Association of methylation marks in monocyte nuclear DNA and platelet mitochondrial DNA with the risk of Myocardial Infarction

Elisa Ruiz Arabí

Final Degree Project
Faculty of Medicine

Clinical tutor: Dr. Ramon Brugada
Methodological tutor: Dr. Rafael Marcos

Girona, November 2016

Quiero hacer una mención especial de agradecimiento a la Dra. Sara Pagans por su gran ayuda y apoyo en la realización de este proyecto.

INDEX

1. ABBREVIATIONS ..... page 5
2. ABSTRACT ..... page 6
3. INTRODUCTION ..... pages 7-19
3.1. CORONARY HEART DISEASE ..... pages 7-12
3.1.1. Epidemiology ..... pages 7-8
3.1.2. Physiopathology ..... pages 8-11
3.1.3. Clinical manifestations ..... pages 11-12
3.2. CARDIOVASCULAR RISK FACTORS ..... pages 12-13
3.3. EPIGENETICS ..... pages 13-16
3.3.1. DNA methylation ..... page 15
3.3.2. Histone modifications ..... page 15
3.3.3. MicroRNAs ..... page 16
3.4. EPIGENETICS AND CARDIOVASCULAR DISEASE ..... pages 16-19
3.4.1. Trained immunity ..... page 16
3.4.2. Interleukin 6 ..... pages 16-17
3.4.3. Monocyte count ..... page 17
3.4.4. LINE-1 . ..... pages 17-18
3.4.5. Platelet mitochondrial DNA methylation ..... pages 18-19
3.5. AGE ..... page 19
3.6. FUTURE APPLICATIONS ..... page 19
4. JUSTIFICATION ..... pages 19-22
5. HYPOTHESIS ..... page 22
6. OBJECTIVES ..... pages 22-23
6.1. MAIN OBJECTIVES ..... page 22
6.2. SECONDARY OBJECTIVES ..... page 23
7. **SUBJECTS AND METHODS ..... pages 23-35**
7.1. STUDY DESIGN ..... page 23
7.2. POPULATION OF INTEREST ..... page 23-24
7.3. SAMPLING ..... pages 24-25
7.4. VARIABLES ..... pages 25-30
7.4.1. Dependent/outcome variable ..... pages 26-27

7.4.2. Independent/predictive variable ……. page 27
7.4.3. Secondary variables ……. page 27
7.4.4. Covariates ……. pages 27-30
7.5. DATA COLLECTION ……. pages 30-35
8. STATISTICAL ANALYSIS …… pages 36-37
9. WORK PLAN …… pages 37-40
9.1. CHRONOGRAM …… page 40
10. FEASIBILITY …… page 41
11. ETHICAL AND LEGAL ASPECTS …… pages 41-42
12. STRENGTHS AND LIMITATIONS …… pages 42-44
13. BUDGET …… pages 45-47
14. IMPACT …… page 48
15. BIBLIOGRAPHY …… pages 49-60
16. ANNEXES
ANNEX 1. REGICOR CORONARY RISK ASSESSMENT FUNCTIONS …… pages 61-64
ANNEX 2. ANKLE BRACHIAL INDEX MESUREMENT …… page 65
ANNEX 3. AUDIT C QUESTIONNAIRE …… page 66
ANNEX 4. PARTICIPANT DATA SHEET …… page 67
ANNEX 5. DIAGENODE MEDIP MANUAL KIT …… pages 68-71
ANNEX 6. **.** PRIMER SEQUENCE AND INFORMATION …… page 72
ANNEX 7. PATIENT INFORMATION SHEET …… pages 73-75
ANNEX 8. INFORMED CONSENT FORM …… page 76

1. ABBREVIATIONS

ABI: Ankle brachial index
ACS: Acute coronary syndrome
AHA: American Heart Association
AUDIT: Alcohol Use Disorder Identification Test
BD: Becton Dickinson
CHD: Coronary heart disease
c-HDL: High-density lipoprotein cholesterol
c-LDL: Low-density lipoprotein cholesterol
CpG: Cytosine-phosphate-guanine dinucleotide
CRF: Coronary risk factor
CRP: C reactive protein
CVD: Cardiovascular disease
CVRF: Cardiovascular risk factor
DM: Diabetes mellitus
EKG: electrocardiogram
HbA1c: Glycated hemoglobin
HT: hypertension
IdIBGi: Institut d'Investigació Biomèdica de Girona
IHD: Ischemic heart disease
IL-1: Interleukin 1
IL-6: Interleukin 6
IP'd: immunoprecipitated
JTH: Josep Trueta Hospital
MCP-1: monocyte chemoattractant protein-1
MeDIP: Methylated DNA Immunoprecipitation
meDNA: Methylated DNA
MI: Myocardial infarction
miRNA: microRNA
mLDL: Modified low-density lipoprotein
mtDNA: Mitochondrial DNA
NSTE-ACS: Non-ST-segment elevation acute coronary syndrome
PBL: Peripheral blood leukocyte
PBMC: Peripheral blood mononuclear cell
PRP: platelet rich plasma
qPCR: Quantitative Polymerase Chain Reaction
RCF: Relative Centrifugal Force
SMC: Smooth muscle cell
STE-ACS: ST-segment elevation acute coronary syndrome
TNF: Tumor necrosis factor
unDNA: unmethylated DNA

2. ABSTRACT

Background: Epigenetics is one of the fastest growing research fields in biomedicine. Recently, numerous studies have analyzed the involvement of epigenetic mechanisms, especially DNA methylation, in the development and progression of cardiovascular diseases. It is well known that atherosclerotic lesions often precede the clinical manifestation of myocardial infarction. Therefore, studying the role of two key blood cell types, monocytes and platelets, in the development of MI, may provide greater insight into the identification of individuals at risk of suffering a coronary event. Since DNA methylation marks are cell-specific, many studies have analyzed the association of DNA methylation levels in myocardium or coronary atherosclerotic plaques in association with coronary heart disease. However, these techniques are highly invasive. Alternatively, studying of epigenetic marks in blood cells obtained by non-invasive techniques as venipuncture shows a promising landscape for coronary risk assessment in addition to classical risk functions.

Purpose: We aim to study the association between DNA methylation levels in IL-6 and MCP-1 genes as well as LINE-1 in monocytes, platelet DNA methylation levels, and the risk of MI.

Design: An observational case-control study consisting in patients after a first episode of a myocardial infarction as well as age and gender-frequency matched healthy control subjects.

Participants: Patients who suffered from a first episode of myocardial infarction hospitalized in the Coronary Unit of Josep Trueta Hospital in Girona.

Key words: Epigenetics, mitochondrial epigenetics, DNA methylation, platelet, myocardial infarction, interleukin-6, monocytes, coronary heart disease, LINE-1, MCP-1, blood biomarker, prognostic biomarker, cardiovascular risk assessment, atherosclerosis.

3. INTRODUCTION

3.1. Coronary heart disease

3.1.1. Epidemiology

Cardiovascular disease (CVD) remains the most common cause of death worldwide, with the 2013 Global Burden of Disease study estimating that CVD caused 17.3 million deaths globally. It accounted for 31.5% of all deaths and 45% of all non-communicable disease deaths, more than twice that caused by cancer, as well as more than all communicable, maternal, neonatal and nutritional disorders combined (1,2). Among different forms of CVD, coronary heart disease (CHD), stands as the first cause of mortality and one of the main causes of disability worldwide (3,4), being responsible for almost one third of all deaths in people older than 35 years old (5). In figures, CHD accounts for 1.8 million of deaths each year only in Europe (6).

Effects of CHD are not limited to developed countries; in fact mortality from ischemic heart disease (IHD) is expected to increase in developing countries (China, India, sub-Saharan Africa, Latin America, and the Far East), from an estimated 9 million in 1990 to an expected 19 million in 2020. The causes of this potential increase are social and economic changes, increased life expectancy, sedentariness, smoking, and a ''westernization'' of diet (7). Hence, although the incidence of CHD continues to stabilize or even decrease in developed countries, immigration and progressive population aging suggest that the absolute number of coronary events and, consequently, the prevalence of CHD will not decrease and may even increase in the near future. With similar numbers of men and women dying from CHD for all ages, the greatest differences between them are in the number of premature deaths, which are higher in men than women (6). Regarding racial differences, mortality from IHD is higher in Afro-Americans than in Caucasians, although these differences disappear at the age of around 75 years. In the Hispanic population, the coronary mortality rate is not as high as that in African-American and Caucasian populations (7).

In Spain, CHD is the first specific cause of death in men and the second one, after stroke, in women and represented 9.7% and 4.7% of the total mortality, respectively, in 2013 (3).

Even thought the high figures, mortality from CVD have been decreasing for the last decades and, as a result of the observed data, a number of countries now record a greater number of deaths from cancer than from CVD annually. According to this, more men die from cancer than CVD in 12 countries, Spain included, specifically Catalonia (6,8).

Population-based studies show a great variability in myocardial infarction (MI) incidence between countries. Hence, in Spain, MI incidence is among the lowest ones in the world, lower than northern European countries and the USA, and similar to other Mediterranean southern European countries such as France (2). The annual MI accumulative incidence tax in population between 25 and 74 years is approximately of 200/100.000 in men and 50/100.000 in women, with slight variability between regions and with a tendency to stabilize during the last two decades. In population older than 75 years, incidence is greater in both sexes (3). Although most studies have shown a downward trend in the incidence of myocardial infarction, some studies have shown contradictory results, above all those that cover the period after 2000, when physicians began to use troponin as a marker of myocardial necrosis (9). The introduction of this much more sensitive biomarker into the diagnosis criteria could have masked a reduction in the infarction rate (10).

In-hospital acute coronary syndrome (ACS) mortality in Spain was 4.1% in 2012 (11), which follows the decreasing tendency observed during the last years. This decline in mortality while incidence appears to remain stable has led to an increased prevalence of CHD (12), together with the progressive aging of population.

Total prevalence of people reporting heart or circulation problems in the last 12 months in the European Social Survey, for all countries combined, was the same for both sexes at 9.2% (6).

In relation to ACS types, in the last 20 years the incidence of ACS with ST-segment elevation (STE-ACS) has decreased notably, whereas ACS without ST-segment elevation (NSTE-ACS) incidence has doubled. The former reflects a larger efficacy in prevention of the disease, whereas the latter is related to treatment improvement, with a notable reduction in mortality and life expectancy enlargement, and use of new necrosis markers like cardiac troponins (cTn), much more sensitive in the detection of small infarcts. It has been estimated that 32.8% of ACSs are infarcts with ST elevation; 55.8% without elevation and 6% non-classifiable ACS, with these figures expected to increase in the next years (12).

3.1.2. Physiopathology

Myocardial ischemia

Myocardial ischemia is the result of the disequilibrium between coronary offer and myocardium demand of oxygen. There are multiple causes of myocardial ischemia, but all of them act through two mechanisms: blood flow reduction by coronary arteries obstruction and rising of oxygen necessities from the myocardium. The main causes of coronary flow

reduction are the progressive obstruction due to atherosclerotic lesion in the big coronary arteries of the epicardium and acute coronary thrombosis, which produces total or partial blockage of the arteries (13).

Coronary atherosclerosis

Atherosclerosis, an arterial disease that affects the tunica intima of large and medium-sized elastic and muscular arteries, is characterized by the accumulation of lipid and cellular elements, especially macrophages and smooth muscle cells (SMCs). The current hypothesis on the origin of atherosclerosis, considers the process a specialized inflammatory response against different forms of arterial wall lesions. The chronicity of the inflammatory process results in the formation of plaques, which in advanced phases can occlude the artery (13), leading to ischemia of the brain, the extremities or the heart depending on their location. In fact, these lesions represent a series of highly specific cellular and molecular responses that can best be described as an inflammatory disease, more than based on lipid accumulation (14).

Endothelial dysfunction is considered the first step in the process of atherogenesis. Possible causes of endothelial dysfunction leading to atherosclerosis include elevated and modified low density lipoproteins (mLDL), free radicals caused by cigarette smoking, hypertension (HT), diabetes mellitus (DM), genetic alterations or proinflammatory molecules as tumor necrosis factor (TNF) or interleukin 1 (IL-1) (14). The different forms of injury increase the adhesiveness of the endothelium with respect to circulating leukocytes or platelets, as well as its permeability. Monocyte and T cell receptor specific adhesion molecules are expressed (E-selectin, P-selectin), together with vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecules 1 and 2 (ICAM-1 and 2). The injury also induces the endothelium to have prothrombotic instead of antithrombotic properties, leading to an increase in platelet adhesion and the formation of vasoactive molecules, cytokines, and growth factors that play a role as chemotactic factors and SMCs proliferation stimulants. Such substances are platelet-derived growth factor (PDGF), IL-1, TNF-α or transforming growth factor β (TGF-β) (13).

In the process of atherosclerotic plaque formation, circulating monocytes bind to activated endothelial cells, and transmigrate into the subendothelial space of the arterial wall where maturation into macrophages occurs. Macrophages express scavenger receptors for uptake of mLDL, leading to foam cell formation (**Figure 1**). In addition, resident macrophages are major contributors to the local inflammatory response through secretion of proinflammatory

mediators (15), which can induce further damage and eventually lead to focal necrosis. Cycles of accumulation of mononuclear cells, migration and proliferation of SMCs, and formation of fibrous tissue lead to further enlargement and restructuring of the lesion, so that it becomes covered by a fibrous cap that overlies a core of lipid and necrotic tissue, a so-called advanced, complicated lesion. At some point, the artery can no longer compensate by dilation; the lesion may then intrude into the lumen and alter the blood flow (14), initiating the thrombogenic process.

ACS is produced by plaque rupture or erosion (70% and 30%, respectively) leading to thrombus formation, which could contribute asymptomatic plaque's growth or originate clinical manifestations (13).

Thrombosis

Atheroma plaques, not necessary obstructive but with a great lipid component, a thin fibrous capsule and signs of inflammation with monocyte and macrophage infiltration, can suffer from rupture or erosion of the tunica intima and induce the platelet adhesion, aggregation and activation, together with coagulation activation, leading to the formation of a thrombus that

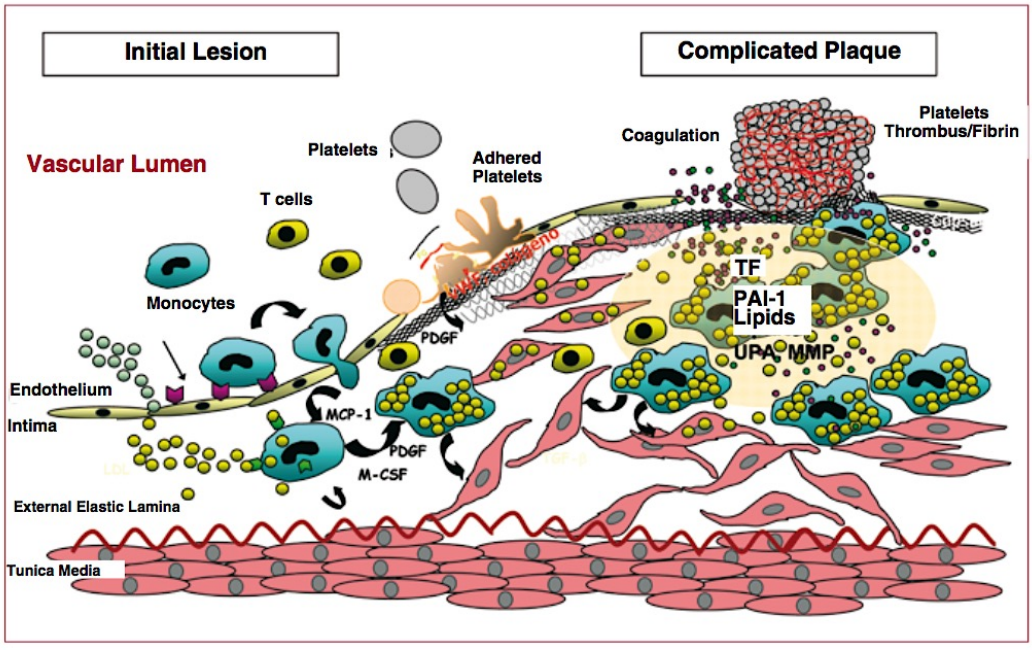


Figure 1. Schematic representation of the progression of atherosclerotic plaque from initial stages of endothelial dysfunction to advanced stages with the presence of complicated plaques. M-CSF indicates macrophage colony stimulating factor; MCP-1, monocyte chemotactic protein 1; MMP, metalloproteinases; PAI-1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; TF, tissue factor; UPA, urokinase plasminogen activator (102).

could occlude total or partially the vessel lumen acutely. Coronary thrombus formation plays a fundamental role in MI development. Hence, the knowledge of platelets and coagulation role and their inhibition pathways, are crucial for treatment of clinical ischemic complications associated with atherosclerosis, also called atherothrombotic events (13).

Platelets can adhere to dysfunctional endothelium, exposed collagen, and macrophages. When activated, platelets release their granules, which contain cytokines and growth factors that, together with thrombin, may contribute to the migration and proliferation of smooth-muscle cells and monocytes (14).

3.1.3. Clinical manifestations

CHD can adopt different clinical forms, with stable chronic angina and MI being the most frequent ones. Half the patients present with MI or sudden death as a first manifestation of the disease (13). Angina is an exclusively clinical concept known as the condition that presents with pain, oppression or discomfort, generally thoracic, due to transitory myocardial ischemia.

Acute coronary syndrome

ACS conforms the acute phase of CHD. Clinical manifestations depend on the gravity of the ischemia and the previous status of the patient, from the asymptomatic form to unstable angina, MI and sudden death. Atherosclerosis is the cause of more than 90% of coronary disease cases. Between the different factors associated with plaque rupture and ACS, stand out smoking, cocaine and amphetamine consumption, intense physical efforts in non-trained subjects, sexual activity, acute stress, aggressiveness, depression, anxiety, acute alcohol intake, atmosphere pollution, cold and respiratory infections.

In the diagnostic process, fundamentally clinical, anamnesis is essential to correctly evaluate a possible ACS. So, here coronary risk factors (CRFs) acquire an important role in evaluating the probability of the pain to be coronary.

The electrocardiogram (EKG) allows classifying ACS in different types: persistent ST segment elevation and thoracic pain after nitroglycerin administration, will lead the diagnosis to STE-ACS, candidate to rapid reperfusion therapy. Presence of transitory ST-segment descent during pain could be suspicious of unstable angina or NSTE-ACS; therefore, due to the urgency of applying a correct reperfusion treatment, patients are classified in two groups: those with and without ST-segment elevation. Unstable angina and non-ST-segment elevation infarct are considered parts of the same entity and are grouped under the name of NSTE-ACS.

At present, MI is defined as an elevation and fall of necrosis biomarkers (cTn preferentially) together with myocardial ischemia evidence by one of the following: ischemia symptoms, EKG changes indicative of ischemia (ST segment alterations, T wave and left branch blockage not evidenced before), appearance of new Q waves in the EKG, evidence by image techniques of viability loss or dyskinesia of a myocardial segment (13).

Nontroponin biomarkers are being studied intensely in this moment, in order to add them to classic risk functions, such as brain-type natriuretic peptide (BNP/NT-proBNP) (16,17).

3.2. Cardiovascular risk factors

The term *coronary risk factor* was firstly introduced in the USA based on Framingham study results (18). It is defined as the condition (biological, lifestyle or acquired habit) that increases the probability of suffering from a coronary pathology and enables the recognition of the most exposed population group. The factors are divided in: a) non-modifiable: age (more than 55 years in men and 65 in women), sex, menopause, family history (premature CVD [men < 55 years and women < 65 years]), ethnic group; b) potentially modifiable: dyslipidemia, HT, smoking, DM, obesity, personality, alcoholism, sedentary lifestyle, diet, oral contraception and left ventricular hypertrophy; and c) other risk factors, such as homocysteine levels, lipoprotein [a] levels, C reactive protein (CRP) serum levels, cardiac frequency, albumin urine excretion or postprandial glycaemia (13).

In comparison to MI, the prevalence of risk factors is higher among adult male and female population in Spain (19). For example, hypercholesterolemia, defined as total cholesterol blood levels equal or superior to 190mg/dL, is present in 81% of men and 79% of women; and if defined as total cholesterol equal or superior to 250mg/dL, is presented in 43% and 41%, respectively. HT is presented in 47% and 39%, and obesity is at 29% both men and women (20).

The impact in disease appearance of the different risk factors can be estimated by population attributable risk calculation, which depends on incremental risk associated with the presence of the factor and the prevalence of it in the population. Individually, the risk of having a MI in the next ten years can be estimated depending on the exposition to the different cardiovascular factors described before by risk functions. In Spain, there are some cardiovascular risk functions, but the only validated is the one adapted by the group *Registre Gironí del Cor* (REGICOR) from the Framingham function. Framingham's equation adaptation considering events tax and risk factors prevalence in Girona population, provided estimations very close to the real numbers and is the most valid one available in Spain to population

between 35 and 74 years old (21). It allows making an estimation of the risk to have a coronary event, angina and MI (mortal or not) in the next 10 years. Risk assignment is based depending on the presence of diabetes, smoking, sex and age. Moreover, it also has into consideration HT, total cholesterol and high-density lipoprotein (HDL) levels (**Annex 1**).

During the last decade, great efforts have been made in order to incorporate new biomarkers that enables to improve the prediction of risk functions (i.e. high sensitivity CRP or oxidized LDL) and, more recently, with punctuations of the presence of adverse genetic characteristics to CHD (22,23) and ankle brachial index (ABI) (24). In all cases, improvement is modest and tends to be measured in percentages of patients reclassified to greater risk levels than those assigned by risk functions based on classic risk factors.

Population cardiovascular risk stratification is important in order to make cardiovascular primary prevention as effective as possible directing it to groups of individuals of greater risk. This is why, efforts are directed in improving classic risk functions, in order to classify more efficiently a great amount of individuals of moderate risk that eventually will develop the disease and who will be specially benefited of an intense program of primary prevention (25).

Recently the American Heart Association (AHA) published recommendations which define the different stages in the evaluation of new risk biomarkers and their subsequent application in clinical practice (26). In the first stage, known as "*Proof of Concept*", it is necessary to establish whether the level of exposure to the biomarker is different in individuals with and without the disease. In subsequent stages, cohort studies should be carried out to analyze any improvement in ability to discriminate, determine the effect on daily clinical practice and evaluate cost effectiveness.

3.3. Epigenetics:

Epigenetics is one of the fastest growing research fields in biomedicine. Epigenetic changes may explain why subjects with similar genetic backgrounds and risk factors for particular diseases can differ greatly in clinical manifestation and therapeutic response.

The role of epigenetics has been mainly evaluated in cancer, diabetes, neurological and imprinting disorders, autoimmune diseases and aging. Recently, numerous studies have analyzed the involvement of epigenetic mechanisms in the development and progression of CVD (27–31). In addition, some solid experimental data has proved that epigenetic changes have also an important role in regulation of the different pathways related to the atherosclerosis process (endothelial integrity, lipid inflammatory response, SMCs proliferation, cholesterol synthesis) and lipoproteins metabolism (32,33).

It is estimated that genetic factors could explain between 40% and 55% of the existing variability among the population (inheritability) in the development of CHD (34,35), showing the necessity for a further study of the interactions between genome and environment that can be achieved by epigenetics. Epigenetics relevance can be also demonstrated taking into account the negligible proportion of the total human genome coding for proteins.

Epigenetic modifications refer to modifications in chromatin state or non-coding microRNAs (miRNAs) that may lead to reprogramming in gene expression without changing the nucleotide sequence of the DNA itself (36). These modifications are heritable mechanisms, in case of methylation, that amend the genotype to differentially modulate the phenotype without changing the underlying DNA sequence. Moreover, epigenetic marks are not uniform within an organism and can locally regulate silencing or transcription of genes to manifest a phenotypic adaptation in a specific tissue.

Although epigenetic modifications are well established as one of the underlying causes in cancer development (37), only recently has the link between epigenetics and metabolic diseases been recognized giving to ambient its important role in disease development by gene expression modulation. Earlier studies suggested that epigenetic modifications are established during development and maintained over the lifetime. However, recent research provides evidence that epigenetic mechanisms can be both rigid and flexible, allowing rapid adaptations to a changing environment. Therefore, these mechanisms may exacerbate the epidemic of metabolic diseases like obesity or type 2 DM, which are well known cardiovascular risk factors CVRFs (38). Epigenetic methylation marks are erased in early embryogenesis and reset during development. Environmental influences can lead to stable changes in these marks that alter the individual's susceptibility to disease.

Two major epigenetic mechanisms are DNA methylation and histone modifications. Modulation of gene transcription and translation by non-coding RNAs, including miRNAs, is also a mechanism of epigenetic control. DNA modifications can operate on a genome-wide level, such that a uniform pattern exists across the entire organism, or on a tissue-specific level. Tissue-specific epigenetic regulation may be very important for disease development.

3.3.1. DNA methylation

DNA methylation is the heritable and reversible attachment of a methyl group to a cytosine nucleotide. In mammals, DNA methylation appears to be specific to cytosine, predominantly to CpG (cytosine-phosphate-guanine) dinucleotide. In gene promoter regions, CpG-sites are often found as clusters called CpG islands. Methylation of promoter CpGs can introduce stable changes in gene expression, which might lead to silencing of that gene (39,40). Thus, the primary function of DNA methylation is to actively silence genes and DNA regions in which transcription is not desired. High levels of DNA methylation are found in telomeres, centromeres, and inactive X-chromosomes (41). DNA methylation is crucial for cell development and differentiation and loss of the methylation-establishing enzymes, DNA methyltransferases, results in embryonic lethality, probably due to compromised genomic organization and orchestration (42,43). Likewise, disturbances in DNA methylation are associated with cancer development. DNA methylation can indeed occur at various locations spanning a given gene, not only the promoter region, but also within the gene body introns or exons, intergenic and non-translated regions. DNA methylation within a gene promoter can silence the associated gene due to the presence of methyl groups that can allosterically inhibit the binding of transcription factors or transcriptional enhancers and thus result in decreased gene transcription. Conversely, the role of intragenic DNA methylation, compared with gene promoter methylation, is incompletely resolved and may enhance rather than silence gene activity (44).

DNA methylation states regulate biological processes underlying CVD, such as atherosclerosis, inflammation, hypertension, and diabetes (45).

3.3.2. Histone modifications

DNA is organized into chromatin, the proper function and maintenance of which is crucial for cell identity and survival. Histones are essential proteins of chromatin that facilitate the packaging of DNA into nucleosomes and possess tails that can be post-translationally modified to alter the accessibility of the DNA. Such histone modifications include acetylation, methylation, phosphorylation, adenosine diphosphate (ADP)-ribosylation, and ubiquitination (46). The effect of histone modifications on transcription is highly diverse and is contingent on the type of modification. Thus, histone modifications can function to either silence or activate genes and evidence indicates that these modifications may be altered following changes in the environment (47).

3.3.3. MicroRNAs

miRNAs are short (20–22 nucleotides) non-coding RNAs modulating gene expression further by downregulating the translation of target messenger RNAs (mRNAs) by the inhibition of post-transcriptional events, transcript degradation or direct translational suppression (48). Non-coding RNAs were found to be important in the pathogenesis of CVD and also offer the possibility of operating as diagnostic and prognostic biomarkers (49). However, as each miRNA may post-transcriptionally regulate 100 different mRNAs, it is difficult to connect any particular miRNA to a specific disease (50,51).

3.4. Epigenetics and cardiovascular disease

3.4.1. Trained immunity

Recent discoveries in the field of innate immunity have shed light on how monocytes and macrophages adopt a long-term proinflammatory phenotype in patients with risk factors for atherosclerosis. It has been posed that these cells, guardians of non-specific host defense, could also build up long-term memory via epigenetic reprogramming, a process termed 'trained immunity' (52). Circulating monocytes that have not yet infiltrated the plaque are exposed to environmental signals that may induce epigenetic proatherosclerotic reprogramming of the cells. Oxidized LDL (53) and other endogenous compounds associated with metabolic diseases, such as cholesterol crystals, free fatty acids or advanced glycation end products, which also act as CVRFs, could be such inductors (36). Therefore, insights into mechanisms controlling (long-term) activation of innate immune cells, especially in CVD, are of crucial importance for understanding the molecular features underlying atherosclerotic plaque development and progression.

Clinical evidence for the concept of trained immunity in CHD has been recently provided by a study. This investigation evaluated whether monocytes derived from patients with CHD are primed differently, resulting in proinflammatory cytokine production upon entering the tissue space, by comparing ex vivo-differentiated macrophages from patients and controls (54). Patient-derived monocytes showed increased production of proinflammatory cytokines interleukin 6 (IL-6) and IL-1b after stimulation, which persisted once cells differentiated into macrophages, suggesting a different reprogramming of the cells.

3.4.2. Interleukin 6

IL-6 is a proinflammatory cytokine that is triggered by vulnerable plaque or necrotic myocardium and is correlated with the severity of CHD. In patients with CHD, elevated

circulating IL-6 concentrations may be involved in the function of macrophage/foam cells present in atheromatous plaques (55).

IL-6 demethylation is a common epigenetic basis for the pathogenesis of inflammation-associated diseases (56). DNA hypomethylation of 2 CpGs, located downstream in the proximity of the IL-6 gene promoter, has been associated with air pollution exposure, a risk factor for IHD (56). Moreover, DNA hypomethylation of IL-6 gene measured in blood leukocytes was associated with increased risk of IHD and these levels of methylation were inversely associated with serum IL- 6 levels. Therefore, IL-6 demethylation may upregulate its expression, whereby exerting its risk effect on the development of CHD (57). It has also been found higher expression of IL-6 receptor in different subsets of monocytes in association with CHD (58).

3.4.3. Monocyte count

High total monocyte count is a strong predictor of high risk of CHD or MI. Recent data suggests that a high monocyte count was a significant and independent predictor of cardiovascular events in CHD patients (59), which correlates with other studies showing that high monocyte counts in healthy and middle-aged subjects could predict a future coronary event (60,61). Even though these studies differ in the cutoff values of monocyte counts for predicting a cardiovascular event, varying according to clinical characteristics of the patient. Hence, together with data about different cutoff value for plaque progression in patients during acute phase of MI (62), it seems that monocyte count may vary according to the severity of atherosclerosis. Moreover, monocyte figures have also been reported to be an independent predictor of common carotid atherosclerosis in healthy subjects and they also positively correlate with both mean and maximum intima-media thickness of the common carotid artery in subjects with type 2 diabetes (63). In high-risk populations, the amount of circulating monocytes independently predicts the risk for CHD (64–66). Taken together, these studies suggest that, of the leukocyte fractions, the monocyte count is the most useful for the evaluation of atherosclerosis, and is involved in all stages of its progression.

3.4.4. LINE-1

About 55% of the human genome consists of repetitive elements, including approximately 500.000 Long Interspersed Nucleotide Elements (LINE-1), which are heavily methylated and represent approximately 17% of the total genome. Because of this high representation throughout the genome, LINE-1 methylation correlates with global genomic DNA methylation content (67,68) and has been used as a surrogate marker for estimating global DNA

methylation levels. A global loss of DNA methylation has been related to atherosclerosis (69) in a variety of tissues, including peripheral blood leukocytes (PBLs) (70). In human investigations, although the range of variation in healthy subjects is relatively narrow, LINE-1 methylation or global genomic methylation content measured in blood DNA has been found to be lower in people with risk factors for atherosclerotic disease, such as older age, smoking and air pollution (71–75). Hence, blood DNA hypomethylation represent an easily measurable marker reflecting the presence and the progression of atherosclerosis. However, it has not yet been established how this global DNA hypomethylation affects gene expression and future investigations must shed some light on this issue.

LINE-1 hypomethylation in PBLs was associated with increased risk for IHD in both cross-sectional and longitudinal analyses, in a cohort of 712 elderly men from American population (76). Moreover, a recent study found that LINE-1 hypomethylation was associated with the risk of CHD in Chinese population (77).

3.4.5. Platelet mitochondrial DNA methylation

Abnormal platelets, either quantitatively or qualitatively, are associated with CVD (78–81). Platelets have a greater rate of ATP turnover than resting mammalian muscle that contains high levels of mitochondria (82), suggesting an essential role for mitochondria as energy source in platelet function. Mitochondrial DNA (mtDNA), as with nuclear DNA, can be methylated by machinery existing inside of the mitochondria and can mediate the control of mitochondrial gene expression (83). Platelets lack a nucleus, and therefore, the mitochondrial genome is the only genetic material in these cells. Therefore, the epigenetic regulation of mitochondrial genes in platelets is critical to understanding their implication in the development of CVD (84).

The mitochondria contain their own DNA molecules, which are copies of a genome of approximately 17 kilobase (kb) in circular form. Mitochondrial genome contains 37 genes, 13 of which are protein encoding while 24 encode transference RNAs (tRNAs) and ribosomal RNAs (rRNAs). Among several aspects that distinguish mitochondrial from nuclear genome, mitochondrial genome lacks LINE-1 and histone complexes (85).

Association between mtDNA methylation in PBLs and environmental air pollution exposure has been observed in healthy humans (86). Indeed, mtDNA methylation has been proposed as a next-generation biomarker and diagnostic tool (87). As every cell type contains different number of mitochondria and different levels of their activity, it is expected that mtDNA epigenetic patterns will differ by tissue and cell type. Therefore, studying mitochondrial

epigenetic patterns by single cell type is critical to understand the effects of mtDNA methylation on disease.

Even thought the efficacy of using mtDNA as a biomarker has been controversial (87,88), a recent study has found association between specific mtDNA genes methylation in platelets and CVD (84).

3.5. Age

A growing body of research has reported associations between age and the state of the epigenome, the set of epigenetic modifications in an organism. In particular, DNA methylation associates with chronological age over long time scales and changes in methylation have been linked to complex age-associated diseases such as metabolic disease, cancer and CHD(77,89).

3.6. Future applications

Apart from diagnostic and prognostic purposes, research is being directed to the potential use of epigenetically active compounds to treat CVD pathologies such as atherosclerosis, heart failure, MI and cardiac hypertrophy. However, it is only preliminary and the complex relationship between epigenetic regulation and CVD development clearly demands further studies (50). Current available therapies, such as statins are being used to promote epigenetic-based control in CVD prevention through histone modifications. Indeed, studies show that pretreatment of endothelial cells in atherosclerotic plaques of human coronary arteries with simvastatin or fluvastatin for 24h reduces oxidized LDL-related release of IL-8 and monocyte chemoattractant protein-1 (MCP-1) (90). Even though clinical practice does not yet use epigenetically active molecules in the therapy of atherosclerosis-related CVD.

4. JUSTIFICATION:

Taking into account the current trends in disease burden, CHD is going to acquire even more relevance during the next years due to the progressive aging of population and CVRFs prevalence increase. A decrease in disease's incidence is needed in order to also achieve prevalence lowering, because diagnosis and treatment improvements have had success in mortality diminishing, increasing the total number of patients among population. In order to achieve this incidence reduction, which will translate in prevalence lowering, efforts should be directed to primary prevention, where current risk stratification functions have demonstrated to be insufficient in proper risk graduation of patients. Moreover, the improvement of risk equations by including new biomarkers is clinically significant since

existing risk equation sensitivity is low and a large number of cases affect intermediate-risk patients.

With this intention, novel biomarkers are being assessed to evaluate its potential in better stratification of in-risk patients through their inclusion in classic risk functions. Among such biomarkers are genetic variations, which have showed modest results. This can be explained due to the small proportion of heritability of complex diseases and the importance of environment and its interactions with the genome. Hence, it seems plausible that epigenetic biomarkers could better explain different patients' risk of disease development and increase the capacity of classic risk functions in patients' stratification.

Studying two key blood cell types for atherosclerosis development such as monocytes and platelets may provide greater insight into developing biomarkers for CHD risk assessment. Because atherosclerotic lesions often precede the clinical manifestation of MI, blood DNA methylation patterns might be used to identify individuals at risk of cardiovascular events.

As we already said, methylation marks are tissue specific, which is why research is being addressed to determine differential methylation profiles in heart and atherosclerotic arteries in relation to distinct pathologies such as stroke, cardiomyopathy, heart failure and atherosclerosis (91–95). Considering its invasive nature, samples are difficult to obtain, obstructing studies' development. These studies also lead with ethical aspects due to their bloodiness and are often restricted to post-mortem analysis. For this reason, study of epigenetic biomarkers obtained by non-invasive techniques as venipuncture is of great importance and needs to be well investigated.

This tissue-specificity makes it difficult to know whether circulating blood leukocyte DNA reflects the correct epigenetic signature. DNA methylation markers in the nuclear genome of unfractionated PBLs associated with CVD risk have produced inconsistent results and have been particularly challenging to develop. A possible reason for this difficulty could be that PBLs are a mixture of multiple leukocyte subtypes, and therefore the observed changes in molecular markers in at-risk individuals, which may differ in each of the cellular subtypes, may simply reflect differences in the proportions of circulating cellular subtypes. Furthermore, each leukocyte subtype has a variable risk-predictive relationship with CVD, as we already seen with monocyte count. Thus, investigating single-cell epigenome, such as platelets and monocytes, provides a unique opportunity to solve the biological riddle that arises with the use of a mixed population of blood cells (PBLs/buffy coat). Platelet mitochondria can be obtained non-invasively and can be easily isolated from both fresh and

frozen plasma samples, so they provide an easy-to-handle biospecimen for clinical and preventive applications (84), as well as monocytes.

Even though epigenetic marks have been well studied in PBLs, little or none has been done in relation to specific changes in monocytes, so the investigation of particular epigenetic modifications in these cells would be of great interest not only because epigenetic mechanisms have been shown to be cell-specific, but also because of their important role in disease development. Thus, particular study of LINE-1, IL-6 and MCP-1 methylation state in monocytes are presented to be of enormous relevance in the study of epigenetic associations to MI and may help identify individuals at risk of developing CHD. Specifically, only 2 CpG sites in IL-6 gene were analyzed in the Chinese study (57), and given that gene transcription has been speculated to be regulated by a combination of loss and gain of methylation at different regulatory regions, further studies covering all CpG sites across the IL-6 regulatory regions are required to investigate the combined regulation effect of DNA methylation on IL-6 expression and CHD risk. Moreover, taking into consideration that plasma levels of IL-6, as CRP levels, are known for its low specificity, determining the specific epigenetic modifications in IL-6 gene sequence associated with MI could become of great value also in disease diagnosis.

The monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes. From different cell types, monocyte/macrophages are found to be the major source of MCP-1 (96). Examination of the relationship between monocyte count and this inflammatory biomarker blood levels, and also its epigenetic modifications in monocyte genome would be of great relevance because there is no such data published yet.

Global DNA methylation levels, assessed in repeat regions from leukocyte-derived DNA, have been reported to be associated with risk of CHD in American, Singapore and Indian populations, with inconsistent observations (76,97,98). The different lifestyle, environmental exposures, and genetic backgrounds among the populations, and differences in sample size and characteristics, together with different repetitive elements of DNA targeted for measuring global hypomethylation levels, might contributed to the different findings across these studies. For example, due to the American study (76) can only address to older white men in the Boston area, additional investigations should address the role of LINE-1 hypomethylation among women, as well as in various age and ethnic groups. Furthermore, because they measured methylation in blood DNA, their results might have reflected shifts in the

proportions of white blood cell subsets caused by alterations related to impeding disease onset.

The recent published investigation about platelet mtDNA methylation (84) lacks of a proper definition of cases and its sample size is small. However, it shows some promising results that are worth to further investigate due to its future perspectives. From the entire platelet mtDNA, we are going to study methylation levels of genes associated with adenosine triphosphate (ATP) synthesis: three protein-encoding cytochrome c oxidase (COX) genes associated with the respiratory electron transport chain complex (MT-CO1, MT-CO2 and MT-CO3). ATP-synthesis disturbance will affect platelets function, which could be in association with CHD.

In conclusion, considering that CHD is one of the most common diseases, along with its severity, the risk factor of global hypomethylation, IL-6 and MCP-1 epigenome in monocytes, as well as platelet mtDNA methylation pattern could have a sizable impact in public health. Because DNA methylation is a reversible epigenetic mechanism, these blood-based markers could offer exciting new opportunities for population-based CHD prevention as well as risk assessment.

5. HYPOTHESIS

Specific methylation patterns in IL-6 and MCP-1 gene sequence, together with LINE-1 methylation level in monocytes DNA, as well as platelet mtDNA methylation state, are associated with the risk of MI.

6. OBJECTIVES

6.1. Main objectives

- To assess whether LINE-1 methylation, IL-6 and MCP-1 gene methylation in monocytes DNA is associated with the risk of MI in Girona population.
- To asses whether platelet mtDNA methylation is associated with the risk of MI in Girona population

6.2. Secondary objectives

- To evaluate the association between MCP-1 gene methylation and serum MCP-1 level.
- To assess the association between IL-6 gene methylation and serum IL-6 level.
- To determine the association between MCP-1 gene methylation and monocyte counts in patients with MI.
- To establish the correlation of LINE-1 methylation in monocyte DNA and mtDNA methylation in platelets.

7. SUBJECTS AND METHODS

7.1. Study design

This study will be designed as an observational analytical case-control study consisting in patients after a first episode of a MI as well as age and gender-frequency matched healthy control subjects, in order to study their differential DNA methylation state in monocyte and platelet genomes. The total length of the study period will be 29 months and it will be performed in the Josep Trueta Hospital (JTH) in Girona and in the *Institut d'Investigació Biomèdica de Girona* (IdIBGi) Cardiovascular genetics laboratory.

7.2. Population of interest

The study population will include patients who suffered from a first episode of MI (incident cases) hospitalized in the Coronary Unit of JTH in Girona. Controls will be recruited from patients attending to the Emergency Services of the same hospital during the same period when the cases are recruited. The selection criteria for controls must be the same as in cases in order to maintain the same probability of exposition to the risk factor. The only difference between both groups is the presence or absence of CHD (MI, chronic angina) and the other main clinical manifestations of atherosclerosis in controls (stroke, peripheral artery disease).

A) Inclusion criteria:

- Patients between 18 and 80 years old.
- Patients (men and women) with diagnostic criteria of MI, according to clinical guidelines of Spanish Society of Cardiology.
- Patients after a first episode of MI.
- Patients who agree to participate in the study. They have to understand and sign an

informed consent form.

In controls, they must be diagnosed free of CAD (MI, chronic angina).

B) Exclusion criteria:

- Patients diagnosed with hypertrophic or dilated cardiomyopathy.
- Patients diagnosed with cardiac insufficiency.
- Patients with previous history of congestive heart failure and/or heart failure at admission.
- Patients with active infectious disease (axillary body temperature >37°C), local or systemic inflammatory conditions, history of malignancy and/or auto-immune-related diseases that could alter the immune system response.
- Patients with any type of blood dyscrasia.
- Patients at end-stage of renal disease (estimated glomerular filtration rate [eGFR] <15mL/min/1.73m^2).

In controls, we have decided to include history of stroke, carotid artery stenosis ≥50% and peripheral artery disease as exclusion criteria in order to collect individuals free of main clinical manifestations of atherosclerosis. We have not include these criteria in cases due to sampling difficulties and based on the knowledge that atherosclerosis is a systemic process that when produces clinical manifestations in one organ (i.e. heart), is common that it already have produce alterations in other regions, even though it has not yet produce clinical manifestations in those ones.

Even though smoking, obesity, DM and HT have been independently linked to specific changes in the epigenome (36,38,75,81,86,99,100) and are well known risk factors for coronary disease, we have decided not to exclude them from the study.

7.3. Sampling

The sampling method will be non-probabilistic consecutive. Every patient hospitalized in the Coronary Unit of JTH in Girona that fulfills the criteria of inclusion and not exclusion will be enrolled in the study as long as he/she agrees to participate. On the other hand, controls will be obtained also consecutively from patients attending to Emergency Services of the same hospital and accomplishing the inclusion and not exclusion criteria to enter the study, with previous agreement to participate. This sampling will be stratified for age and gender-

frequency in order to guarantee proper variables representation in both groups. This is important because, as we already explained, MI is more frequent in men and older people and also the methylation patterns vary with age.

Taking into account that epigenetic marks are very influenced by environment, we would like to stratify for other risk factors related to changes in methylation profile such as smoking, HT, obesity and DM. However, we finally rejected the idea in order to preserve sample representativeness and results generalization as much as possible.

The sampling will last until we obtain the number of subjects we calculated for the study.

Sample size

Assuming an alpha risk of 0.05 and a beta risk lower than 0.2 in a bilateral contrast, we need 454 cases and 454 controls to detect a minimum difference (Odds Ratio, OR) in methylation proportion of 1.5. This difference has been determined to be significant based on previous data about the issue (57,76,77) and the opinion of experienced members of the Cardiovascular genetics team of the IdIBGi. We have estimated that the tax of follow up loses will be 10%, due to problems in different steps of sample processing. Sample size has been calculated using the approach of POISSON through the "Calculadora de Grandària Mostral GRANMO"(101).

Acknowledging that the number of hospitalizations due to a MI in the Coronary Unit of the Josep Trueta Hospital are around 800 cases per year, and taking into account that we want incident cases, not prevalent, which are 86% of these 800, together with the selection criteria and patients denial to participate, we estimate, being conservative, a period of 2 years for reaching the sample size.

7.4. Variables

Data will be collected at JTH in Girona, where the patients will be recruited from the Coronary Unit (cases) and the Emergency Services (controls) and analytical procedures will take place mainly in the Cardiovascular genetics laboratory of IdIBGi, but also in the JTH clinical analysis laboratory.

7.4.1. Dependent/outcome variable

Myocardial infarction

Patients will be classified as MI from the diagnosis of the medical team of the Coronary Unit in the JTH. The diagnostic criteria of MI, according to the European Society of Cardiology /AHA (9) are the following:

Non-fatal MI

- Development of pathologic Q waves on the ECG.
- ECG changes indicative of ischaemia (ST segment elevation or depression).
- Typical rise and gradual fall (troponin) or more rapid rise and fall (creatine-phosphokinase-MB fraction).
- Ischaemic symptoms.

The diagnosis can be ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) or unstable angina in relation to findings in the ECG and serum cardiac enzymes.

First episode of MI

Patients diagnosed with MI by JTH Coronary Unit medical team should not have been previously documented of MI and/or myocardial revascularization with percutaneous coronary intervention (PCI) or by-pass surgery (CABG), demonstrated by recent medical registers, together with the absence of ancient necrosis Q waves in the ECG, sign of a silent past MI. Documented previous MI should have occur at least 28 days before the date of actual episode symptoms beginning to be considered as past MI in the medical history. If not, current symptoms will be considered as a complication of the same episode and patient can be included in the study.

Free of MI

In controls, patients are determined to be free of CHD (MI, chronic angina) by medical history, clinical examinations and ECG. Not-previous history of stroke needs to be evidenced by medical history too and carotid artery stenosis <50% by ECO-Doppler. Peripheral artery disease is rejected by an Ankle Brachial Index (ABI) > 0.9 (**Annex 2**).

ECO-Doppler of the carotid arteries will be performed by trained medic personal and calibrated and certificated instruments in the Emergency Services of JTH, together with ABI determination following standardized instructions.

7.4.2. Independent/predictive variable

DNA methylation (%5mC) (mean ± SD)[1]

Levels of DNA methylation for each gene will be expressed as percentage of 5-methylated cytosines (%5mC) over the sum of methylated and unmethylated cytosines among the total gene length. The result will be expressed as a mean with standard deviation (SD) in controls and cases separately. This process will be done for IL-6, MCP-1 and LINE-1 in nuclear DNA of monocytes; and for cytochrome c oxidase genes (MT-CO1, MT-CO2 and MT-CO3) in platelets mitochondrial DNA.

7.4.3 Secondary variables

IL-6 level (mean ± SD)[1]

IL-6 level will be measured from plasma samples. Results will be expressed as a mean ± SD in controls and cases separately.

MCP-1 level (mean ± SD)[1]

From plasma samples, MCP-1 concentration level will be expressed as a mean ± SD in controls and cases separately.

Monocytes count (mean ± SD)[1]

Monocyte count from cases serum samples will be expressed as mean ± SD number of monocytes per mm^3 of serum sample ($/mm^3$).

7.4.4. Covariates

Epidemiological characterization of patients will be made by these covariates that will be

[1] Results will be expressed as mean ± SD if we can assume a normal distribution. If not, using the median, first and third quartile.

collected at the hospitalization in the Coronary Unit (cases) or Emergency Services (controls) of JTH by clinical interview, physical exploration and clinical analysis.

❖ **Baseline characteristics [n (%)]:**

- **Age**[2]: years
- **Gender**: Female or Male
- **Ethnicity:** African/Caucasian/Hispanic/Asiatic
- **Body mass index (BMI)**[2](kg/m^2)
- **Waist-hip ratio (WHR)**[2](cm)
- **Cigarette smoking** (Non-smoker/Smoker)

An ever-smoker was defined as a smoker of at least 1 cigarette per day for at least 6 months.

- **Oral contraception** (Yes/No)
- **Alcohol drinking** (Non-drinker/Drinker)

A drinker was considered with an AUDIT (Alcohol Use Disorder Identification Test) C (reduced version) punctuation ≥5 in men and ≥4 in women (**Annex 3**).

- **History of hypertension** (Yes/No)

HT will be considered when:

- A personal history of hypertension is reported.
- A treatment for hypertension is followed.

- **History of diabetes** (Yes/No)

Diabetes will be considered when:

- A personal history of DM is reported.
- A treatment for DM is followed.
- Unique glycaemia is ≥ 200mg/dL in patients neither diagnosed nor treated with typical symptoms (polyuria, polydipsia, polyphagia), weight loss or ketoacidosis.
- Unique glycaemia is ≥ 126 mg/dL and glycated hemoglobin (HbA1c) ≥ 6,5% in patients neither diagnosed nor treated.

- **Statin use** (Yes/No)
- **Fasting lipoproteins** (mg/dL) (mean ± SD)
    - Total cholesterol
    - c-LDL concentration
    - c-HDL concentration
    - Triglycerides

[2] Age, BMI and WHR quantitative variables will be categorized in terciles based on values among control subjects and expressed as n, %.

- **Family history of premature cardiovascular disease** (Yes/No)

Premature CV disease in first-degree familiars (Male < 55 years; Female < 65 years): stroke, peripheral arteriopathy and MI

VARIABLE	TYPE	MESUREMENT UNIT
Age	Discrete quantitative	Years
Gender	Dichotomous Nominal qualitative	Male/Female
Ethnicity	Nominal qualitative	African Caucasian Hispanic Asiatic
Body Mass Index (BMI)	Continuous quantitative	kg/m^2
Waist-hip ratio (WHR)	Continuous quantitative	cm
Cigarette smoking	Dichotomous Nominal qualitative	Yes/No
Oral contraception	Dichotomous Nominal qualitative	Yes/No
Alcohol drinking	Dichotomous Nominal qualitative	Yes/No
History of hypertension	Dichotomous Nominal qualitative	Yes/No
History of diabetes	Dichotomous Nominal qualitative	Yes/No
Statin use	Dichotomous Nominal qualitative	Yes/No
Total cholesterol	Discrete quantitative	mg/dL
c-LDL	Discrete quantitative	mg/dL
c-HDL	Discrete quantitative	mg/dL
Triglycerides	Discrete quantitative	mg/dL
Family history of premature CV disease	Dichotomous Nominal qualitative	Yes/No
IL-6 methylation	Continuous quantitative	%5mC
LINE-1 methylation	Continuous quantitative	%5mC

MCP-1 methylation	Continuous quantitative	%5mC
MT-CO1 methylation	Continuous quantitative	%5mC
MT-CO2 methylation	Continuous quantitative	%5mC
MT-CO3 methylation	Continuous quantitative	%5mC
IL-6 serum level	Continuous quantitative	pg/mL
MCP-1 serum level	Continuous quantitative	pg/mL
Monocyte count *	Discrete quantitative	n/mm^3

Table 1. Cases and controls characteristics. **Monocyte count will be only analyzed in cases samples.*

7.5. Data collection

Prior starting this study, a course will be performed in order to form all members of the team in the proper collection of the data. This course will be performed in the JTH Coronary Unit as well as in the Emergency Services in the same hospital. Specific medical team will be formed in participants' selection criteria, together with calibration of diagnostic tools (Ecographs, scales, sphygmomanometers, thermometers, electrocardiographs) and standardization of protocols (ABI measurement, carotid arteries echographic study). In front of a candidate, proper information of the study should be given, not only verbally, but also by an information sheet and informed consent should be signed before any intervention.

Specific members of the nursing team of both services will be formed and trained in venipuncture technique and blood sample management, together with structured questionnaires to follow in participant data collection. Blood samples will be labeled in specific numeration in order to prevent laboratory members to know if the sample they are analyzing is from a control or a case. This is especially important in methylation analysis.

Formed medical team members of Coronary Unit and Emergency Services of JTH will follow inclusion and exclusion criteria in order to decide participants' inclusion to the study. Data will be collected as soon as participants sign the informed consent before hospital discharge. Structured questionnaires will be used by trained nursing interviewers to collect information about demographic variables, lifestyle habits and medical history. All the information collected will be written in the participant Data Sheet (**Annex 4**). The identification code will follow a particular numeration in order to differentiate cases, from Coronary Unit (#1-) and controls, from Emergency Services (#2-). In this way, we will guarantee blindness of operators in charge of methylation and plasma analysis in Cardiovascular genetics laboratory.

According to this form, data will be reported to the study database.

Anthropometric measurements

A precision scale of easy calibration will be used for weight measurement. Participants will wear underwear. Height will be measured in centimeters without shoes. Measurements shall be rounded up to whole centimeters. BMI will be determined as weight divided by squared height (kg/m^2). We will also measure waist-hip ratio (WHR) with a measuring tape in centimeters (cm).

Blood samples collection

For each selected participant three venous blood samples will be collected for:

A) Peripheral Blood Mononuclear Cells (PBMCs) isolation:

Collect blood sample into 4mL Vacutainer® Cell Preparation Tubes (CPT™) with anti-coagulant solution (sodium citrate). BD (Becton Dickinson) CPT™ is a vacuum driven drawing tube containing anti-coagulant and a cell separation medium. The cell separation medium is comprised of a polyester gel and a density gradient liquid. This configuration permits cell separation during a single centrifugation step. Therefore it enables blood to be drawn and PBMCs to be separated in the same tube, without the need for blood dilution or transfer to other containers. This simplified device is designed to avoid operator-dependence of classic separation technics such as Ficoll, making PBMCs isolation accessible to even inexperienced laboratory workers. Hence, the process will follow the next steps:

1. The BD Vacutainer® CPT™ Tube with Sodium Citrate should be stored at room temperature (18-25°C) and properly labeled for patient identification.
2. Collect 4mL of blood into the tube using the standard technique for BD Vacutainer® Evacuated Blood Collection Tubes following manufacturer's instructions.
3. After collection, store tube upright at room temperature until centrifugation. Blood samples should be centrifuged within two hours of blood collection for best results.
4. Centrifuge tube/blood sample at room temperature in a horizontal rotor (swing-out head) for a minimum of 20 minutes at 1500 to 1800 RCF (Relative Centrifugal Force).
5. After centrifugation, mononuclear cells will be in a whitish layer just under the plasma layer. Aspirate approximately half of the plasma without disturbing the cell layer. Collect cell

layer with a Pasteur pipette and transfer to a Falcon™ 5 mL conical centrifuge tube with cap. Collection of cells immediately following centrifugation will yield best results. Finally, store at -80°C isolated PBMCs samples until transferring to Cardiovascular genetics laboratory for monocytes isolation and methylation analysis.
JTH nurses will be trained in using these tubes. Once collected, blood samples will be transferred to clinical analysis laboratory at the first floor of the same hospital. Samples will be stored until transferring to Cardiovascular genetics laboratory.

B) Plasma collection:

Collect 4 mL of fasting venous blood samples in labeled EDTA-Vacutainer (lavender tops, anticoagulant-treated tubes) following standard venipuncture JTH nursing protocol. Transfer samples to clinical analysis lab (first floor in the same hospital) where plasma will be collected. In samples we can distinguish three levels:

- **Bottom layer**: Red blood cells (accounting for 50–80% of the total volume)
- **Middle layer:** Very thin band of white blood cells (buffy coat)
- **Top layer:** Straw-colored PRP (platelet rich plasma)

Hence, we can transfer two thirds of the PRP into a new plastic tube (Falcon™ 5 mL conical centrifuge tube with cap) using a transfer pipette (wide orifice) without disturbing the buffy coat layer, in order to avoid contamination. Store plasma samples at -80°C until transferring to Cardiovascular genetics laboratory for platelet isolation and methylation analysis, together with IL-6 and MCP-1 plasma level measurement.

C) Serum collection:

Total cholesterol, c-HDL, triglycerides, c-LDL, basal glycaemia and HbA1c are analyzed from participants' serum samples.

Whole blood samples are collected in serum tubes (red topped tubes, no anticoagulant) of 4 mL (BD Vacutainer). After collection of the whole blood, allow the blood to clot by leaving it undisturbed 15-30 minutes at room temperature. Transfer the sample to clinical analysis laboratory where the clot is removed by centrifuging at 1.000-2.000 RCF 10 minutes in a refrigerated centrifuge. The resulting supernatant is designated serum. Following centrifugation, it is important to immediately transfer the liquid component (serum) into a

clean polypropylene tube (Falcon™ 5 mL tube) using a Pasteur pipette. Samples should be maintained at 2-8°C while handling. If the serum is not analyzed immediately, it will be aliquoted into 0.5 mL aliquots and stored at -20°C or lower until laboratory analysis.

Additional analysis of serum monocyte count in cases samples is performed by Flow Cytometry following standard protocols at JTH clinical analysis laboratory.

Results will be communicated to corresponding investigator in charge of data collection.

Monocyte isolation

Monocyte isolation will be performed according to manufacturer's protocol by trained members of the Cardiovascular genetics lab team.

From PBMCs samples, we isolate monocytes using Monocyte Isolation Kit II (MACS Miltenyi Biotec), an indirect magnetic labeling system for the isolation of untouched monocytes from human PBMCs. Using this kit, human monocytes are isolated by depletion of non-monocytes (negative selection). Non-monocytes are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. In between the two labeling steps no washing steps are required. The magnetically labeled non-monocytes are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled monocytes pass through the column.

Plasma analysis

From plasma samples, centrifuge 400 µL at 2.000 RCF for 15 minutes at room temperature in order to remove all the cells. Transfer plasma to aliquots. IL-6 and MCP-1 levels will be measured with sensitive enzyme-linked immunosorbent assay (Quantikine® HS Immunoassay Kit, R&D Systems) according to the manufacturer's instructions. The microtiter plates is read at a wavelength of 490nm with an ELISA microplate reader.

Methylation analysis

We use the Diagenode Methylated DNA Immunoprecipitation (MeDIP) kit, which includes methylated DNA (meDNA) and unmethylated DNA (unDNA) controls to be used together with

the DNA sample. The MeDIP kit includes three modules, they are used for: genomic DNA preparation, immunoprecipitation of methylated DNA and qPCR (quantitative polymerase chain reaction) analysis of the immunoprecipitated (IP'd) DNA. Each module is provided with adapted buffers and detailed protocols.

1). A Genomic DNA module is included and optimized for the preparation of DNA ready-to-use. An optimized protocol for DNA shearing is provided as well.

2) In the Methyl DNA Immunoprecipitation module, an antibody directed against 5-methyl Cytidine is provided as well as methylated and unmethylated DNA controls (positive meDNA control - that is IP'd - and negative unDNA control - that is not IP'd). The immunoprecipitation has been optimized to specifically select and precipitate the meDNA by using of the antibody, buffers and protocol. The technique efficiency can be double-checked with the internal controls.

3) The qPCR module includes validated primer pairs specific to four types of DNA: a, the meDNA control (meDNA positive ctrls #1 and #2); b, the unDNA control (unDNA negative ctrls #1 and #2); c, one methylated human DNA region (X-linked alpha satellites) and d, one unmethylated human DNA region (GAPDH promoter).

Complete protocol is specified in **Annex 5** and qPCR primers are specified in **Annex 6**.

Samples from JTH are stored at -80°. This allows to perform methylation analysis gradually without depending on sample expiration and in an investigator established rhythm that allows samples' correct processing.

Platelet mitochondrial DNA methylation:

Centrifuge 400 µL of plasma samples at 1.400 RCF for 10 minutes at room temperature to obtain platelet pellets. Resuspend these pellets by gently vortexing or tapping tube with index finger and start MeDIP manual kit protocol from step 3.

Monocyte nuclear DNA methylation:

Once monocytes are isolated from PBMCs, start MeDIP manual kit protocol from step 3.

During the study, a member of the team will look after data quality and consistency obtained

at the moment. This review will be done as soon as possible in order to detect errors and solve them. In addition, in order to assess coordination of different members of the study, especially between nurses and clinical analysis laboratory and the latter with Cardiovascular genetics laboratory, a pilot test will be performed.

A member of the team will be in charge of transporting samples from JTH clinical analysis laboratory to Cardiovascular genetics lab periodically.

In terms of analytical data quality, techniques will be performed preserving investigator blindness about the sample origin (case or control), each sample will be analyzed by duplicate and all the techniques will count with positive and negative controls. A unique team member will conduct methylation analysis in order to prevent inter-observer bias, as well as IL-6 and MCP-1 plasma level assessment.

Josep Trueta Hospital		Clinical analysis laboratory of Josep Trueta Hospital	Cardiovascular genetics laboratory of IdIBGi
Coronary Unit (cases)	**Emergency Services (controls)**		
1.Candidate selection criteria evaluation (anamnesis + physical examination + medical history) 2. Fill in "participant data sheet" + "information sheet" + "informed consent" 3. Allocate a numeric code 4. Blood venipuncture		1. PBMCs isolation 2. Serum analysis (Total cholesterol, c-LDL, c-HDL, Triglycerides, basal glycaemia and HbA1c) & monocyte count (only in cases). 3. Plasma preparation	1. Monocyte isolation and methylation analysis. 2. Platelet isolation from plasma samples and methylation analysis. 3. IL-6 and MCP-1 plasma levels determination.

Table 2. Summary of data collection

All data collection will be done as long as participants enter to the study. Frozen stored samples in JTH clinical analysis lab will be collected and transferred to Cardiovascular genetics lab in groups of 50 samples or monthly.

8. STATISTICAL ANALYSIS

Sample size calculation has been described in subjects and methods section (**see 7.3: Sampling**). Statistical analysis will be held with Statistical Package for the Social Sciences (SPSS) software for Windows®. The *p* value less than 0.05 in two sides will be considered statistically significant.

Univariate

The results will be shown as percentages for categorical variables, while the univariate analysis of the quantitative variables will be represented by mean of ± SD if we can assume a normal distribution or if not, using the median, first and third quartile.

Bivariate

Student's t-test for the differences in means of continuous variables and Chi-square ($\chi 2$) test for differences in the distributions of categorical variables will be used to examine differences between cases and controls in age, gender, ethnicity, BMI, WHR, total cholesterol, triglycerides, c-HDL, c-LDL, cigarette smoking, alcohol drinking, history of HT, history of DM, oral contraception, statin use and family history of premature CV disease (covariates included in the study) together with differences in IL-6, MCP-1, LINE-1, MT-CO1, MT-CO2 and MT-CO3 methylation level.

Logistic regression will be used to estimate Odds Ratio (OR) for MI and 95% confidence interval (CI). We will perform a model for each studied gene (IL-6, MCP-1, LINE-1, MT-CO1, MT-CO2 and MT-CO3). Results will be expressed in quartile and median cut-points based on methylation distributions among controls.

Serum IL-6 levels will be log-transformed prior to analysis to improve normality. Linear regression will be applied to determine the association between methylation and serum levels of IL-6, where methylation as a covariate. The same process will be made for MCP-1.

To assess association between MCP-1 gene methylation and monocyte counts in MI cases, data will be plotted and Pearson or Spearman Rank correlation coefficient will be calculated depending on normal distribution of the variables, together with linear regression analysis.

To establish the correlation between LINE-1 methylation in monocytes and mitochondrial DNA methylation in platelets (average MT-CO1, MT-CO2 and MT-CO3 methylation level) we will plot the data and calculate Pearson or Spearman Rank depending on variables distribution.

To assess the precision of DNA methylation determination, as well as to confirm the possible differences between controls and cases, Spearman Rank correlation coefficient will be performed between technical replicates.

Multivariate

Multivariate logistic regression will be used to estimate the associations between IL-6 methylation and CAD risk by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) with adjustment for age, BMI, WHR (these variables will be expressed in tertiles based on values among controls), mean values of total cholesterol, triglycerides, c-HDL, c-LDL and for categorical variables gender, cigarette smoking, ethnicity, alcohol drinking, history of HT, history of DM, oral contraception, statin use and family history of premature CV disease. ORs will be calculated for quartile and median cut-points of IL-6 methylation based on distribution among controls.

The same process will be followed for each analyzed gene: MCP-1, LINE-1, MT-CO1, MT-CO2 and MT-CO3.

This multivariate logistic regression analysis will be performed in order to appraise the contribution of the covariates or possible confusion variables in the results.

9. WORK PLAN

Researchers:

- Dr. Ramon Brugada (RB) – Main researcher of IdIBGi Cardiovascular genetics.
- Elisa Ruiz (ER) – Post-graduate student in medicine and human biology.

Collaborators:

- Dr. Sara Pagans (SP) – methylation analysis consultant (Cardiovascular genetics group researcher).

- Dr. Francesc Xavier Albert (XA) – JTH cardiologist.
- Dr. Francesc Xavier Queralt i Moles (XQ) – JTH clinical analysis laboratory director and collaborator in research and teaching projects.
- JTH Emergency Services medical team (E-MT).
- JTH Emergency Services nursing staff (E-NS).
- JTH Coronary Unit nursing staff (C-NS).
- JTH Coronary Unit medical team (C-MT).
- Mr. Ferran Picó (FP) – Cardiovascular genetics laboratory technician.

Stage 0. Preparation [1 month]

- Conducted by: RB, ER
- Date: November 2016
- **Objective**: protocol processing, variables definition and presentation to Ethics Committee of Clinical Research.

Stage 1. Coordination [1 month]

- Conducted by: RB, ER, XA, XQ, SP.
- Date: December 2016
- **Objective**: inform the team about the working plan, schedule and methods of data collection. Separated meetings with E-MT/E-NS and C-MT/C-NS will be hold.

Stage 2. Field research [22 months]

- Recruitment of patients [January 2017-November 2018]
    - Inclusion to the study every participant that meets inclusion and not exclusion criteria.
    - Conducted by: E-MT, C-MT

Stage 3. Data collection [24 months]

- Conducted by: RB, ER, ST, SP, XQ, E-MT/NS, C-MT/NS.
- Date: January 2017-January 2019

- **Objective**: during the study data will be registered in the database and reviewed regularly by an external collaborator to control its evolution and verify that the protocol is being followed.

Data collection includes blood samples and participant covariates obtaining, JTH clinical analysis laboratory procedures, samples transferring and Cardiovascular genetics laboratory analysis.

During January and February of 2017, a data collection pilot test will be conducted in order to depurate data procedures and detect possible errors. Definitive protocol will be sent to all study collaborators.

Stage 4. Data analysis [1 month]

- Conducted by: Statistical team (ST)
- Date: January 2019
- **Objective**: analysis of the data using the appropriate statistical test.

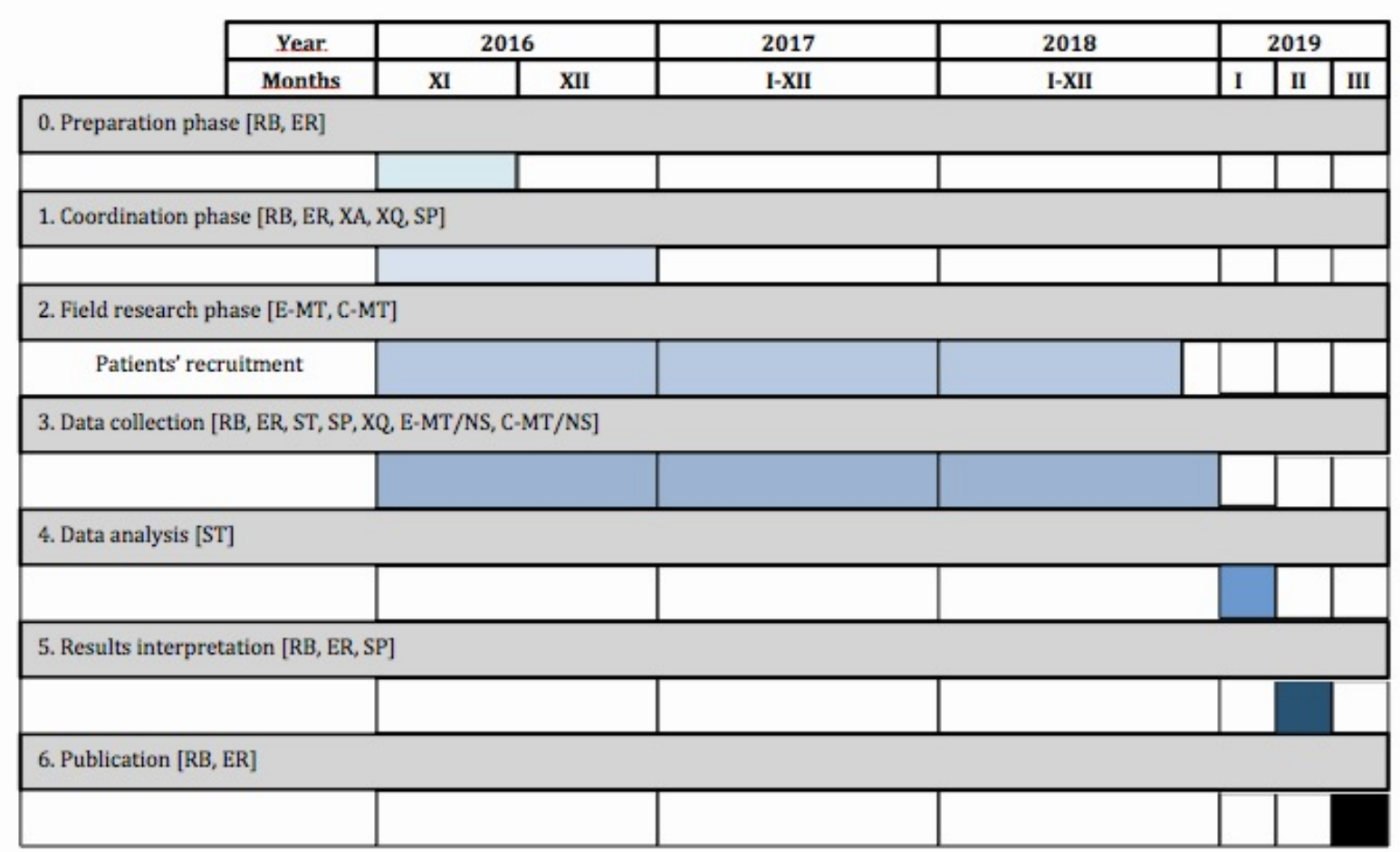
Stage 5. Results interpretation [1 month]

- Conducted by: RB, ER, SP.
- Date: February 2019
- **Objective**: interpretation of the results, conclusions drawing.

Stage 6. Publication [1 month]

- Conducted by: RB, ER.
- Date: March 2019
- **Objective**: write an article according to the results and send it to different journals for its publication. In order to avoid data publication bias, results will be published independently they agree or not our investigation hypothesis.

9.1. Chronogram



10. FEASIBILITY

Taking into account the study work plan, in just three years we could be managing data of great scientific value and statistical power due to big sample size. In relation with team expertise and investigation facilities, the main researcher has the sufficient capacity to conduct this project, as well as the team members. The investigators of IdIBGi Cardiovascular genetics laboratory have actively participated in the field of cardiovascular diseases for more than 20 years through clinical research as well as basic research in genetics, molecular biology, biophysics and biochemistry. Furthermore, its main researcher, Dr. Ramon Brugada, is known worldwide due to its work in the field of genetics and cardiac sudden death, by which has received many awards.

Cardiovascular genetics laboratory counts with almost all the necessary equipment for data analysis such as Microplate readers for plasma protein levels determination; centrifuges; a Bioruptor® for DNA sonication, basic in DNA methylation analysis; incubators; fume hoods; quantitative PCR facilities and reagents; and agarose gel apparatus. Other necessary reagents are relatively affordable in our study like specific detection kits or PCR/conical tubes.

Moreover, in the laboratory there is an expert, Dr. Sara Pagans, who dedicates exclusively to epigenetics procedures like methylation analysis.

In relation to serum analysis and other procedures conducted in JTH, the center counts with enough experience in data collection and clinical analysis being the reference hospital of the province of Girona.

11. ETHICAL AND LEGAL ASPECTS

The most important thing whilst carrying on this study, is to respect the ethical principles that characterize a good clinical practice. In accordance with provisions of the organic law 15/1999 of the 13th of December about data protection, confidentiality and protection of personal data shall be guaranteed. All information obtained during the study will be treated in a homogenous way during the data collection process.

All participants will be well informed about the purpose and all the details of this investigation. They will be given an Information Sheet (**Annex 7**) and an Informed Consent for the Inclusion in the Study (**Annex 8**). It is imperative that patients read and understand the

information sheet and sign the informed consent form voluntarily without any pressure. Moreover, investigators will be well formed in giving proper and honest information to study participants. Patients will have the right to deny participation in the study, with no impact on the health care they receive. In this way, together with data confidentiality protection, the principle of autonomy will be respected.

In this study the right of health protection will be respected as per article 43 of the Spanish Constitution of 1978.

As regulated by the law 14/2007 of the 3rd of July, RD 1716/2011, about investigation on biologic samples, this study will have to be approved by the Clinical Research Ethics Committee (CEIC) of the JTH, in accordance to the RD 1090/2015 about biomedical investigation in Spain.

The ethical principles for experimentation in humans will be equally respected as provided by the Declaration of Helsinki (1964).

This study implies very low risks or discomforts to the participant because it only requires a simple blood sample obtained by venipuncture and the potential benefits and the importance of generated knowledge would be considerable. No maleficence principle is guaranteed due to researchers and centers experience and formation. According to selection criteria, discrimination of vulnerable populations is prevented and we are committed to punctual and accurate results publication independently their agreement or not to our hypothesis.

12. STRENGTHS AND LIMITATIONS

According to the study design, the case-control configuration allows us to carry out a very efficient study with relatively low cost in terms of money and time comparing with a large cohort study, ideal to study diseases with a large period of latency like CHD. Furthermore, the design let us to evaluate multiple risk factors associated with MI, which is of additional importance taking into account the great influence that the environment exercises over epigenome. However, this kind of study design tends to overestimate the effect sizes of real associations. Moreover, this is a design very vulnerable to biases, such as selection and information bias. In our case, information bias is solved by objective measurements of covariates based on medical history and techniques without an open interpretation. For example, we apply an objective measure for alcohol consumption calculation like is the AUDIT C questionnaire. Even more, internal validity is guarded by quality controls during study

developing, using of validated and standardized techniques, duplicate analysis and positive and negative controls applying, together with blindness in analytical procedures.

One possible limitation of our study could be the technique used for methylation quantitative analysis, especially in measuring subtle changes in mitochondrial DNA methylation because other studies have described pyrosequencing methodology as the most reliable, sensitive and robust technique for studying DNA methylation. We have discarded this technique due to its high price and the inexperience in its use of our laboratory team. Alternatively, MeDIP method presents acceptable levels of sensibility and specificity in comparison with pyrosequencing and is a well-known technique in our laboratory, converting it as good as the former one to be used in the study.

One of the strengths of our study, in comparison with related published data, is the precise definition of what we consider MI, as well as the discrimination between incident and prevalent cases.

Identifying causality is difficult due to the unstable nature of the epigenome and the case-control design and it remains uncertain if the previous observed associations of epigenetic profiles with disease are causative or just epiphenomena. However, the study is needed in order to investigate the association between MI and distinct epigenetic marks as a previous step for future longitudinal studies, which may help provide stronger evidence toward causation performing repeated measurements of DNA methylation before disease development and after.

In this study we have also control confusion bias, especially in statistical analysis. Majority of the studies lack in the adjustment for basic covariates such as sex and age and also the established CVRFs. Unlike genetic association studies, which are resistant to confounding, controlling for different confounders and mediators is of importance in epigenetic analysis. Assignation of a specific methylation mark to a concrete process like MI is difficult because this epigenome is also influenced by other processes such as HT, DM or obesity, but it is worth studying due to health implications. Ideally, we would like to apply more restriction to our sampling in order to control this known confusion variables, but we only stratify for age and sex trying not to lose sample representation and to be capable of extrapolate our results to the target population keeping in mind that results from a very stratified sample are difficult to extrapolate.

With a non-probabilistic consecutive sampling, we are conscious that individuals of the population do not have the same probability to be part of the control sample and exists the possibility that subjects who go to the Emergency Services are indeed different to the rest of the population. However, by using this method of sampling, we avoid the volunteer bias. In cases, it exists a survival bias due to the fact that patients who die of MI do not enter to our study and they could have some distinct epigenetic marks that we are not able to analyze.

In our sampling, we stratify for age and gender, which is important not only due to differences in MI incidence, but also because differential methylation modifications have been linked to age. In addition, our sample size is big enough to detect slight differences in methylation pattern, making our study of great reliability in comparison with other published studies about the issue.

The main strength of our study is that we are studying epigenetic differences in blood cells that can be easily obtained by a non-invasive procedure like is venipuncture, in comparison of other published data which study myocardial tissue or atherosclerotic plaques obtained by invasive procedures. Taking into account that we want to apply this new knowledge to primary prevention, non-invasive techniques are necessary in order to not increase subject basal risk.

There is no such published study carried out in our country, even worldwide, that had studied monocyte nuclear DNA methylation and platelet mitochondrial DNA pattern in relation to MI. There are only few studies that have investigated differential methylation marks in PBMCs, but any of them have analyzed the monocyte epigenome. Hence, taking into account that epigenetic changes are cell specific, it seems plausible that the study of monocytes epigenome could reveal some interesting knowledge about the disease. We also have as strength that we associate DNA methylation analysis with serum protein levels

Only one study has showed promising results about the study of platelets mitochondrial DNA methylation pattern in relation to CVD. Our study is the first to investigate these differences with a concrete definition of cases and using a wide sample.

The study results can only be applied to the population of Girona, but it will be interesting to compare the results with other related investigations made in other countries and could conform the basis for further studies around the world.

Finally, this study is also the first in the field of using the WHR as a covariate in coronary risk evaluation, which has showed to be more intensely associated with cardiovascular risk than BMI because the former expresses the subcutaneous and visceral fat.

13. BUDGET

Supplies (materials and reagents)

Blood samples analysis will require supplies and reagents.

In relation to analysis performed in Cardiovascular genetics laboratory, assuming the equipment and reagents that already has the center such as centrifuges, Bioruptor® (for sonication of DNA in methylation analysis), qPCR facilities and incubators, we expect different costs depending on the analysis performed:

- For PBMCs isolation, Vacutainer CPT™ Glass Molecular Diagnostics Tubes will be required, with a total price of 8.758,95€ (1 case of 60 costs 583,93€).
- For monocyte isolation, a total cost of 10.328,5€ is expected (1135€ per kit that allows the analysis of 100 samples).
- For plasma collection, 4 mL capacity DB Vacutainer™ Plastic Blood Collection Tubes with K_2EDTA will cost 316€ (31,6€ each pack of 100 tubes). 1000 Falcon™ 5 mL conical centrifuge tubes will cost 263€.
- For methylation analysis an estimated budget of 91.000€ is calculated (MeDIP Manual Kit, 1000€ per 10 IP's).
- For determination of plasma IL-6 and MCP-1 level, the immunoassay kit for each protein analysis costs 2290€ approximately, with a total cost of 4.580€.

Subcontracting (services)

JTH clinical analysis services will be contracted in order to determine controls lipid profile and basal glucose concentration and HbA1c. We assume a total cost of 9.080€ (20€ per sample) considering serum collection procedures and laboratory analysis. We assume routine determination of lipid and glucose profile in MI cases hospitalized in the JTH Coronary Unit in order to assess their cardiovascular risk previous to hospital discharge. Hence, we do not estimate any budget for this purpose. However, JTH clinical analysis services will be also required for determination of monocyte count by Flow Cytometry in cases serum samples, with an approximated cost of 450€ (50€/h, assuming 9h).

Determination of carotid artery stenosis by echography and ABI in controls for exclusion criteria will be performed in the JTH Emergency Services facilities, with measure tools (echograph and sphygmomanometer) that already have the center, so we await for

coordination meetings in order to agree a final price for techniques performance. An estimated total budget of 20.500€ (45€ per patient).

A Statistician will be necessary to analyze the results due the amount of samples and our team lack of expertise. The estimated budget for this is 2.100€ (35€/h, assuming 60h).
We assume 273€ cost for printing information sheets for patients, informed consent forms and participant data sheets.

Publication costs and dissemination of results

We expect a cost of 2500€ for article publication in a high profile open access journal. In addition, we aim to present our results at a local conference (Annual Congress of the Catalan Society of Cardiology), a national conference (Annual Congress of the Spanish Society of Cardiology) and an international conference (European Society of Cardiology Congress).

	QUANTITY	COST	SUBTOTAL
1. Services and material			
JTH clinical analysis services	454 serum samples	20€/sample	9.080€
JTH Flow cytometry	454 serum samples	50€/h	450€
BD 362761 Vacutainer CPT™ Glass Molecular Diagnostics Tube with Blue/Black Conventional closure	910	1 pack of 60 costs 583,93€	8.758,95€
Monocyte Isolation Kit II, human (MACS, Myltenil Biotec)	910	11,35€ per unit	10.328,5€
4 mL capacity DB Vacutainer™ Plastic Blood Collection Tubes with K_2EDTA	1000	31,6€ each pack of 100 tubes	316€
Falcon™ 5 mL conical centrifuge tubes	1000	0,263€ per unit	263€
MeDIP Manual Kit	910	1000€ per kit	91.000€

Human MCP-1 Quantikine® HS Immunoassay Kit, R&D Systems	910	1 pack of 6 plates (100 samples per plate)	2290€
Human IL-6 Quantikine® HS Immunoassay Kit, R&D Systems	910	1 pack of 6 plates (100 samples per plate)	2290€
Carotid artery echography and sphygmomanometer	454	53€ per patient	24.062€
Statistical analysis	60h	35€/h	2.100€
Office supplies	910	0,3€	273€
2. Publication and presentation costs			
Article publication in open access journal	1	2.500€	2.500€
Annual Congress of the Catalan Society of Cardiology assistance			
Registration	2	255€ per person	510€
Travel	2	30€ per person	60€
Accommodation	2	75€ per person	150€
Annual Congress of the Spanish Society of Cardiology			
Registration	2	569€ per person	1.138€
Travel	2	150€ per person	300€
Accommodation	2	75€ per person	150€
European Society of Cardiology Congress			
Registration	2	665€ per person	1.330€
Travel	2	250€ per person	500€
Accommodation	2	150€ per person	300€

TOTAL AMOUNT	**158.149,45€**

14. IMPACT

The study of epigenetic markers is emerging as one of the most promising molecular strategies for risk stratification in complex diseases, including CVD, and when implemented it will have a sizable public health impact. This epigenetic markers study is complex because usually requires tissue analysis obtained by invasive techniques. In this project, on the contrary, we use peripheral blood, which is easy to access and reflects multiple metabolic and inflammatory pathways. Therefore, methylation profiling in peripheral blood to identify CVD-related methylated regions is of great interest since it could allow clinicians to identify high-risk individuals who may benefit from preventive and therapeutic interventions, promising high potential clinical utility. In addition, knowing that epigenetics marks are cell specific, the study of particular blood cell types such as monocytes and platelets, which also have an important role in atherosclerosis, presents outstanding perspectives. Besides, as epigenetic DNA modifications are potentially reversible and may be influenced by nutritional–environmental factors and through gene–environment interactions, future therapies targeting the epigenome can be a novel preventive strategy and treatment for CVD. For example, some studies show that supplementation with methyl donors such as folate, choline and vitamin B12 may influence DNA methylation and may have a beneficial effect on CVD risk, but results are still inconsistent. Therefore, in the background of the high burden of CVD despite great advances in its prevention and treatment, transfer of these novel therapeutic avenues on the field of CVD should become a research priority in the future.

The improvement of CHD risk estimation is a priority for research as long as the majority of CV events occur in individuals who are classified as having low or intermediate risk. With this purpose the AHA has proposed the essential steps for assessing the potential value of such novel biomarkers in estimating risk and considering the modest results obtained by addition of genetic variants, epigenetics present as a promising alternative.

15. BIBLIOGRAPHY

1. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet (London, England) [Internet]. 2015 Jan 10 [cited 2016 Oct 14];385(9963):117–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25530442
2. Nichols M, Townsend N, Luengo-Fernandez R, Leal J, Gray A SP. European Cardiovascular Disease Statistics 2012. Eur Hear Network, Brussels Eur Soc Cardiol. 2012;
3. Dégano IR, Salomaa V, Veronesi G, Ferriéres J, Kirchberger I, Laks T, et al. Twenty-five-year trends in myocardial infarction attack and mortality rates, and case-fatality, in six European populations. Heart [Internet]. 2015 Sep [cited 2016 Oct 4];101(17):1413–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25855798
4. WHO. Estadísticas sanitarias mundiales. 2014. Una mina de información sobre salud pública mundial [Internet]. 2014. Available from: http://www.who.int/gho
5. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al. Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation [Internet]. 2010 Feb 23 [cited 2016 Oct 6];121(7):948–54. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20177011
6. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J [Internet]. 2016 Aug 14 [cited 2016 Oct 11]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/27523477
7. Ferreira-González I. The epidemiology of coronary heart disease. Rev española Cardiol (English ed) [Internet]. 2014 Feb [cited 2016 Oct 6];67(2):139–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24795124
8. Departament S. Anàlisi de la mortalitat a Catalunya 2014. Avanç de resultats. Barcelona; 2016.
9. Alpert JS, Thygesen K, Antman E, Bassand JP. Myocardial infarction redefined--a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. J Am Coll Cardiol [Internet]. 2000 Sep [cited 2016 Oct 6];36(3):959–69. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/10987628

10. Agüero F, Marrugat J, Elosua R, Sala J, Masiá R, Ramos R, et al. New myocardial infarction definition affects incidence, mortality, hospitalization rates and prognosis. Eur J Prev Cardiol [Internet]. 2015 Oct [cited 2016 Oct 6];22(10):1272–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25139771
11. Barrabés JA, Bardají A, Jiménez-Candil J, del Nogal Sáez F, Bodí V, Basterra N, et al. Prognosis and management of acute coronary syndrome in Spain in 2012: the DIOCLES study. Rev española Cardiol (English ed) [Internet]. 2015 Feb [cited 2016 Oct 5];68(2):98–106. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25623429
12. Dégano IR, Elosua R, Marrugat J. Epidemiología del síndrome coronario agudo en España: estimación del número de casos y la tendencia de 2005 a 2049. Rev Española Cardiol [Internet]. 2013 Jun 1 [cited 2016 Sep 28];66(6):472–81. Available from: http://www.revespcardiol.org/es/epidemiologia-del-sindrome-coronario-agudo/articulo/90202772/
13. Cardellach F, Rozman C, Farreras Valentí P, Domarus A von. Medicina interna [Internet]. Madrid [etc.] : Elsevier; 2016 [cited 2016 Sep 30]. Available from: http://cataleg.udg.edu/record=b1384575~S10*cat
14. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med [Internet]. 1999 Jan 14 [cited 2016 Sep 21];340(2):115–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9887164
15. Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol [Internet]. 2012 Sep [cited 2016 Sep 29];32(9):2045–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22895665
16. Millard RW, Tranter M. Complementary, alternative, and putative nontroponin biomarkers of acute coronary syndrome: new resources for future risk assessment calculators. Rev española Cardiol (English ed) [Internet]. 2014;67(4):312–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24774594
17. Blankenberg S, McQueen MJ, Smieja M, Pogue J, Balion C, Lonn E, et al. Comparative impact of multiple biomarkers and N-Terminal pro-brain natriuretic peptide in the context of conventional risk factors for the prediction of recurrent cardiovascular events in the Heart Outcomes Prevention Evaluation (HOPE) Study. Circulation [Internet]. 2006 Jul 18 [cited 2016 Sep 21];114(3):201–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16831981

18. Dawber T, Meadors G, Moore F. Epidemiological approaches to heart disease: the Framingham Study. Am J Public Health Nations Health [Internet]. 1951 Mar [cited 2016 Oct 3];41(3):279–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14819398
19. Bertomeu V, Cequier A, Bernal JL, Alfonso F, Anguita MP, Muñiz J, et al. In-hospital mortality due to acute myocardial infarction. relevance of type of hospital and care provided. RECALCAR study. Rev española Cardiol (English ed) [Internet]. 2013 Dec [cited 2016 Oct 5];66(12):935–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24774106
20. Grau M, Elosua R, Cabrera de León A, Guembe MJ, Baena-Díez JM, Vega Alonso T, et al. [Cardiovascular risk factors in Spain in the first decade of the 21st Century, a pooled analysis with individual data from 11 population-based studies: the DARIOS study]. Rev española Cardiol [Internet]. 2011 Apr [cited 2016 Oct 3];64(4):295–304. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21397375
21. Marrugat J, Vila J, Baena-Díez JM, Grau M, Sala J, Ramos R, et al. [Relative validity of the 10-year cardiovascular risk estimate in a population cohort of the REGICOR study]. Rev española Cardiol [Internet]. 2011 May [cited 2016 Oct 4];64(5):385–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21482004
22. Lluis-Ganella C, Subirana I, Lucas G, Tomás M, Muñoz D, Sentí M, et al. Assessment of the value of a genetic risk score in improving the estimation of coronary risk. Atherosclerosis [Internet]. 2012 Jun [cited 2016 Oct 3];222(2):456–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22521901
23. Lluís-Ganella C, Lucas G, Subirana I, Sentí M, Jimenez-Conde J, Marrugat J, et al. Additive effect of multiple genetic variants on the risk of coronary artery disease. Rev Esp Cardiol [Internet]. 2010 Aug [cited 2016 Oct 24];63(8):925–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20738937
24. Velescu A, Clara A, Peñafiel J, Ramos R, Marti R, Grau M, et al. Adding low ankle brachial index to classical risk factors improves the prediction of major cardiovascular events. The REGICOR study. Atherosclerosis [Internet]. 2015 Aug [cited 2016 Oct 5];241(2):357–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26071658
25. Marrugat J, D'Agostino R, Sullivan L, Elosua R, Wilson P, Ordovas J, et al. An adaptation of the Framingham coronary heart disease risk function to European Mediterranean areas. J Epidemiol Community Health [Internet]. 2003 Aug [cited 2016 Oct

24];57(8):634–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12883073

26. Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, Elkind MS V, et al. Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association. Circulation [Internet]. 2009 May 5 [cited 2016 Oct 24];119(17):2408–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19364974
27. Napoli C, Infante T, Casamassimi A. Maternal-foetal epigenetic interactions in the beginning of cardiovascular damage. Cardiovasc Res [Internet]. 2011 Dec 1 [cited 2016 Oct 6];92(3):367–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21764886
28. Udali S, Guarini P, Moruzzi S, Choi S-W, Friso S. Cardiovascular epigenetics: from DNA methylation to microRNAs. Mol Aspects Med [Internet]. 2012 [cited 2016 Oct 14];34(4):883–901. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22981780
29. Schleithoff C, Voelter-Mahlknecht S, Dahmke IN, Mahlknecht U. On the epigenetics of vascular regulation and disease. Clin Epigenetics [Internet]. 2012 [cited 2016 Oct 14];4(1):7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22621747
30. Portela A, Esteller M. Epigenetic modifications and human disease. Nat Biotechnol [Internet]. 2010 Oct [cited 2016 Oct 14];28(10):1057–68. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20944598
31. Handy DE, Castro R, Loscalzo J. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. Circulation [Internet]. 2011 May 17 [cited 2016 Oct 14];123(19):2145–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21576679
32. Guardiola M, Vallvé JC, Zaina S, Ribalta J. [Epigenetics in atherosclerosis]. Clínica e Investig en Arterioscler publicación Of la Soc Española Arterioscler [Internet]. [cited 2016 Oct 14];28(2):102–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26088002
33. Fish JE, Matouk CC, Rachlis A, Lin S, Tai SC, D'Abreo C, et al. The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. J Biol Chem [Internet]. 2005 Jul 1 [cited 2016 Oct 14];280(26):24824–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15870070
34. Freson K, Izzi B, Van Geet C. From genetics to epigenetics in platelet research. Thromb Res [Internet]. 2012 Mar [cited 2016 Oct 17];129(3):325–9. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/22192152

35. Elosua R, Lluís C LG. Estudio del componente genético de la cardiopatía isquémica: de los estudios de ligamiento al genotipado integral del genoma. Rev Española Cardiol. 2009;9(Suppl B):B24-38.
36. Van Diepen JA, Thiem K, Stienstra R, Riksen NP, Tack CJ, Netea MG. Diabetes propels the risk for cardiovascular disease: sweet monocytes becoming aggressive? Cell Mol Life Sci [Internet]. 2016 Jul 28 [cited 2016 Sep 29]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/27469259
37. Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. Nat Rev Cancer [Internet]. 2011 Oct [cited 2016 Oct 14];11(10):726–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21941284
38. Kirchner H, Osler ME, Krook A, Zierath JR. Epigenetic flexibility in metabolic regulation: disease cause and prevention? Trends Cell Biol [Internet]. 2013 May [cited 2016 Oct 6];23(5):203–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23277089
39. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science [Internet]. 2001 Aug 10 [cited 2016 Oct 17];293(5532):1068–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11498573
40. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature [Internet]. 2007 May 24 [cited 2016 Oct 17];447(7143):425–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17522676
41. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. Cell [Internet]. 2007 Feb 23 [cited 2016 Oct 14];128(4):669–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17320505
42. Mahmoud SA, Poizat C. Epigenetics and chromatin remodeling in adult cardiomyopathy. J Pathol [Internet]. 2013 Oct [cited 2016 Oct 14];231(2):147–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23813473
43. Duygu B, Poels EM, da Costa Martins PA. Genetics and epigenetics of arrhythmia and heart failure. Front Genet [Internet]. 2013 [cited 2016 Oct 14];4:219. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24198825
44. Rideout WM, Coetzee GA, Olumi AF, Jones PA. 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. Science [Internet]. 1990 Sep 14 [cited 2016 Oct 14];249(4974):1288–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1697983

45. Voelter-Mahlknecht S. Epigenetic associations in relation to cardiovascular prevention and therapeutics. Clin Epigenetics [Internet]. 2016 [cited 2016 Oct 6];8:4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26779291
46. Li B, Carey M, Workman JL. The role of chromatin during transcription. Cell [Internet]. 2007 Feb 23 [cited 2016 Oct 14];128(4):707–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17320508
47. Strahl BD, Allis CD. The language of covalent histone modifications. Nature [Internet]. 2000 Jan 6 [cited 2016 Oct 14];403(6765):41–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10638745
48. Kaikkonen MU, Lam MTY, Glass CK. Non-coding RNAs as regulators of gene expression and epigenetics. Cardiovasc Res [Internet]. 2011 Jun 1 [cited 2016 Oct 14];90(3):430–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21558279
49. Mattick JS, Makunin I V. Non-coding RNA. Hum Mol Genet [Internet]. 2006 Apr 15 [cited 2016 Oct 14];R17-29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16651366
50. Schiano C, Vietri MT, Grimaldi V, Picascia A, De Pascale MR, Napoli C. Epigenetic-related therapeutic challenges in cardiovascular disease. Trends Pharmacol Sci [Internet]. 2015 Apr [cited 2016 Oct 6];36(4):226–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25758254
51. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature [Internet]. 2005 Feb 17 [cited 2016 Oct 14];433(7027):769–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15685193
52. Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science [Internet]. 2016 Apr 22 [cited 2016 Sep 29];352(6284):aaf1098. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27102489
53. Bekkering S, Quintin J, Joosten LAB, van der Meer JWM, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. Arterioscler Thromb Vasc Biol [Internet]. 2014 Aug [cited 2016 Sep 29];34(8):1731–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24903093
54. Shirai T, Nazarewicz RR, Wallis BB, Yanes RE, Watanabe R, Hilhorst M, et al. The

glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. J Exp Med [Internet]. 2016 Mar 7 [cited 2016 Sep 29];213(3):337–54. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26926996

55. Tang J-N, Shen D-L, Liu C-L, Wang X-F, Zhang L, Xuan X-X, et al. Plasma levels of C1q/TNF-related protein 1 and interleukin 6 in patients with acute coronary syndrome or stable angina pectoris. Am J Med Sci [Internet]. 2015 Feb [cited 2016 Oct 7];349(2):130–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25635749
56. Bind M-A, Lepeule J, Zanobetti A, Gasparrini A, Baccarelli A, Coull BA, et al. Air pollution and gene-specific methylation in the Normative Aging Study: association, effect modification, and mediation analysis. Epigenetics [Internet]. 2014 Mar [cited 2016 Oct 7];9(3):448–58. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24385016
57. Yang Q, Zhao Y, Zhang Z, Chen J. Association of interleukin-6 methylation in leukocyte DNA with serum level and the risk of ischemic heart disease. Scand J Clin Lab Invest [Internet]. 2016 Jul [cited 2016 Oct 7];76(4):291–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26986049
58. Shantsila E, Tapp LD, Wrigley BJ, Pamukcu B, Apostolakis S, Montoro-García S, et al. Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. Atherosclerosis [Internet]. 2014 May [cited 2016 Oct 7];234(1):4–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24583499
59. Yamamoto E, Sugiyama S, Hirata Y, Tokitsu T, Tabata N, Fujisue K, et al. Prognostic significance of circulating leukocyte subtype counts in patients with coronary artery disease. Atherosclerosis [Internet]. 2016 Aug 25 [cited 2016 Oct 7]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/27612676
60. Chapman CML, Beilby JP, McQuillan BM, Thompson PL, Hung J. Monocyte count, but not C-reactive protein or interleukin-6, is an independent risk marker for subclinical carotid atherosclerosis. Stroke [Internet]. 2004 Jul [cited 2016 Oct 17];35(7):1619–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15155967
61. Johnsen SH, Fosse E, Joakimsen O, Mathiesen EB, Stensland-Bugge E, Njølstad I, et al. Monocyte count is a predictor of novel plaque formation: a 7-year follow-up study of 2610 persons without carotid plaque at baseline the Tromsø Study. Stroke [Internet]. 2005 Apr [cited 2016 Oct 17];36(4):715–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15746459
62. Nozawa N, Hibi K, Endo M, Sugano T, Ebina T, Kosuge M, et al. Association between

circulating monocytes and coronary plaque progression in patients with acute myocardial infarction. Circ J [Internet]. 2010 Jul [cited 2016 Oct 17];74(7):1384–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20467155

63. Matsumura T, Taketa K, Motoshima H, Senokuchi T, Ishii N, Kinoshita H, et al. Association between circulating leukocyte subtype counts and carotid intima-media thickness in Japanese subjects with type 2 diabetes. Cardiovasc Diabetol [Internet]. 2013 Dec 27 [cited 2016 Oct 17];12:177. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24373412

64. Olivares R, Ducimetière P, Claude JR. Monocyte count: a risk factor for coronary heart disease? Am J Epidemiol [Internet]. 1993 Jan 1 [cited 2016 Sep 29];137(1):49–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8434572

65. Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, et al. CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. J Am Coll Cardiol [Internet]. 2012 Oct 16 [cited 2016 Sep 29];60(16):1512–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22999728

66. Coller BS. Leukocytosis and ischemic vascular disease morbidity and mortality: is it time to intervene? Arterioscler Thromb Vasc Biol [Internet]. 2005 Apr [cited 2016 Sep 29];25(4):658–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15662026

67. Yang AS, Estécio MRH, Doshi K, Kondo Y, Tajara EH, Issa J-PJ. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res [Internet]. 2004 Feb 18 [cited 2016 Oct 17];32(3):e38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14973332

68. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res [Internet]. 2005 [cited 2016 Oct 17];33(21):6823–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16326863

69. Turunen MP, Aavik E, Ylä-Herttuala S. Epigenetics and atherosclerosis. Biochim Biophys Acta [Internet]. 2009 Sep [cited 2016 Oct 17];1790(9):886–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19233248

70. Castro R, Rivera I, Struys EA, Jansen EEW, Ravasco P, Camilo ME, et al. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. Clin Chem [Internet]. 2003 Aug [cited 2016 Oct 17];49(8):1292–6.

Available from: http://www.ncbi.nlm.nih.gov/pubmed/12881445

71. Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, et al. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. Ann Hum Genet [Internet]. 2004 May [cited 2016 Oct 17];68(Pt 3):196–204. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15180700
72. Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev [Internet]. 2009 Apr [cited 2016 Oct 17];130(4):234–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19150625
73. Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, et al. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev [Internet]. 2007 Jan [cited 2016 Oct 17];16(1):108–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17220338
74. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, et al. Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med [Internet]. 2009 Apr 1 [cited 2016 Oct 17];179(7):572–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19136372
75. Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. DNA methylation in white blood cells: association with risk factors in epidemiologic studies. Epigenetics [Internet]. 2011 Jul [cited 2016 Oct 17];6(7):828–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21636973
76. Baccarelli A, Wright R, Bollati V, Litonjua A, Zanobetti A, Tarantini L, et al. Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology [Internet]. 2010 Nov [cited 2016 Oct 17];21(6):819–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20805753
77. Wei L, Liu S, Su Z, Cheng R, Bai X, Li X. LINE-1 hypomethylation is associated with the risk of coronary heart disease in Chinese population. Arq Bras Cardiol [Internet]. 2014 May [cited 2016 Oct 17];102(5):481–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24918913
78. Gregg D, Goldschmidt-Clermont PJ. Cardiology patient page. Platelets and cardiovascular disease. Circulation [Internet]. 2003 Sep 30 [cited 2016 Oct 17];108(13):e88-90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14517153
79. Kottke-Marchant K. Importance of platelets and platelet response in acute coronary

syndromes. Cleve Clin J Med [Internet]. 2009 Apr [cited 2016 Oct 17];76 Suppl 1:S2-7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19332590

80. Salky N, Dugdale M. Platelet abnormalities in ischemic heart disease. Am J Cardiol [Internet]. 1973 Oct [cited 2016 Oct 17];32(5):612–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4744691
81. Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. Eur J Cardiovasc Nurs [Internet]. 2002 Dec [cited 2016 Oct 17];1(4):273–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14622657
82. Akkerman JW. Regulation of carbohydrate metabolism in platelets. A review. Thromb Haemost [Internet]. 1978 Jun 30 [cited 2016 Oct 17];39(3):712–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/705700
83. Feng S, Xiong L, Ji Z, Cheng W, Yang H. Correlation between increased ND2 expression and demethylated displacement loop of mtDNA in colorectal cancer. Mol Med Rep [Internet]. 2012 Jul [cited 2016 Oct 17];6(1):125–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22505229
84. Baccarelli AA, Byun H-M. Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. Clin Epigenetics [Internet]. 2015 [cited 2016 Oct 17];7:44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25901189
85. Shock LS, Thakkar P V, Peterson EJ, Moran RG, Taylor SM. DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. Proc Natl Acad Sci U S A [Internet]. 2011 Mar 1 [cited 2016 Oct 17];108(9):3630–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21321201
86. Byun H-M, Panni T, Motta V, Hou L, Nordio F, Apostoli P, et al. Effects of airborne pollutants on mitochondrial DNA methylation. Part Fibre Toxicol [Internet]. 2013 May 8 [cited 2016 Oct 17];10:18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23656717
87. Iacobazzi V, Castegna A, Infantino V, Andria G, Lee T, Zhai J, et al. Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool. Mol Genet Metab. 2013;110(1–2):25–34.
88. Maekawa M, Taniguchi T, Higashi H, Sugimura H, Sugano K, Kanno T. Methylation of mitochondrial DNA is not a useful marker for cancer detection. Clin Chem [Internet]. 2004 Aug [cited 2016 Oct 17];50(8):1480–1. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15277367

89. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol Cell [Internet]. 2013 Jan 24 [cited 2016 Oct 7];49(2):359–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23177740

90. Dje N'Guessan P, Riediger F, Vardarova K, Scharf S, Eitel J, Opitz B, et al. Statins control oxidized LDL-mediated histone modifications and gene expression in cultured human endothelial cells. Arterioscler Thromb Vasc Biol [Internet]. 2009 Mar [cited 2016 Oct 14];29(3):380–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19122173

91. Castillo-Díaz SA, Garay-Sevilla ME, Hernández-González MA, Solís-Martínez MO, Zaina S. Extensive demethylation of normally hypermethylated CpG islands occurs in human atherosclerotic arteries. Int J Mol Med [Internet]. 2010 Nov [cited 2016 Oct 26];26(5):691–700. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20878091

92. Movassagh M, Choy M-K, Goddard M, Bennett MR, Down TA, Foo RS-Y. Differential DNA methylation correlates with differential expression of angiogenic factors in human heart failure. PLoS One [Internet]. 2010 Jan 13 [cited 2016 Oct 26];5(1):e8564. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20084101

93. Movassagh M, Choy M-K, Knowles DA, Cordeddu L, Haider S, Down T, et al. Distinct epigenomic features in end-stage failing human hearts. Circulation [Internet]. 2011 Nov 29 [cited 2016 Oct 26];124(22):2411–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22025602

94. Zaina S, Gonçalves I, Carmona FJ, Gomez A, Heyn H, Mollet IG, et al. DNA methylation dynamics in human carotid plaques after cerebrovascular events. Arterioscler Thromb Vasc Biol [Internet]. 2015 Aug [cited 2016 Oct 26];35(8):1835–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26088578

95. Zaina S, Heyn H, Carmona FJ, Varol N, Sayols S, Condom E, et al. DNA methylation map of human atherosclerosis. Circ Cardiovasc Genet [Internet]. 2014 Oct [cited 2016 Oct 26];7(5):692–700. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25091541

96. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res [Internet]. 2009 Jun [cited 2016 Oct 7];29(6):313–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19441883

97. Kim M, Long TI, Arakawa K, Wang R, Yu MC, Laird PW. DNA methylation as a biomarker for cardiovascular disease risk. PLoS One [Internet]. 2010 Mar 15 [cited 2016 Oct 17];5(3):e9692. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20300621

98. Sharma P, Kumar J, Garg G, Kumar A, Patowary A, Karthikeyan G, et al. Detection of altered global DNA methylation in coronary artery disease patients. DNA Cell Biol [Internet]. 2008 Jul [cited 2016 Oct 17];27(7):357–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18613790

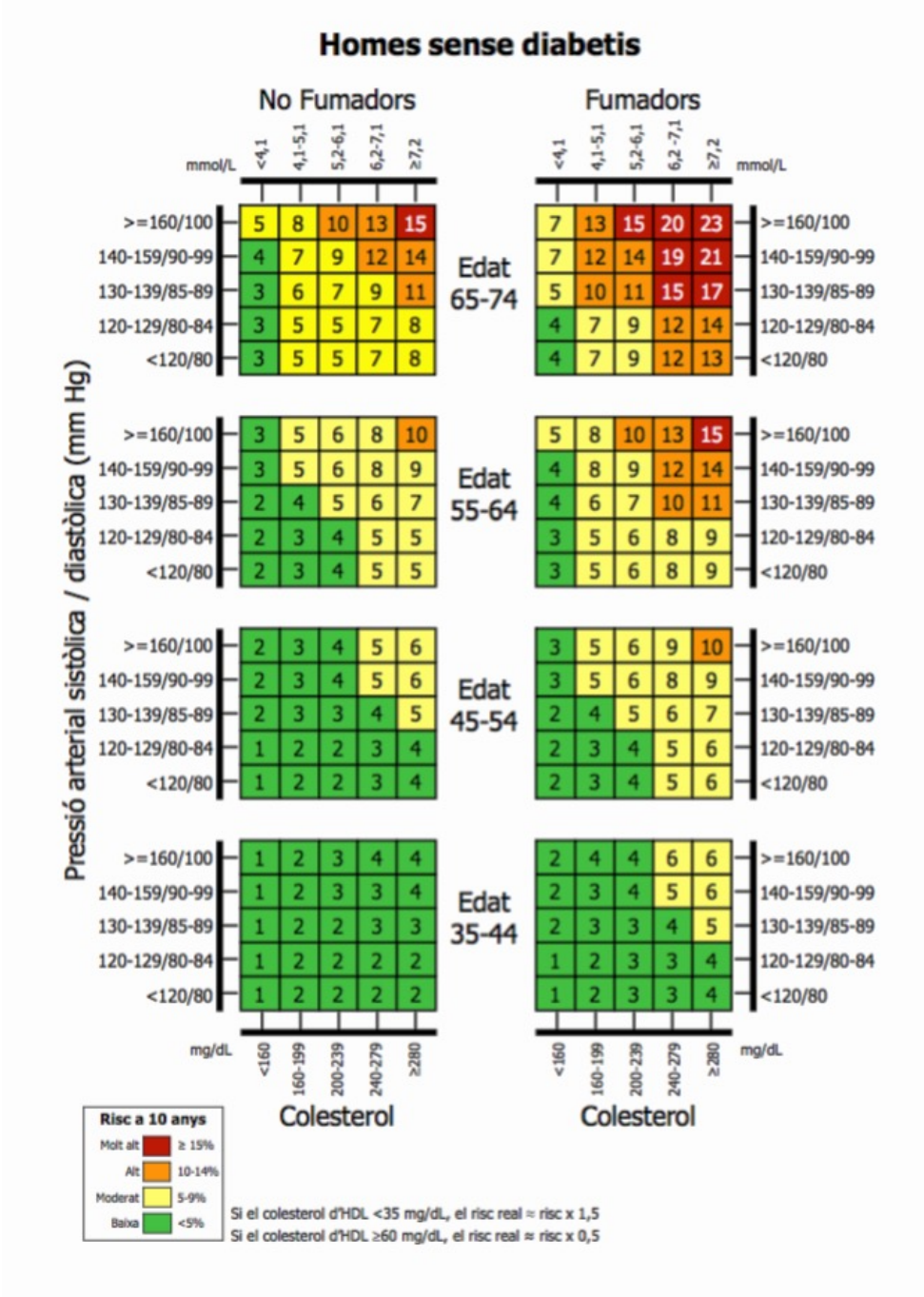
99. Wise IA, Charchar FJ. Epigenetic Modifications in Essential Hypertension. Int J Mol Sci [Internet]. 2016 Mar 25 [cited 2016 Oct 27];17(4):451. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27023534

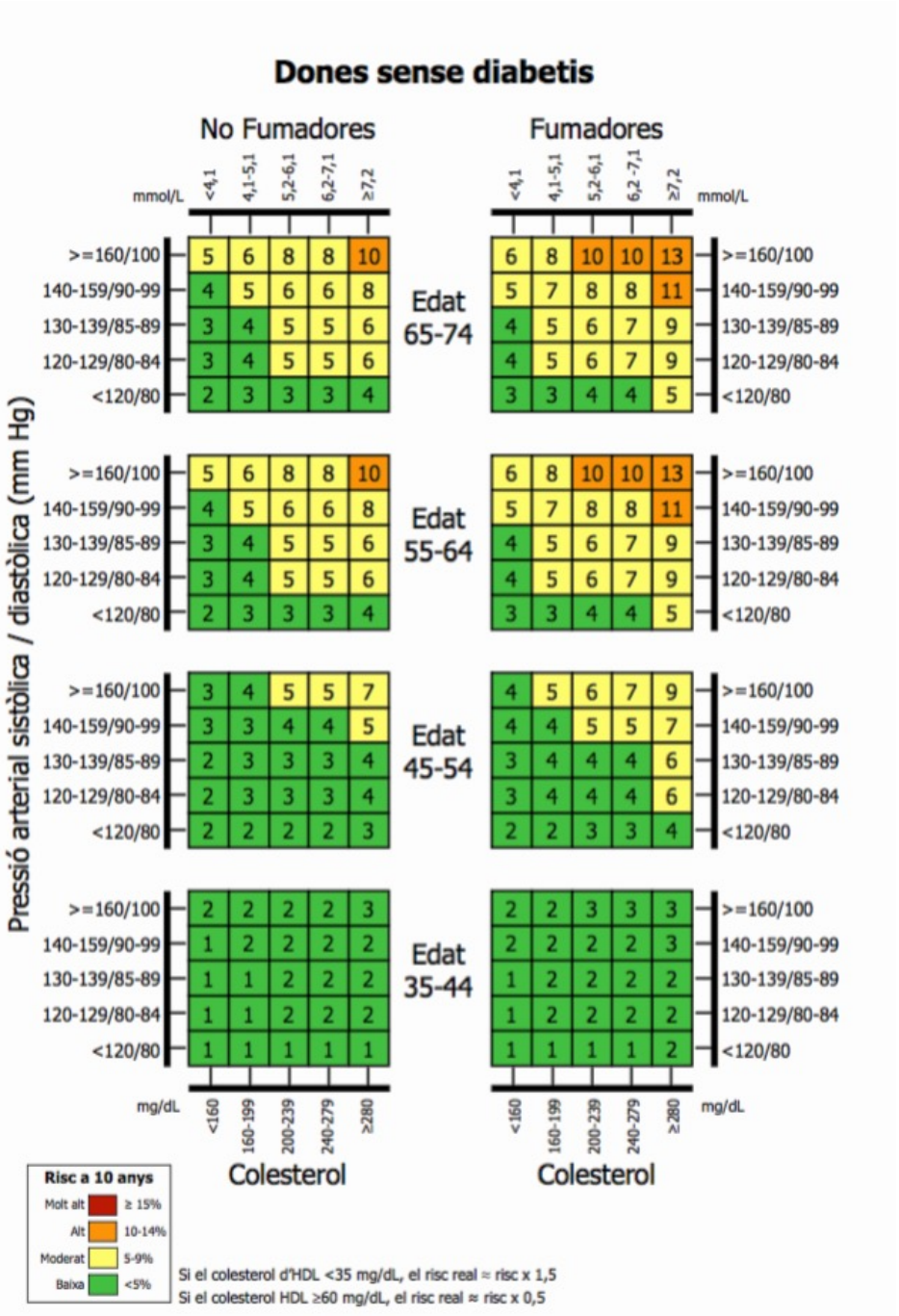
100. Benton MC, Johnstone A, Eccles D, Harmon B, Hayes MT, Lea RA, et al. An analysis of DNA methylation in human adipose tissue reveals differential modification of obesity genes before and after gastric bypass and weight loss. Genome Biol [Internet]. 2015 Jan 22 [cited 2016 Oct 27];16:8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25651499

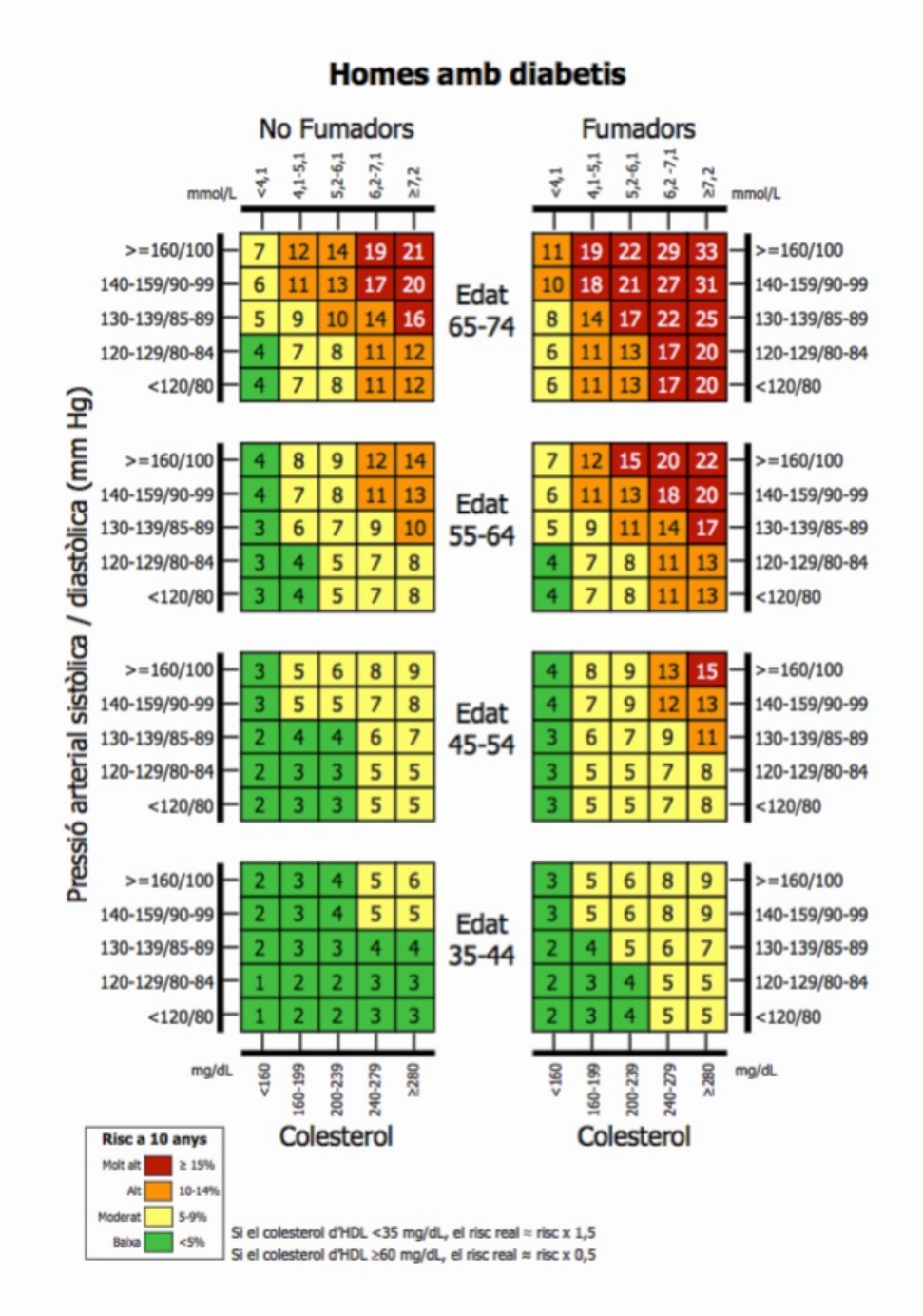
101. Marrugat J. Calculadora de Grandària Mostral GRANMO [Internet]. 2012 [cited 2016 Sep 26]. Available from: http://www.imim.cat/ofertadeserveis/software-public/granmo/

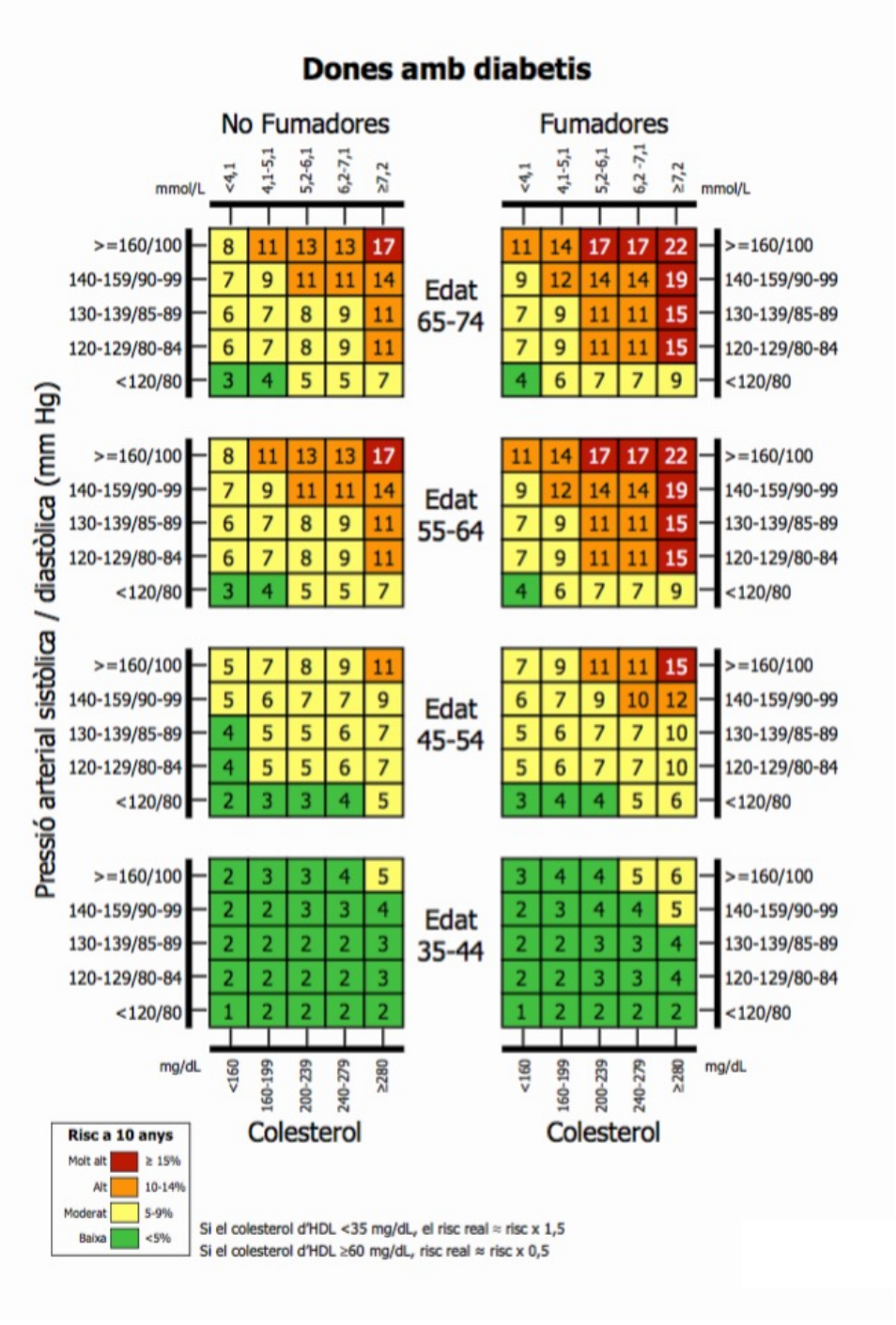
102. Badimón L, Vilahur G, Padró T. Lipoproteins, Platelets, and Atherothrombosis. Rev Española Cardiol (English Ed [Internet]. 2009 Oct [cited 2016 Oct 3];62(10):1161–78. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1885585709733316

ANNEX 1. REGICOR CORONARY RISK ASSESSMENT FUNCTIONS




Dones sense diabetis
No Fumadores
Fumadores
mmol/L
<4,1
4,1-5,1
5,2-6,1
6,2-7,1
≥7,2
Pressió arterial sistòlica / diastòlica (mm Hg)
>=160/100
140-159/90-99
130-139/85-89
120-129/80-84
<120/80
Edat 65-74
5 6 8 8 10
4 5 6 6 8
3 4 5 5 6
3 4 5 5 6
2 3 3 3 4
6 8 10 10 13
5 7 8 8 11
4 5 6 7 9
4 5 6 7 9
3 3 4 4 5
Edat 55-64
5 6 8 8 10
4 5 6 6 8
3 4 5 5 6
3 4 5 5 6
2 3 3 3 4
6 8 10 10 13
5 7 8 8 11
4 5 6 7 9
4 5 6 7 9
3 3 4 4 5
Edat 45-54
3 4 5 5 7
3 3 4 4 5
2 3 3 3 4
2 3 3 3 4
2 2 2 2 3
4 5 6 7 9
4 4 5 5 7
3 4 4 4 6
3 4 4 4 6
2 2 3 3 4
Edat 35-44
2 2 2 2 3
1 2 2 2 2
1 1 2 2 2
1 1 2 2 2
1 1 1 1 1
2 2 3 3 3
2 2 2 2 3
1 2 2 2 2
1 2 2 2 2
1 1 1 1 2
mg/dL
<160
160-199
200-239
240-279
≥280
Colesterol
Risc a 10 anys
Molt alt ≥ 15%
Alt 10-14%
Moderat 5-9%
Baixa <5%
Si el colesterol d'HDL <35 mg/dL, el risc real ≈ risc x 1,5
Si el colesterol HDL ≥60 mg/dL, el risc real ≈ risc x 0,5


Homes amb diabetis
No Fumadors
Fumadors
mmol/L
<4,1
4,1-5,1
5,2-6,1
6,2-7,1
≥7,2
Pressió arterial sistòlica / diastòlica (mm Hg)
>=160/100
140-159/90-99
130-139/85-89
120-129/80-84
<120/80
Edat 65-74
7 12 14 19 21
6 11 13 17 20
5 9 10 14 16
4 7 8 11 12
4 7 8 11 12
11 19 22 29 33
10 18 21 27 31
8 14 17 22 25
6 11 13 17 20
6 11 13 17 20
Edat 55-64
4 8 9 12 14
4 7 8 11 13
3 6 7 9 10
3 4 5 7 8
3 4 5 7 8
7 12 15 20 22
6 11 13 18 20
5 9 11 14 17
4 7 8 11 13
4 7 8 11 13
Edat 45-54
3 5 6 8 9
3 5 5 7 8
2 4 4 6 7
2 3 3 5 5
2 3 3 5 5
4 8 9 13 15
4 7 9 12 13
3 6 7 9 11
3 5 5 7 8
3 5 5 7 8
Edat 35-44
2 3 4 5 6
2 3 4 5 5
2 3 3 4 4
1 2 2 3 3
1 2 2 3 3
3 5 6 8 9
3 5 6 8 9
2 4 5 6 7
2 3 4 5 5
2 3 4 5 5
mg/dL
<160
160-199
200-239
240-279
≥280
Colesterol
Risc a 10 anys
Molt alt ≥ 15%
Alt 10-14%
Moderat 5-9%
Baixa <5%
Si el colesterol d'HDL <35 mg/dL, el risc real ≈ risc x 1,5
Si el colesterol d'HDL ≥60 mg/dL, el risc real ≈ risc x 0,5


Dones amb diabetis
No Fumadores
Fumadores
mmol/L
<4,1
4,1-5,1
5,2-6,1
6,2-7,1
≥7,2
Pressió arterial sistòlica / diastòlica (mm Hg)
>=160/100
140-159/90-99
130-139/85-89
120-129/80-84
<120/80
Edat 65-74
8 11 13 13 17
7 9 11 11 14
6 7 8 9 11
6 7 8 9 11
3 4 5 5 7
11 14 17 17 22
9 12 14 14 19
7 9 11 11 15
7 9 11 11 15
4 6 7 7 9
Edat 55-64
8 11 13 13 17
7 9 11 11 14
6 7 8 9 11
6 7 8 9 11
3 4 5 5 7
11 14 17 17 22
9 12 14 14 19
7 9 11 11 15
7 9 11 11 15
4 6 7 7 9
Edat 45-54
5 7 8 9 11
5 6 7 7 9
4 5 5 6 7
4 5 5 6 7
2 3 3 4 5
7 9 11 11 15
6 7 9 10 12
5 6 7 7 10
5 6 7 7 10
3 4 4 5 6
Edat 35-44
2 3 3 4 5
2 2 3 3 4
2 2 2 2 3
2 2 2 2 3
1 2 2 2 2
3 4 4 5 6
2 3 4 4 5
2 2 3 3 4
2 2 3 3 4
1 2 2 2 2
mg/dL
<160
160-199
200-239
240-279
≥280
Colesterol
Risc a 10 anys
Molt alt ≥ 15%
Alt 10-14%
Moderat 5-9%
Baixa <5%
Si el colesterol d'HDL <35 mg/dL, el risc real ≈ risc x 1,5
Si el colesterol d'HDL ≥60 mg/dL, risc real ≈ risc x 0,5

ANNEX 2. ANKLE BRACHIAL INDEX MESUREMENT

Ankle Brachial Index (ABI) reflects the degree of obstructive arteriopathy in lower limbs. After 10 minutes of resting in supine position, the sleeve is placed in the arm at the middle point of the studied artery. The catheter is placed at 45-60° at the pedis/posterior tibial artery and at the humeral artery. The sleeve is swelled 20-30mmHg above the Doppler signal and it deflates 2-4mmHg per second. The first arterial sign is registered and we calculate the Systolic Blood Pressure (SBP) with the Doppler in both brachial arteries. We also calculate the SBP in both pedis or posterior tibial arteries and the greater one of each limb is selected. The bigger ankle value is divided by the brachial one. In normal conditions, the SBP should not be much different among arms and ankles.

The ABI is inversely proportional to disease gravity. A resting ABI > 0.9 is normal; 0.7-0.9 reflects mild obstruction; 0.4-0.69 moderate obstruction and < 0.4 means a severe obstruction.

ANNEX 3. AUDIT C QUESTIONNAIRE/ CUESTIONARIO AUDIT C

1). ¿Con qué frecuencia toma alguna bebida alcohólica?

(0) Nunca
(1) Una o menos veces al mes
(2) 2-4 veces al mes
(3) 2-3 veces a la semana
(4) 4 o más veces a la semana

2). ¿Cuántas consumiciones de bebidas con contenido alcohólico suele hacer normalmente un día que bebe?

(0) 1-2
(1) 3-4
(2) 5-6
(3) 7-9
(4) 10 o más

3). ¿Con qué frecuencia toma 6 o más consumiciones alcohólicas en un solo día?

(0) Nunca
(1) Menos de una vez al mes
(2) Mensualmente
(3) Semanalmente
(4) Diariamente o casi cada día

ANNEX 4. PARTICIPANT DATA SHEET/INFORMACIÓN SOBRE EL PARTICIPANTE

**La información considerada confidencial es: nombre y apellidos, dirección, población de residencia, teléfono y número de historia clínica; dichas variables (1-5) pueden registrarse utilizando una etiqueta del Hospital Josep Trueta e ir en un documento a parte.*

- Fecha:
- Persona responsable:
- Código de identificación del caso:

6. **Fecha de nacimiento**: _ _ /_ _ / _ _ _
7. **Sexo**:
   - □ Mujer
   - □ Hombre
8. **Raza**:
   - □ Caucásica
   - □ Asiática
   - □ Africana
   - □ Hispana
9. **Peso** (kg):
10. **Talla** (m):
11. **Perímetro cintura-cadera** (cm):
12. **Hábito tabáquico**:
    - □ Fumador
    - □ No fumador
13. **Anticonceptivos orales**:
    - □ Sí
    - □ No
14. **Consumo de alcohol**:
    - □ Sí
    - □ No
15. **Historia de hipertensión**:
    - □ Sí
    - □ No
16. **Consumo de estatinas**:
    - □ Sí
    - □ No
17. **Historia de Diabetes Mellitus**:
    - □ Sí

    En caso afirmativo:
    - □ Tipo I
    - □ Tipo II
    - □ No
18. **Colesterol total** (mg/dL):
19. **c-LDL** (mg/dL):
20. **c-HDL** (mg/dL):
21. **Triglicéridos** (mg/dL):
22. **Historia familiar de enfermedad cardiovascular prematura**:
    - □ Sí
    - □ No

ANNEX 5. DIAGENODE MEDIP MANUAL KIT

STEP 1. Cell collection and lysis

1. Pellet suspension culture out of its serum-containing medium. Trypsinize adherent cells and collect cells from the flask. Centrifuge at 300 g for 5 min at 4°C.

2. Discard the supernatant. Resuspend cells in 5 to 10 ml ice-cold PBS. Centrifuge at 500 g for 5 min. Discard the supernatant. Repeat this resuspension and centrifugation step once more.

3. Meanwhile, place the GenDNA Digestion buffer at room temperature (RT) and the GenDNA proteinase K on ice.

4. Add GenDNA proteinase K to the GenDNA Digestion buffer before use. The stock of provided proteinase K is 200 X. e.g. add 5 µl per 1 ml of Digestion buffer.

5. Resuspend cells in complete Digestion buffer (point 4). For 3 million cells, use 300-µl complete Digestion buffer. For 10 million cells, use 500-µl complete Digestion buffer. If necessary: for 3 million cells, use up to 600 µl of buffer. For 10 million cells, use up to 1,000 µl of buffer.

6. Cell lysis: Incubate the samples with shaking at 50°C for 12 to 18 hours in tightly capped tubes.

STEP 2. Extraction of nucleic acids and DNA purification

1. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol. Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1). One volume is about 500 µl. It is possible to incubate the samples at RT for 10 min on a rotating wheel before centrifugation. Use gentle rotation and do not vortex. Work under a fume-hood.

2. Centrifuge at 1.700 g for 10 min in a swinging bucket rotor.

3. Transfer the aqueous (top) layer to a new tube. Increase volume if necessary (see above) and pipette slowly.

4. Add 1 volume of chloroform/isoamyl alcohol (24:1). It is possible to incubate the samples at RT for 10 min on a rotating wheel before centrifugation. Use gentle rotation and do not vortex. Work under a fume-hood.

5. Centrifuge at 1.700 g for 10 min in a swinging bucket rotor

6. Transfer the aqueous (top) layer to a new tube.

7. Add 1/2 volume of GenDNA precipitant and 2 volumes of 100% ethanol. One volume is about 500 µl and corresponds to the original amount of top layer. Add therefore 250 µl of

precipitant and 1.000 µl of 100% ethanol. The DNA should immediately form a stringy precipitate.

8. Recover DNA by centrifugation at 1.700 g for 2 min.

9. Rinse the pellet with 70% ethanol. Decant ethanol and air-dry the pellet. It is important to rinse extensively to remove any residual of salt and phenol.

10. Resuspend the pellet of DNA at ~1 mg/ml in GenDNA TE until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C. From 3 million cells, ~20 to 30 µg of DNA can be expected (in a volume of 20 to 30 µl). From 10 million cells, ~50 to 100 µg of DNA can be expected (in a volume of 200 to 300 µl).

11. If necessary, residual RNA can be removed at this step by adding 2 µl of GenDNA RNase (DNase-free) per ml of DNA sample and incubating 1 hour at 37°C, followed by phenol/chloroform extraction and ethanol precipitation (similar to above).

12. For DNA analysis, run samples on a 1% agarose gel along with DNA size marker to visualize the DNA preparation efficiency.

STEP 3. DNA shearing

1. In a 1.5-ml tube, dissolve the DNA sample in TE to reach 0.1 µg/µl.

2. Use a final volume of 300 µl of DNA sample in 1.5-ml tubes.

3. Shear the DNA by sonication using the Bioruptor®. - Bioruptor Pico: 6 cycles of 30 sec ON / 90 sec OFF (1.5 ml microtubes with caps) - other Bioruptor models: 10 cycles of 15 sec ON / 15 sec OFF; Low Power

4. Sheared DNA can be analyzed on agarose gel.

STEP 4 and 5. Methylated DNA Immunoprecipitation and washes

1. Prepare the IP incubation mix w/o DNA sample as follows. For one IP: 24.00 µl Buffer A, 6.00 µl Buffer B, 1.5 µl of positive meDNA control and 1.5 µl negative unDNA control and 45 µl water.

2. Label new 1.5-ml tubes. Add the DNA sample to the IP incubation mix.

3. Add per labeled "IP" tube: the IP incubation mix. Then, add 1 µg of DNA sample per tube. Using DNA samples at a concentration of 0.1 µg/µl: add 65 µl of IP incubation mix and 10 µl of DNA per tube. The total volume per IP is 75 µl.

4. Add per "input sample" tube: 20% of what is used per IP above. Using DNA samples at a concentration of 0.1 µg/ µl: add 13 µl of IP incubation mix and 2 µl of DNA per tube. The total volume for 20% input is 15 µl.

5. Incubate at 95°C for 3 min.
6. Quickly chill on ice (it is best to use ice-water).
7. Quickly perform a short spin at 4°C.
8. In a new tube, prepare the Diluted Antibody mix. For one IP: prepare a 1:10 antibody dilution as follows: (0.30 µl antibody, 0.60 µl Buffer A and 2.10 µl water). Then, add 2.00 µl of Buffer C. Final volume is 5.00 µl.
9. Add 5 µl of Diluted Antibody mix per IP tube (3. above). Antibody is added to the IP tubes, which contain IP incubation mix and your DNA sample.
10. Add 20 µl of beads (resuspend beads before use) to all tubes. That is the IP incubation which comprises the IP samples, the Diluted antibody mix and the beads. The final volume: 100 µl.
11. Place on a rotating wheel at 4oC for 4 hours or overnight.
12. The IP samples are then washed as follows: add 450 µl of ice-cold Wash buffer to each IP tube, starting with Wash buffer-1. Place the four Wash buffers on ice and perform the washes in a cold room.
13. Rotate for 5 min at 4°C.
14. Centrifuge at 6.000 rpm for 1 min at 4°C.
15. Discard the supernatant. Keep the pellet.
16. Wash the pellet again (as described above: Point 13. to 16.) as follows: perform one more wash with Wash buffer-1, then one wash with Wash buffer-2, and one wash with Wash buffer-3. Finally perform two more washes using the Wash buffer-4.
17. After the last wash, discard the last traces of Wash buffer (using a P200 pipet). Keep the bead pellets. These are the Methyl DNA IP samples. The Immunoprecipitated Methylated DNA is bound to the beads.

STEP 6. DNA elution and purification

1. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.
2. Prepare the complete elution buffer by mixing Buffer D, E and F as follows. For one IP: 360 µl of Buffer D, 40 µl of Buffer E, 16 µl of Buffer F. The total volume is of 416 µl.
3. Add 416 µl of freshly prepared complete elution buffer to the bead pellets (the Methyl DNA IP samples).
4. Add 416 µl of freshly prepared complete elution buffer to the input samples.
5. Incubate in a thermo-shaker for 10 min at 65oC at 1.000 to 1.300 rpm.

6. Cool down samples to room temperature, add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1).
7. Centrifuge for 2 min at 14,000x g (13.000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml-tube.
8. Add 1 volume of chloroform/isoamyl alcohol (24:1).
9. Centrifuge for 2 min at 14.000 x g (13.000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml-tube.
10. Thaw on ice the DNA co-precipitant.
11. Per tube: add 5 µl of the provided meDNA-IP co-precipitant and 40 µl of the meDNA-IP precipitant. Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -80°C for 30 min.
12. Centrifuge for 25 min at 14.000 x g (13.000 rpm) at 4°C. Carefully remove the supernatant and add 500 µl of ice- cold 70% ethanol to the pellet.
13. Centrifuge for 10 min at 14.000 x g (13.000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 min at room temperature to evaporate the remaining ethanol. The pellets are: 1/ DNA that has been purified from the sheared DNA (input sample(s)) and 2/ DNA that has been isolated by IP (Methyl DNA IP samples).
14. Add 50 µl TE to the IP and input samples. Suspend the DNA evenly: place the tubes in a shaker for 30 min at 12,000 rpm at room temperature to dissolve the pellets.

STEP 7. qPCR analysis of IP'd DNA

1. Make aliquots of the purified DNA and prepare dilutions. Use the purified DNA from Methyl DNA IPs and DNA input(s). From the 50 µl of purified DNA: transfer 10 µl into a new tube (keep 40 µl for Methyl DNA IP-on-chip analysis, or further PCR analysis). For the first PCR analysis, dilute 10 µl of each purified DNA sample as follows: to 10 µl of purified DNA sample (from IP and input), add 35 µl of water. Final volume is 45 µl. Use 5 µl per PCR. Note: when testing the hum meDNA primer pair (AlphaX1): dilute the DNA sample 1:1,000.
2. Prepare your qPCR mix using SYBR PCR Green master mix. qPCR mix (total reaction volume is 25 µl: 1.00 µl of provided primer pair (stock: 10 µM each: reverse and forward), 12.50 µl of master mix (e.g.: iQ SYBR Green supermix), 5.00 µl of diluted purified DNA sample (see above for DNA dilutions) and 6.50 µl of water.
3. PCR cycles: amplification: 1x 95°C for 7 min, 40 cycles of (95°C for 15 seconds, 60°C for 1 min and 95°C for 1 min).
4. When the PCR is done, analyze the results.

ANNEX 6. PRIMER SEQUENCE AND INFORMATION

ASSAY	NAME	SEQUENCE	GENOMIC LOCATION	ANNEALING TEMPERATURE (°C)
MT-CO1	Forward	TATTAATTGGTTTTTTAGGGTTTAT	Chromosome MT: 5.904-7.445	52
	Reverse	CAACAAATCATTTCATATTACTTCC		
MT-CO2	Forward	TTTATGAGTTGTTTTTATATTAGGTTTAAA	Chromosome MT: 7.386-8.229	52
	Reverse	ACTCCACAAATTTCAAAACATTAAC		
MT-CO3	Forward	TATATTATTTGTTTAAAAAGGTTTT	Chromosome MT: 9.207-9.990	52
	Reverse	AATAAAAAACTCAAAAAAATCCTAC		
LINE-1	Forward	TTTTGAGTTAGGTGTGGGATATA	n/a	56,3
	Reverse	AAAATCAAAAAATTCCCTTTC		
IL-6	Forward	TATTTTAGTTTTGAGAAAGGAGGTG	Chromosome 7: 22,725,884-22,732002	57
	Reverse	CAATACTCTAAAACCCAACAAAAAC		
MCP1	Forward	GCTTAATGGCACCCCATCCT	Chromosome 17: 32,255,277-34,257,203	57
	Reverse	GTGGGTACCACGTCTGCTTG		

**Gene sequence data was obtained from ensembl databe (ensembl.org). Primers sequence were produced with the NCBI tool "Primer BLAST"(www.ncbi.nlm.nih.gov/tools/primer-blast/).*

ANNEX 7. PATIENT INFORMATION SHEET

Title: Association of methylation marks in monocyte nuclear DNA and platelet mitochondrial DNA with the risk of Myocardial Infarction

Investigators: Ramon Brugada, Elisa Ruiz.

Location: Josep Trueta Hospital in Girona, IdIBGi Cardiovascular genetics laboratory.

Introduction

You have been offered the possibility of entering to a study where an epigenetic test will be performed by using a sample of your blood. The aim of this study is to determine the existence of epigenetic modifications associated to myocardial infarction risk.

The analysis results, coupled with other clinical and pathological factors, are of prognostic value for the development of this condition. The test is performed by IdIBGi Cardiovascular genetics laboratory, a genetic analysis service that analyzes the genes involved in the heart disease. Cardiovascular genetics laboratory will analyze your epigenetic characteristics and produce data that will help your physician to assess your risk of having a myocardial infarction in future. Additionally, your lipid profile and glycaemia levels will be determine from the blood sample.

Purpose

The goal of this study is to identify your epigenetic modifications in order to gain a better knowledge and understanding of your potential risk of developing myocardial infarction. Your physician will assess your epigenetic data, together with other biochemical, clinical and functional factors, and provide you with the best treatment recommendations and lifestyle advice.

Volunteer participation

Your participation in the study is totally voluntary. You are free to decide whether to participate or not. If you decide to take part we will ask you to sign a consent form. Your decision will not affect the healthcare treatment you receive.

You have been chosen because you have been diagnosed with a first episode of myocardial infarction or free of Coronary Artery Disease and you meet all the inclusion criteria and none of the exclusion ones.

Procedure

If you agree to participate in this study, your physician will explain the steps required to take three samples of 4 ml each from peripheral blood. Your physician will send the sample to clinical analysis laboratory of Josep Trueta Hospital and from there, the sample will also be transfer to Cardiovascular genetics laboratory in Girona. The epigenetic analysis will not involve any further examination, control or medical procedure, or any risk or additional discomfort.

After the analysis is performed, the sample will be frozen and stored. Should you wish to perform a counter-analysis; the sample will be available to you and your physician for 1 year. After this period of time, and unless you state otherwise, the sample may only be retained if it is disassociated from your personal data and remains anonymous.

Associated risks

There are no significant risks associated with obtaining blood samples. Drawing a blood sample may cause some discomfort, it may be slightly painful sometimes and it rarely causes fainting. Blood samples will be drawn only by qualified professionals.

Confidentiality and management of personal information

Confidentiality and privacy will be respected at all times. No information that could reveal your identity will be published without your consent. Your identity will not be used in any analysis reports. In all the records created by the laboratory you will be identified only by a code. Your physician and their team will be the only individuals with access to your personal data, and they are bound to an obligation of confidentiality. Your personal data and epigenetic data will be reversely coded so you can only be identified by your physician and their team.

Your personal and epigenetic data will be kept for a period of 5 years. After this period, the data will be retained for as long as it is necessary to preserve your health, provided you have not exercised your cancellation right. Notwithstanding the above, the data may be retained for research purposes, in which case it will remain completely anonymous.

Your personal data will be stored in a file managed by researcher Ramon Brugada and Elisa Ruiz and will only be used for the above mentioned purposes, unless you state otherwise.

You have a right to access, modify, erase and object your data by writing to researchers Ramon Brugada or Elisa Ruiz at the following address: Parc Hospitalari Martí i Julià; Carrer Dr. Castany, s/n Edifici Mancomunitat 2, 17190 –Salt-tlf 872.98.70.87 ext. 63.

Thank you for reading this. Try to keep this information sheet for your records until you finish your participation in the study. Any queries, questions or doubts, do not hesitate to ask us. If you decide to participate in the study, sign the consent form below.

ANNEX 8. INFORMED CONSENT FORM to participate in the study named**:** *"Association of methylation marks in monocyte nuclear DNA and platelet mitochondrial DNA with the risk of Myocardial Infarction"*

□ I have received a copy of the Patient Information Sheet and I understand the information contained therein.

□ I have had sufficient time to make a decision.

□ I authorize my physician to send a biologic sample of my blood, as well as the relevant clinical information required by Cardiovascular genetics laboratory and JTH clinical analysis laboratory for its processing, since the clinical information may be relevant for the interpretation of the epigenetic results.

□ I understand that, occasionally, the laboratory may have difficulties analyzing the sample and will require a second sample.

□ My consent is voluntary and will not affect my relationship with my treating physician. My data will remain strictly confidential according to Organic Law 15 of 13th December 1999 on Personal Data Protection and in accordance with Law 14 of 3 July 2007 on biomedical research. My consent does not free from any liability the persons and/or entities involved in the analysis process. My legal rights will remain safeguarded at all times.

□ I understand that, in the interest of science, a summary of the analysis results may be published in scientific settings. However, under no circumstances will the information reveal confidential information about me, unless I state otherwise in writing.

□ I authorize my sample to be kept for research purposes provided my data remain confidential and I cannot be identified.

□ I want to be informed of any new results obtained through research. I may change my opinion at anytime and withdraw my authorization for this epigenetic study and, therefore, revoke my decision to continue with the test.

- Patient's name: _____
  Signature: _____

- Person appointed by the clinician to participate in the informed consent process:
  Name: _____
  Signature: _____

- Physician's name: _____ Role/Post:_____

Signature: _____ ***Girona, __/_____/20__***