in our studied population, as inflammatory pathways are being modulated by the anti-inflammatory therapy taken by CVD patients, which could have decreased ICAM-1 levels. Though, further studies are necessary to confirm this interaction.

Also CRP levels seem to induce the expression of ICAM-1 and VCAM-1, which are inversely correlated with HDL levels in monocytes[187,196] and in HUVEC[211], maybe also influencing the values of these parameters in the studied population.

B.3. H3K27me3 detection in the PBMC of the population

Dysregulation of histone methylation can lead to activation or repression of endothelial pro-inflammatory genes, or of inducible NO synthase genes in a vascular disease scenario[82]. For example, recent work by Dreger and colleagues have shown that, in HUVEC, repression of *NOS2* (gene encoding the inducible NO synthase) was associated with a high H3K27me3 content[212]. As previously stated, EZH2-mediated H3K27me3 mark is responsible for the epigenetic suppression of several endothelial pro-atherogenic genes[213]. As a SAM-dependent histone methyltransferase, EZH2 activity may be inhibited by excess SAH decreasing the H3K27me3 content and promoting atherosclerosis. For this reason, we have investigated the levels of H3K27me3 in PBMC of our studied populations. Our results, as illustrated in Figure 18 show no significant difference between the H3K27me3 content in CVD patients and controls. However, since a significant difference has been found in the PBMC methylation status (SAM/SAH ratio) between CVD patients and controls, we were expecting to observe at least a slight decrease of H3K27me3 content in the patient population. Interestingly, a recent study reported a global H3K27me3 reduction in human vessels of late atherosclerotic lesions comparing with vessels with early lesions[133].

Other report demonstrated that relative H3K27me3 levels decreased significantly in vessels with atherosclerosis compared to healthy ones. Additionally, the causal role of DNA methylation in monocytes and lymphocytes (indicated by the methylation status of LINE-1 sequence) was evaluated and they concluded that there is no difference in the DNA methylation status between atherosclerotic and control vessels[101]. LINE-1 has been identified as a mediator of ischemic heart disease damage[208].

We argue that the absence of a significant difference between the H3K27me3 content in PBMC of CVD patients and controls may be related with the tissue under study. Actually, the histone content in PMBC may not reflect the content in atherosclerotic vascular tissues.

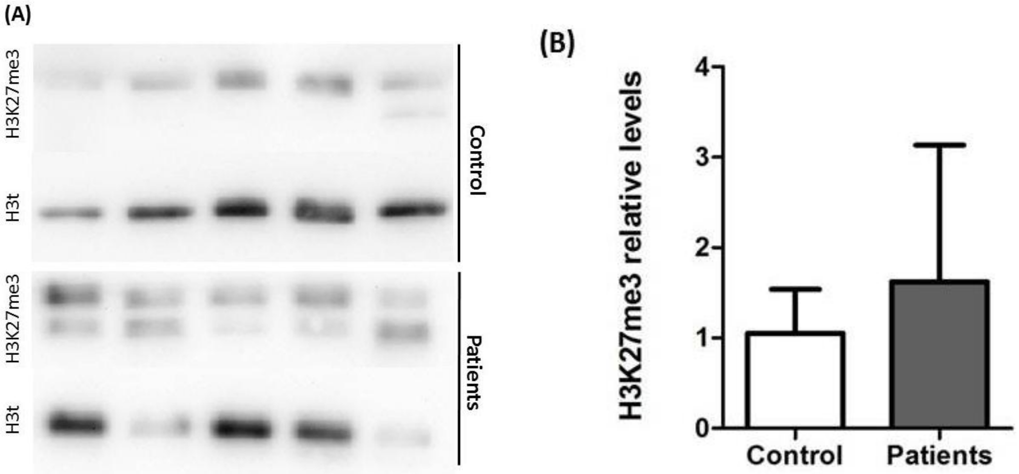

Figure 18. H3K27me3 relative levels in the studied population.
Representative Western blot images upon revelation of H3K27me3 and H3t. (B) H3K27me3 (17 kDa) signal was normalized upon H3t (17 kDa) signal. Results correspond to mean ± SD. Significant difference is inferred at $^{*}p<0.05$ *vs* control using unpaired Student's T test with Welch's correction.
Abbreviations: H3K27me3, trimethylation of histone H3 at lysine 27; H3t, histone H3 total.

B.4. Determination of *EZH2* and *IL-1β* expression

As previously referred, Hcy may be involved in an altered atherosclerosis-related gene expression through epigenetic modifications[50] and its deleterious action might be mediated by SAH, which inhibits most DNA and histone methyltransferases[103]. And histone methylation and DNA methylation have a constant interplay, as histone methylation facilitates DNA methylation, which serves as template for histone modifications[101].

As discussed, H3K27me3 levels were similar in control and CVD patients and this result suggests that the intracellular amount of SAH might not be sufficient to inhibit the activity of EZH2, the histone methyltransferase responsible for H3K27 trimethylation[121]. However, we thought to investigate if SAH levels were sufficient to inhibit DNMT, thus affecting the transcription of two specific genes: *EZH2* and *IL-1β*, the last one encoding a target of EZH2 activity.

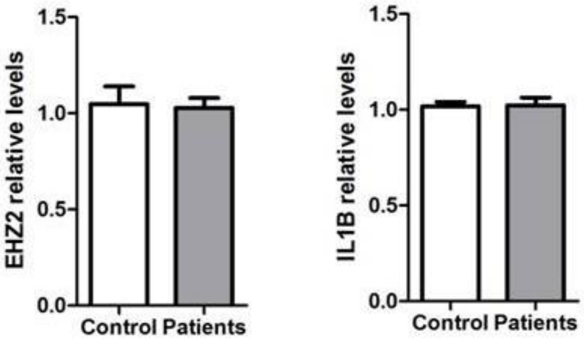

Figure 19. *EZH2* and *IL-1β* relative expressions in the population.
Abbreviations: EZH2, Enhancer of zeste homolog 2; IL-1β, interleukin-1β.

Both genes' expression was evaluated by qPCR and our results, illustrated in Figure 19, demonstrated that neither *EZH2* nor *IL-1β* levels changed in the studied populations. Since EZH2 catalyzes the formation of the H3K27me3 mark[121], these data agree with the Western blot results, which revealed no alteration in the H3K27me3 content. Although IL-1β plasma levels did vary significantly between the studied populations, in what concerns *IL-1β* levels, and as its product is a target of EZH2, its expression is consistent with *EZH2* results, since its expression did not variate amongst the studied groups as well.

A study with human endometrial stromal cells demonstrated that loss of EZH2 activity can lead to chromatin changes[214]. In a study with EC, *EZH2* depletion inhibited endothelial proliferation, an important feature to maintain the normal endothelial quiescent state. The same study has shown that EZH2 can respond to uniform high fluid shear stress, which is atheroprotective, and that EZH2 inhibition or knockdown result in either increase or decrease of gene expression[215]. This study conflicts somehow with our original hypothesis (Figure 4), that suggested that low EZH2 activity, promoted by elevated SAH levels, might be responsible for the activation of inflammatory genes, through diminishing H3K27me3 content. However one thing to take in consideration is that Maleszewska and colleagues[215] studied the effect of EZH2 in EC under uniform fluid shear stress, that is, in normal blood flow conditions. And since we hypothesized that low EZH2 activity mediates the pathogenic state in atherosclerosis, maybe there is a fine line between what are standard levels of EZH2 in normal conditions and in the beginning and development of atherosclerosis. *EZH2* levels or activity diminish so that inhibition of EC proliferation is compromised. Maleszewska and colleagues' work[215] does not invalid that *EZH2* levels or activity may diminish under pathogenic conditions. In fact, our group recently showed that in HCAEC, the loss of EZH2 led to the activation of the inflammatory pathway NF-κB[89], corroborating our working hypothesis.

On the other hand, we performed our studies in human PBMC, so EZH2 might have a different behavior than in EC. PBMC were chosen as study model for their key role in atherosclerosis-related diseases and because they are easy to obtain. Moreover, monocytes' transcriptome seems to reflect genome alterations from the environment[134]. Plus, leukocytes are recruited to the earliest atherosclerotic lesions[216]. More importantly, loss-of-function mutations and deletions in *EZH2* where found in T lymphocytes of acute lymphoblastic leukemia individuals[217], so it could also be a good model to study *EZH2* in CVD scenario.

Our group previously reported that in HCAEC, the expression of *EZH2* and *IL-1β* was significantly increased upon SAH accumulation[89]. We were expecting to have similar results in the present study, since higher circulating levels of tHcy were observed in the patients, leading to SAH accumulation, which could increase the expression of *IL-1β.* Although the expression of *IL-1β* is consistent with *EZH2* expression, we cannot reject that PBMC may behave differently upon the toxicological effects of SAH. Our results showed that IL-1β levels were lower in CVD patients compared with the controls, and again, possibly influenced by the anti-inflammatory therapy prescribed to the patients.

V – CONCLUSION

CVD is the leading cause of death in developed countries. Therefore, it is essential to disclose new underlying molecular mechanisms and markers for the pathology since 15 - 20 % of these subjects develop CVD in the absence of known risk factors. In the present study we postulated that SAH-induced hypomethylating stress would suppress EZH2 to contribute to a pro-atherogenic endothelial phenotype. Nevertheless, we could not establish a role for EZH2 in CVD, since *EZH2* mRNA levels in PBMC were similar in CVD patients and controls. Furthermore, in the same cells, the content of the epigenetic mark that EZH2 establishes, H3K27me3, was also similar in the studied populations. However, we were able to detect the presence of a hypomethylating environment in PBMC of subjects with established CVD. Further, we demonstrated the ability of excess of SAH to decrease an epigenetic mark in human EC. In fact, our *in vitro* study demonstrated that high levels of intracellular SAH led to a decrease in the H3K27me3 content in HUVEC. This observation was important to conclude that SAH build-up may have a globally epigenetic impact, namely through the regulation of histone methylation. These results were reinforced by the low SAM/SAH ratio (a cellular methylation indicator) that we observed in the same cells.

Future studies are needed to clarify the role of EZH2 in the beginning and development of atherosclerosis and CVD as well as the influence of hypomethylation in EC homeostasis. We propose to clarify the role of EZH2 role in atherosclerosis by quantifying the methylation levels in *EZH2* promoter in a CVD scenario *vs* normal one, gauging its active transcriptional state. Cancer cells studies demonstrate that *EZH2* can suffer loss-of-function or gain-of-function mutations[217]. Upcoming EZH2 studies could also include a gene sequencing of *EZH2* in both control and CVD patients, to determinate gene variations that affect *EZH2* enzymatic activity that could result in alteration of H3K27me3 levels.

We cannot exclude that, besides EZH2, other mediators of atherosclerosis, such as ox-LDL-c, deficiencies in the cholesterol efflux, or VSMC proliferation can be important interveners, contributing to the development of a pathological state.

We chose to study PBMC because they have been described as a good cellular model to study CVD. However, we recognize the potential limitations of peripheral blood cells as a source for assessing changes in the endothelium; therefore, future studies should analyze, in addition, vascular tissue, as for example, aortic and saphenous vein samples collected from patients with CVD during elective invasive procedures.

Lastly, upcoming studies should also comprise larger cohorts, with detailed medical history from the participants, and most importantly, with no influence of chronic therapy. In fact, our present results were clearly influenced by the medication of the CVD patients, which affected the circulating levels of most lipids analyzed and the levels of IL-1β, a pro-inflammatory cytokine.

There were some limitations to this study. The sample size was relatively small and the gender distribution was not exactly the same in CVD and control groups.

Our study suggests that controlling the lipid levels is not be effective on reestablishing the homeostasis of a previously injured endothelium, since sVCAM-1 levels, an ED marker, are still higher in the CVD patients, indicating that ED was still present. Avoiding lipid deposition in the blood vessels might not be sufficient to inhibit the atherosclerotic inflammation response. Additionally, modulation of the inflammatory response might not also be sufficient to prevent future CVD events, as already stated. Again, more studies need to be done to elucidate the complex interaction between inflammatory mediators, circulating lipids, ED markers, and other unknown participants in a CVD event.

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