Mitochondrial DNA methylation; cardiovascular diseases risk and response to diet

Sarah Corsi

Human Nutrition Research Centre

Centre for Healthier Lives

Population Health Sciences Institute

Newcastle University

Thesis submitted for the degree of Doctor of Philosophy

September 2020

Abstract

Background: Cardiovascular diseases (CVD) are the leading cause of death worldwide. CVD are complex, multifactorial disorders caused by both genetic and environmental factors. Approximately 80% of CVD burden is attributable to modifiable risk factors, including poor diet, smoking, lack of physical activity, and air pollution.

Higher body mass index (BMI) is considered an intermediate risk factor for the development of CVD. Currently, 66% of men and 58% of women in the UK have overweight or obesity. Although excessive adiposity is a risk factor for CVD, not everyone with overweight or obesity will develop CVD. As yet, there is no established biomarker that distinguishes those adults with overweight and obesity who will develop CVD from those adults who will remain CVD-free.

This project was designed to test the hypothesis that participants with overweight/obesity who will develop CVDs show different mitochondrial DNA methylation pattern compared to those who remain CVD-free.

Mitochondria have a central role in ageing and in the development of age-related diseases, including CVD, which is linked with dysregulated reactive oxygen species (ROS) production, and mitochondrial DNA (mtDNA) instability. Platelets, anucleated cells that contain only mtDNA, have a greater reactivity in adults with overweight and obesity, and are an indicator of CVD risk. Additionally, platelet number and reactivity have been shown to be ameliorated by the Mediterranean diet and by plant extracts. Furthermore, platelets derive from megakaryocytes, which are affected negatively by inflammation, dysregulated adipocytes, and obesity.

Experimental studies: First, I investigated the effects of selected polyphenolic compounds on mtDNA methylation in megakaryocytes *in vitro*. This experiment was devised to test the effects of treatment with specific polyphenols on patterns of mtDNA methylation in the megakaryocyte, MEG-01 cell.

Secondly, I conducted a nested case-control study within the SPHERE cohort of Italian adults with overweight and obesity who were otherwise healthy at Baseline. In a prospective study design, I used a targeted approach to investigate mtDNA methylation at Baseline in those who developed CVD over the following 5 years compared with those who remained disease-free. I identified three specific genomic loci within mtDNA i.e. *MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254, that predicted future CVD risk. MtDNA methylation level at

these loci was independent of conventional adiposity-related CVD risk factors and so may represent an environment-related predictor of future CVD in adults with overweight/obesity. In addition, I investigated the associations between mtDNA methylation in platelets and adherence to the Mediterranean diet. Higher adherence to the Mediterranean diet, as evaluated with the MeDiet Score, was associated with lower methylation at the D-loop nt16383 and higher methylation at *MT-CO2* nt8113.However, the MeDiet Score and the methylation level at these two genes are not associated with future CVD outcome.
In summary, this is the first study investigating patterns of platelet mtDNA methylation in relation to future risk of CVD. I observed that higher mtDNA methylation of *MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254 in platelets was associated with a higher risk of developing CVD within five years. In addition, this appeared to be a dose-dependent relationship so that participants with Score 2 (high methylation at two or three loci) were more likely to develop CVD than the participants with Score 1 (higher methylation at one locus) and Score 0 (no loci with high methylation).

Dedication

“ Ergo vivida vis animi pervicit, et extra

processit longe flammantia moenia mundi

atque omne immensus peragravit mente animoque,

unde refert nobis victor quid possit oriri,

quid nequeat, finite potestas denique cuique

quanam sit ratione atque alte terminus haerens.”

Lucretius, *De Rerum Nature*

I Century B.C.

“Dear comrades-descendants, the labourers of the 20th century are writing to you. […] You have probably already eliminated all harmful bacteria and viruses, and live without ageing or sickness.”

Tiraspol, Soviet Time capsule from 1967 opened December 2017

Acknowledgement

The wonderful and inclusive environment at the Human Nutrition Research Centre made this research possible and evokes this magnificent stream of those passions which sustain science – gratitude and admiration.

I owe human gratitude to those who guided, inspired and supported me during my uneasy path to scientific rigour and inspired me through their example. First and foremost, I am indebted to my principal supervisor Dr Hyang-Min Byun and secondary supervisor Professor John Mathers for their expert guidance and constant dedication throughout these past four years.

I would also like to thank the members of the Mathers group. In particular, a special thanks to Chanachai Sae-Lee, Dr Fiona Malcomson, Dr Stella Breininger, Dr Becca Brown, Dr Azizah Mat Hussin, Dr Ammar Ashor, Abrar Babateen, Inês Mendes, Dr Marga Cifre, and Katie Harrison. Thank you very much for helping me familiarizing with protocols, equipment, and for the constructive feedback when rehearsing my presentations.

I would like to thank the collaborators from Professor Valentina Bollati's lab at Milan University. A special thanks to Valentina Bollati for her guidance and stimulating conversations and Simona Iodice for the support in analysing the mtDNA methylation data from SPHERE. Additionally, I thank Simona Iodice for having written the majority of codes for mtDNA methylation analysis.

I am very grateful to the SPHERE study participants for taking part in the study and donating blood samples and to the SPHERE study team Dr Luisella Vigna, who collected the samples, Dr Raquel Cacace (M.D.) for the calculation of the Framingham Score and HeartScore, and Ms Laura Dioni for the sample shipment. Additionally, I thank Mario Siervo (M.D., PhD) for the classification of diseases into "Mild" and "Severe".

I would like to thanks Santander scholarship (£ 1,395.82) for the travel and accommodation to Milan University.

I would like to express my deepest gratitude and thanks to my current supervisor Dr Ken Raj and all the colleagues from Ken's group at Public Health England: Dr Sylwia Kabacik, Donna Lowe, Gareth Hazel, and Christopher Whiteman.

Lastly, I would like to pay homage to my family, and my partner Dr Andrea Giovannetti. Heartfelt and special thanks go to Gazellino, Mucchetto, and Piratino for the endless support and love, without whom this thesis would not have been possible.

Table Contents

List of Figures

List of abbreviations

Abbreviation	**meaning**
5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
5fC	5-formilcytosine
AA	Arachidonic Acid
BC	Bisulfite converted
BM	Bone Marrow
BMI	Body Mass Index
BS-Seq	BS-Sequencing
CI	Confidence interval
CVD	Cardiovascular diseases
CAD	coronary artery disease
Curcumin	(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione
D-loop	Displacement loop
DBP	diastolic blood pressure
DH_2O	Distilled Water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAm Age	DNA methylation Age
DNMT	DNA-methyl-transferases
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked Immunosorbent assay
ETC	Electron Transport Chain
FFQ	Food frequency questionnaire
GLM	General Linear Model
h	hours
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HR	Hazard ration
HSP1	Heavy-Strand Promoter 1
HSP2	Heavy-Strand Promoter 2
IL	Interleukin
LDL	Light density lipoprotein
LSP	Light-Strand Promoter
MDP	Mitochondrially Derived Peptides
MeDiet	Mediterranean Diet
mt	Mitochondria
MT	Mitochondrially-encoded
MT-CO1	Mitochondrially-encoded cytochrome-C-oxidases I
MT-CO2	Mitochondrially-encoded cytochrome-C-oxidases II
MT-CO3	Mitochondrially-encoded cytochrome-C-oxidases III
MT-OLR	Mitochondrially-encoded light-strand origin-of-replication
MT-TF	Mitochondrially-encoded tRNA phenylalanine
MT-TL1	Mitochondrially-encoded tRNA leucine 1

mtDNA	Mitochondrial DNA
mtDNMT	Mitochondrial DNA-methyl-transferase
MTS	Mitochondrial Targeting sequence
mtSSB	Mitochondrial single-stranded binding protein
NCD	Non-communicable disease
NCR	Non-coding region
nDNA	Nuclear DNA
nt	Nucleotide
O_H	Heavy-strand origin of Replication
O_L	Light-strand origin of Replication
OR	Odds ratio
ORF	Open Reading Frames
PCR	Polymerase Chain Reaction
PD	Parkinson's Diseases
Polγ	polymerase γ
POLMRT	Mitochondrial RNA polymerase
Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one
Resveratrol	5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol
rRNA	Ribosomal RNA
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
SAM	S-Adenosyl L-Methionine
SAH	S-Adenosyl L-homocysteine
SBP	Systolic blood pressure
SD	Standard deviation
ssDNA	Single-strand DNA
TAS	Termination-associated sequence
TFAM	Mitochondrial Transcription Factor A
tRNA	Transfer RNA
xg	Time gravity. It is the unit of relative centrifugal force (RCF).

Chapter 1: General introduction

1.1 Cardiovascular diseases and ageing

1.1.1 Introduction to cardiovascular diseases (CVD)

Cardiovascular disease (CVD) is a general term referring to conditions affecting the heart and/or blood vessels. CVD consist of non-infectious chronic conditions and are part of the non-communicable diseases (NCD) category (World Health Organization, 2016). CVD caused around 17.9 million deaths in 2016 worldwide and this figure is predicted to rise due to urbanisation, unhealthy lifestyle, and population ageing (Meier et al., 2019; Naghavi et al., 2017). It has been estimated that the accumulated cost to the global economy of NCD will be around $ 43 trillion by 2030 (Bloom et al., 2011).

CVD is the single largest cause of death worldwide and is responsible for close to 30% of total deaths (Bloom et al., 2011). However modifiable behavioural risk factors such as unhealthy diet, physical inactivity, smoking habit and harmful use of alcohol account for nearly 80% of the CVD burden (Figure 1.1) (Bloom et al., 2011; Gaziano et al., 2010; Meier et al., 2019; Stanaway et al., 2018; World Health Organization, 2016), which suggests that the large majority of CVD is potentially preventable. The primary solution to this recent global epidemic remains essentially political, inasmuch the availability of affordable healthcare and a cultural and political environment conducive to sustained prevention and equity in opportunities are central macroeconomic aspects (Krueger & Reither, 2015; Lyn et al., 2019). Nevertheless, the effective establishment of the metabolic risk of each individual and the development and implementation of personalised interventions are instrumental in tackling the biological aspects of CVD at an individual level (Gerszten & Wang, 2008; World Health Organization, 2016). One of the biggest challenges in achieving that objective is the definition of a biomarker with high prognostic potential, so that those individuals within the population who are at higher metabolic risk can be identified before CVD is manifested and that allows mitigative countermeasures to be taken (van Holten et al., 2013; Wallace et al., 2016; J. Wang et al., 2017).

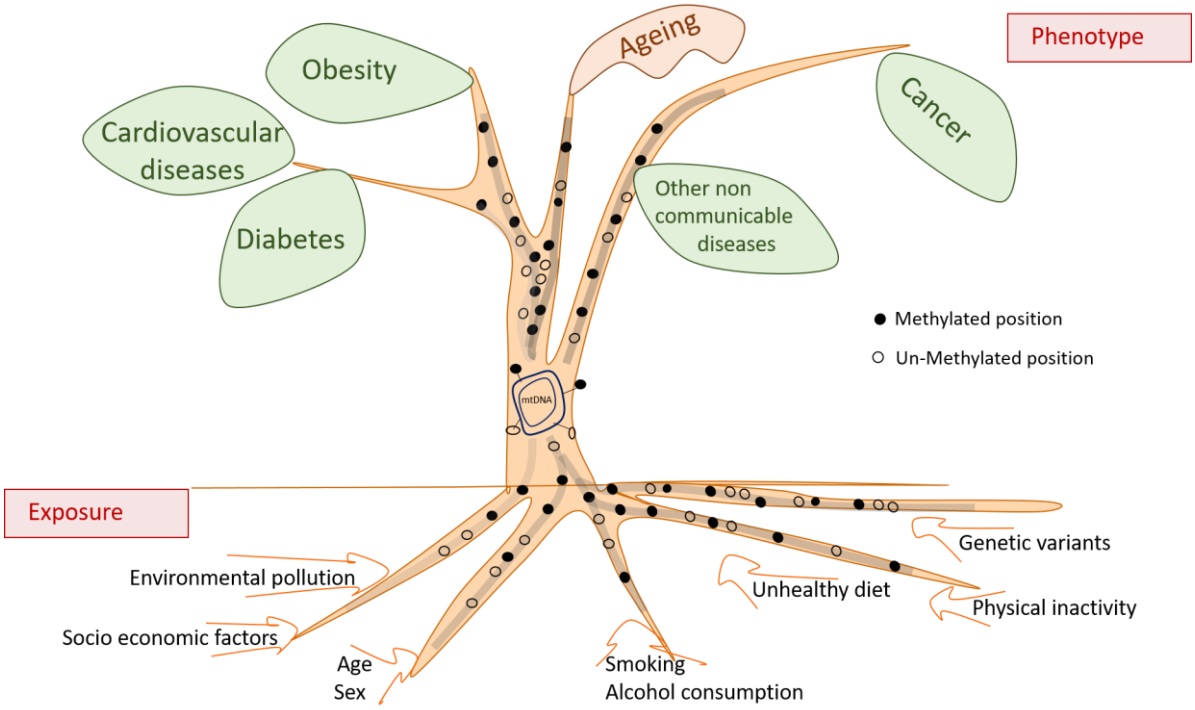


Figure 1.1 Conceptual diagram illustrating how epigenetics biomarkers may link exposure (risk factors) with ageing-related health outcomes

In this conceptual image, I have used the analogy of a tree in which the roots are exposed to the risk factors that provide the substrate for the resulting phenotypes illustrated in the above-ground part of the tree. The risk factors are notably diet, environmental pollution, and physical inactivity. In the foliage part, there are the resulting phenotypes or age-related diseases such as CVD. Since epigenetic alterations, mitochondrial DNA damage, and mitochondrial dysfunction are hallmarks of ageing and that these are modified by the environment, this PhD project focuses on DNA methylation alterations in the mitochondria as an indicator of CVD risk. Therefore mitochondrial DNA (mtDNA) methylation is placed in the centre of the image, where it serves as an indicator of the exposure and of the overall risk of developing CVD.

More generally, I have hypothesised that CVD risk factors modulate mitochondrial epigenetic marks by increasing or decreasing methylation status at specific CpG sites. These risk factors ultimately lead to fully manifested cardiovascular conditions. The pathological processes that lead to CVD also affect the methylation status of mtDNA. Therefore, in this context, the mtDNA methylation pattern can be used both as a marker of a specific phenotype and exposure and as a predictive biomarker for CVD risk.

End of figure legend.

The mechanisms responsible for the ageing process and development of age-related disease are not well characterized. However, at the molecular level, these processes are likely to involve, among others, aberrant gene expression, mitochondrial dysfunction, and epigenetic alterations (López-Otín et al., 2013; Costantino et al., 2016). In particular, epigenetic alterations are gaining momentum as markers for CVD, on the grounds that they mediate the interactions between genes and environment (Wallace et al., 2016; Lind et al., 2018). Hence, I hypothesized that altered mitochondrial epigenetic marks, due to ageing and environmental exposure, is a biomarker of molecular and cellular dysfunction that predisposes to diseases. In figure 1.1, I have used the analogy of a tree whose roots are exposed to environmental factors, such as environmental pollution and unhealthy diet, that cumulatively increase the risk of developing diseases. These risk factors cause damage to the cells and may ignite a pathological process that will lead, eventually, to cardiovascular diseases. Accordingly, mtDNA methylation patterns respond both to environmental exposure and in the presence of CVD.

1.1.2 Ageing as a risk factor for CVD

Ageing is the main risk factor for all common NCD including CVD (Koopman & Kuipers, 2017; North & Sinclair, 2012; Costantino et al., 2016). In addition, both ageing and CVD risk are modified by environmental factors, including physical activity, smoking and diet, and by socioeconomic status (Dor & Cedar, 2018; Estruch et al., 2013; Meier et al., 2019; Stanaway et al., 2018; Gensous et al., 2020; Quach et al., 2017; Fitzgerald et al., 2020). Given the fact that most countries worldwide are experiencing a sustained demographic shift with a rapid increase in the number of older people (North & Sinclair, 2012), the development of novel interventions that reduce CVD risk during ageing is a major public health priority(WHO, 2014).

The mechanisms through which the ageing process drives the development of CVD have not been fully elucidated but the deterioration of the heart and arterial system (North & Sinclair, 2012) may be associated and/or caused by dysregulated gene expression (Mathers & Byun, 2016). During ageing, numerous aspect of metabolism and homeostasis undergo deterioration; in this context, the circulatory system is particularly relevant because it delivers oxygen and nutrients to all the tissues and, therefore, is vital for the performance of all other organs (Deverdun et al., 2016; North & Sinclair, 2012; Wyss-Coray, 2016). At the cellular level, common denominators of ageing are for example epigenetic alterations, mitochondria dysfunction and dysregulated nutrient sensing (López-Otín et al., 2013).

1.1.2.1 Stem cells and ageing

During ageing, the number and phenotype of stem cells change and this lead to a perturbed balance and functionality of the resulting somatic cells (de Haan & Lazare, 2018). Generally, during ageing, stem cells lose their capacity for self-renewal (de Haan & Lazare, 2018). One example is the hematopoietic stem cell (HSC), which resides in the red bone marrow and is responsible for the production of billions of new blood cells daily (Ahmed et al., 2017). With ageing, there is a gradual shift from the "red" bone marrow, or the blood-producing marrow, to the "yellow" marrow, or the fat-containing marrow (Ahmed et al., 2017; Ho & Méndez-Ferrer, 2020). The bone marrow niche supports, and regulates, HPC differentiation. The ectopic accumulation of lipids and increased inflammation within the bone marrow niche during ageing have been associated with changes in the location, function, and regulation of the HPC and their progeny (Ho & Méndez-Ferrer, 2020).
Additionally, with ageing, although HSC increase in number they also deteriorate so that the number of "functional" HPCs that contribute to production of blood cells is reduced (Mejia-Ramirez & Florian, 2020).

1.1.2.2 Molecular aspects of ageing

At the molecular level, the characteristics (or hallmarks) of ageing include DNA damage, telomere attrition, mitochondrial dysfunction, and increased ROS production (López-Otín et al., 2013; Ahmed et al., 2017). Interestingly, both nuclear DNA and mitochondrial DNA is damaged by ROS (Ballinger, 2005). Mitochondria have been the focus of ageing research as not only because mitochondrial dysfunction is associated with age-related diseases (Ballinger, 2005) but also because they are the primary source of ROS within the cell (Pichaud et al., 2019). ROS have a crucial role inside the cells as signalling molecules, but the increased production of ROS during ageing exacerbates DNA damage and leads to the deterioration of mitochondrial homeostasis (Mejia-Ramirez & Florian, 2020). As a consequence, mitochondria have been central in research on biomarkers of ageing.

1.1.3 Obesity as an intermediate risk factor for CVD

Obesity always results from a sustained excess of food energy intake and the resulting excess energy is stored as fat (Camacho & Ruppel, 2017). Obesity is a multifactorial, highly heritable disorder (40-70% heritability), associated with poor diet, inadequate physical activity, mitochondrial dysfunction, altered nutrient sensing, environmental pollution, availability of inexpensive highly processed foods, economical aspects, and infections

(Bournat & Brown, 2010; Chen et al., 2019; Comuzzie & Allison, 1998; Hall et al., 2019; Locke et al., 2015; Patti & Kahn, 2004; Wright & Aronne, 2012).

Obesity is considered an "intermediate risk factor" for CVD (Bloom et al., 2011; Must et al., 1999; Yusuf et al., 2005). As a convention, obesity is often estimated using Body Mass Index (BMI) (Wahl et al., 2017). When BMI (weight in kilograms divided by height^2 in meters) equals or exceeds the threshold of 30 Kg/m2, this condition is defined as obesity in Caucasian populations (Poirier et al., 2006; World Health Organization, 2016; ASRM, 2015). However, at an individual level, BMI may not be the most accurate parameter in predicting obesity-related health risk because it does not distinguish between lean and fatty mass (Janssen et al., 2004; Poirier et al., 2006; Yusuf et al., 2005). Recently, the definition of obesity based on BMI has been challenged on the basis that it is not the best biomarker for CVD risk stratification (Elagizi et al., 2018).

Overweight and obesity are strongly associated with CVD, osteoarthritis, and at least 13 common types of cancer (Butland et al., 2007; Khan et al., 2018; Poirier et al., 2006; Prospective Studies Collaboration, 2009). These comorbidities reduce life expectancy by up to 8 years and reduce healthy-life years by up to 19 years (Grover et al., 2015). In a recent population study in which the participants were followed for over 50 years, Khan et al observed that adults with obesity have reduced longevity, and higher incidence and mortality of CVD, in comparison with the control group with normal BMI (18.5 – 24.9) (Khan et al., 2018). Although adults with overweight had similar life expectancy to healthy weight individuals, they had fewer years of healthy life due to the earlier incidence of CVD (Khan et al., 2018).

Potential mechanisms linking obesity to CVD include insulin resistance (Reaven, 2011), inflammation (Karelis et al., 2005; Van Gaal et al., 2006; Wang & Nakayama, 2010), and the hyperaggregability of platelets (Bordeaux et al., 2010). Whilst the relationship between obesity and CVD is well established (Atkins et al., 2014; Landsberg et al., 2013), it is not inevitable that people with obesity will develop CVD (Karelis et al., 2005; Poirier et al., 2006; Qi & Cho, 2008; Reaven, 2011; Van Gaal et al., 2006). In fact, it has been established that up to 45% of adults with obesity do not develop cardiometabolic complications; such individuals are known as the "metabolically healthy obese" (MHO) (Loos & Kilpeläinen, 2018; Neeland et al., 2018). The reasons why some individuals with obesity do not develop CVD are poorly understood and there are no established biomarkers which distinguish those individuals with obesity who will develop CVD from those who do not.

1.1.4 The role of platelets in CVD

Platelets are anucleate cells required for blood coagulation and for the maintenance of vascular integrity (Ho-Tin-Noé et al., 2011). They are involved in the formation of the blood clot following blood vessel damage, are important coordinators of inflammation as they release numerous inflammatory mediators, and integrate both innate and adaptive immune response (Michelson, 2004; Thomas & Storey, 2015). Abnormal platelet function is responsible for thrombosis and for haemostatic disorders characterized by prolonged bleeding (Patel et al., 2005), and are involved in the development of atherosclerosis, one of the most common inflammatory conditions of the vasculature (Huo & Ley, 2004; Lievens & von Hundelshausen, 2011).

Platelet activity can predict the outcome of CVD (Gurney et al., 2002) and can be used to monitor the efficacy of therapeutic interventions (Michelson, 2004). In fact, due to their central role in inflammation and immune cells recruitment, platelets integrate the numerous discrete inflammatory signals that are found within the bloodstream (Stoner et al., 2013; Thomas & Storey, 2015, 2015; Wallace et al., 2016).

1.1.5 Obesity and platelet function

Obesity-related inflammation, oxidative stress, dyslipidaemia, and ectopic fat accumulation may affect platelet reactivity and function, leading to the development of both arterial and venous thrombosis (Anfossi et al., 2009; Morange & Alessi, 2013; Samad & Ruf, 2013). Also, individuals with obesity have a lower response to preventive anticoagulants therapies (Anfossi et al., 2009; Bordeaux et al., 2010; Keating et al., 2013; Tamminen et al., 2003) which may increase susceptibility to cardiovascular events (Kornblith et al., 2015; Marcucci et al., 2009).

From the early stages of their development, platelets are affected by adipocyte dysregulation. This is because platelets originate from precursor cells called megakaryocytes. Megakaryocytes reside in a specialized environment inside the bone marrow that directs their differentiation (Figure 1.2) (Wang et al., 2018; Naveiras et al., 2009). The bone marrow is a niche for hematopoietic stem cell differentiation in which adipocytes exert an inhibitory role on haematopoiesis (Anthony & Link, 2014; Naveiras et al., 2009). Further, there is evidence for reciprocal regulation between megakaryocytes and adipocytes as well as with other cell types (Adler et al., 2014; Malara et al., 2015). Therefore, it is not surprising that megakaryocytes, in synchrony with platelets, show altered function during inflammation and obesity (Beaulieu et al., 2014; Gerrits et al., 2012; Upadhyay, 2015), implying that platelet

development itself can be distorted by metabolic imbalances. For all these reasons, platelets have a tangible link with the physiological changes occurring in obesity and may be useful for prognostic evaluation of CVD risk (Bacos et al., 2016; Keating et al., 2013; Samad & Ruf, 2013; Wallace et al., 2016; Vilahur et al., 2017) (Figure 1.2 and 1.3 top panel).
Finally, platelet count, function and biochemistry change during ageing (Balduini & Noris, 2014; Cowman et al., 2015; Jones, 2016). Specifically, these modifications lead to the higher reactivity of platelets, which may contribute to the higher incidence of thrombotic events in older people (Cowman et al., 2015; Verdoia et al., 2016).

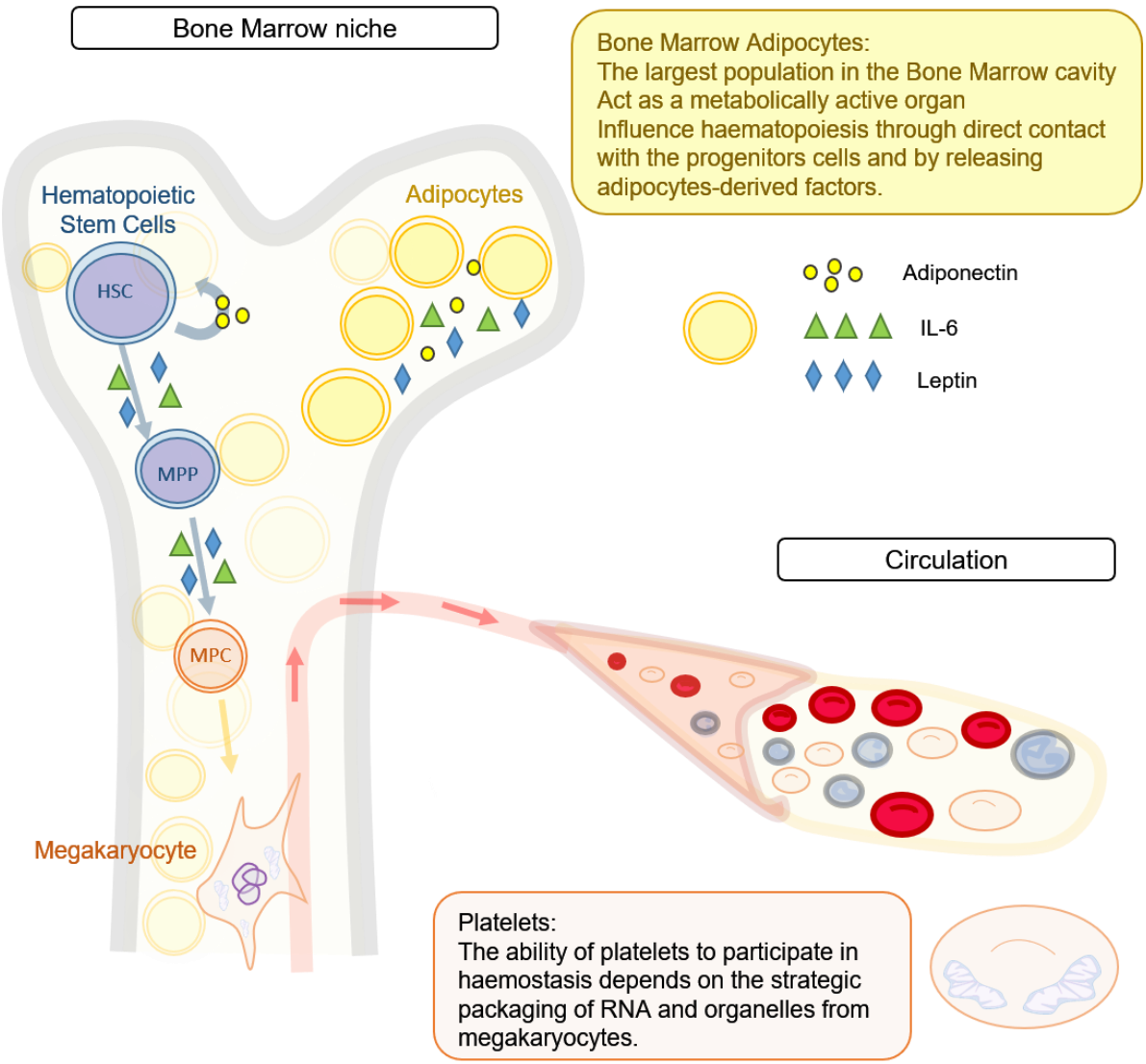


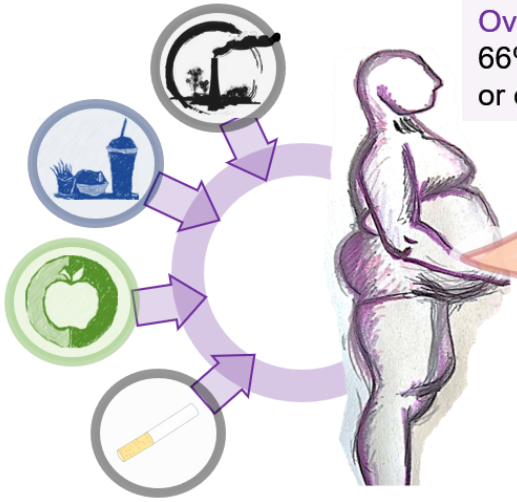
Figure 1.2 Bone Marrow niche, adipocytes and haematopoiesis

This image summarises the influence of Bone Marrow (BM) adipocytes on the differentiation of hematopoietic stem cells into megakaryocytes first, and then into platelets. Adipocytes are the main population of cells inside the BM niche and are in direct contact with the Hematopoietic Stem Cells (HSC), the progenitor cells, and the megakaryocytes.

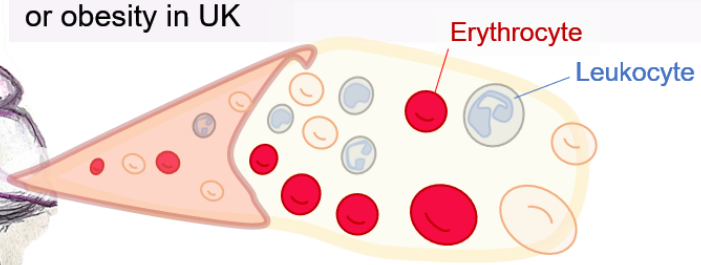
Apart from the direct regulation on megakaryocytes differentiation, adipocytes have also an indirect effect by releasing, among others, adiponectin, IL-6, and Leptin (Wang et al., 2018).

HSC: Hematopoietic Stem cells; MPP: Multipotent Progenitor; MPC: Myeloid Progenitor Cell; Yellow circles: adiponectin; Green triangles: IL-6; Blue rhombus: Leptin.

End of figure legend.



Overweight and Obesity
66% men and 58% of the women have overweight or obesity in UK

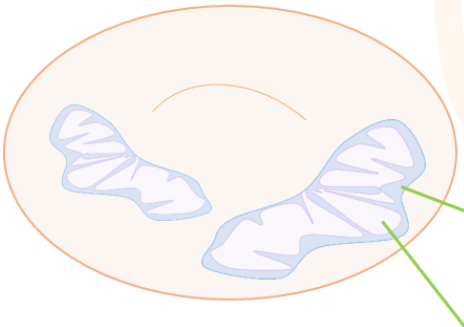


Platelets
Participate in haemostasis
Central role in inflammation
Integrate immune response

Environmental exposure and lifestyle
Highly contribute to the development and progression of CVD

Platelets marker of CVD risk
Greater reactivity in adults with obesity
Less sensitive to anticoagulant drug in adult with obesity

No nucleus
Contains ~2 mitochondria



Mitochondria
Mitochondrial dysfunction: ageing & development of CVD
Regulate Platelets apoptosis
Supply ATP for primary platelets function

Mitochondrial DNA

16,569 bp
No histones
Many copies
DNA repair system is less efficient

MtDNA methylation
Is altered by exposure and diseases
Is a good indicator of CVD risk

MtDNA
Encode for 13 ETC proteins
MtDNA particularly sensitive to environment stress
5-Methylcytosine (altered in adults with CVD)
Independent methylation machinery

Figure 1.3 Potential role of platelets in obesity and the development of cardiovascular diseases.

Environmental pollution and poor lifestyle influence the development of CVD and intermediate phenotypes such as obesity (Bloom et al., 2011). Therefore, the CVD burden is largely preventable (Meier et al., 2019) by modifying the environment and lifestyle (Cosselman et al., 2015). Overweight and obesity are intermediate risk factors for CVD, and, in the UK, individuals with overweight and obesity are the majority of the adult population (CRUK, 2016). A biomarker is required to stratify the population with overweight and obesity between those at higher risk of developing CVD from those who are not. During my study, I investigated a platelet biomarker.

In fact, platelets show hyperaggregability in adults with obesity and are less responsive to anticoagulant drugs – the best available therapy to prevent CVD (Bordeaux et al., 2010; Vilahur et al., 2017). Interestingly, platelets mitochondria have been reported to be dysregulated in those with CVD (Melchinger et al., 2019). More specifically, the mitochondrial DNA is particularly sensitive to ROS and can be used to monitor the exposure and disease progression (Breton et al., 2019).

End of figure legend.

1.2 DNA methylation and CVD risk

1.2.1 Epigenetics

Epigenetics is the science of the marks and molecules which modify DNA accessibility and therefore transcription (Goldberg et al., 2007). This activation or inactivation of genes is highly dynamic (Breton et al., 2017a). Epigenetic mechanisms act primarily as a developmental and adaptive tool which allows the cell to meet its functional requirements and to respond appropriately to the ever-changing environment (Breton et al., 2017b; Gabory et al., 2011). “Epigenetic” refers to any modification of DNA accessibility that affects its transcription. Such modifications can happen at different levels (e.g. chromatin structure of DNA or methylation of DNA double helix). One way the cells use to regulate DNA accessibility and transcription is by modifying the chromatin structure. Chromatin is the complex of DNA and proteins that allows DNA to be packaged appropriately within the nucleus. Chromatin exists in two main forms i.e. euchromatin that is more relaxed and transcriptionally active and heterochromatin that is more compact and transcriptionally silent

(Bannister & Kouzarides, 2011). Chromatin compaction is mostly achieved by modifying the charge on the histones. Histones are a family of basic (alkaline) proteins that pack the DNA double-strand into structures called nucleosomes that can be further packed in more complex structures called "fibers"(Ricci et al., 2015). Histones have long N-terminal tails which are rich in Lysine. Lysines make the histone tails highly basic and this allows a strong interaction with the negatively-charged DNA. Histone tails can be modified post-translationally to relax or compact the chromatin structure. For example, by acetylating lysines the histone tails become "neutral" and this facilitates DNA access. By phosphorylating serines, threonines, and tyrosines, the tails acquire a negative charge that relaxes the tail-DNA interactions (Bannister & Kouzarides, 2011). Further, it has been suggested that histones' methylation is involved in the compaction of the chromatin (Karymov et al., 2001).
Interestingly, methylation occurs not only on the histones around which DNA is wrapped within nucleosomes but also on the DNA itself.

1.2.1.1 DNA methylation

During my studies, I focused on DNA methylation which involves the covalent addition of a methyl group to a cytosine residue. The DNA methylation status of the cell is influenced by age, diseases, and remarkably diet (Baccarelli & Bollati, 2009; Gautrey et al., 2014; Jaenisch & Bird, 2003; Mathers & Byun, 2016; Perna et al., 2016; Quach et al., 2017).

1.2.2 Role of DNMT and TET enzymes

In mammalian cells, methylation of cytosine occurs mostly at a cytosine-guanine dinucleotide or "CpG position". The covalent addition of a methyl group to the 5' position of the cytosine residue is mediated by a family of enzymes called DNA methyltransferases (DNMT) (Moore et al., 2013). This process is highly dynamic and depends on the availability of the universal methyl donor S-adenosyl methionine (SAM) and the regulation of DNMT (Sae-Lee et al., 2018; Link et al., 2013; Zhang, 2015; Burdge & Lillycrop, 2010). In fact, once DNA is *de novo* methylated, this mark can be maintained during replication (maintenance methylation) or not (passive demethylation). In addition, 5mC can be actively demethylated. Active demethylation is a highly regulated process mediated by a class of enzymes called ten-eleven translocations (TET). During this process, 5mC is first oxidised to 5-hydroxymethylcytosine (5hmC), which is further oxidised to 5-formylcytosine (5fC) (Moore et al., 2013). The intermediates 5hmC and 5fC are not only indicative of active demethylation but are also epigenetic marks in their own right and may have a functional role (Zhang & Zhou, 2019; Leenen et al., 2016a). Indeed, the patterns of 5hmC and 5fC in DNA change with ageing and

environmental exposures (Figure 1.4) (Barrow et al., 2018; Zampieri et al., 2015). Because bisulfite sequencing, the gold standard for methylation analysis, does not distinguish between 5mC and 5hmC, antibody-based techniques have been used to measure global concentrations of 5hmC and 5fC (Barrow et al., 2018; Shock et al., 2011).

Evidence suggests that DNMT1 has a "mitochondrial target sequence" (MTS), and that it translocates into the mitochondrial compartment where it interacts directly with the mtDNA (Shock et al., 2011; Saini et al., 2017). The presence of DNMT and TET enzymes in the mitochondria support the presence of mtDNA methylation as a tightly regulated epigenetic process, as summarised in figure 1.4.

Transcription

TFAM

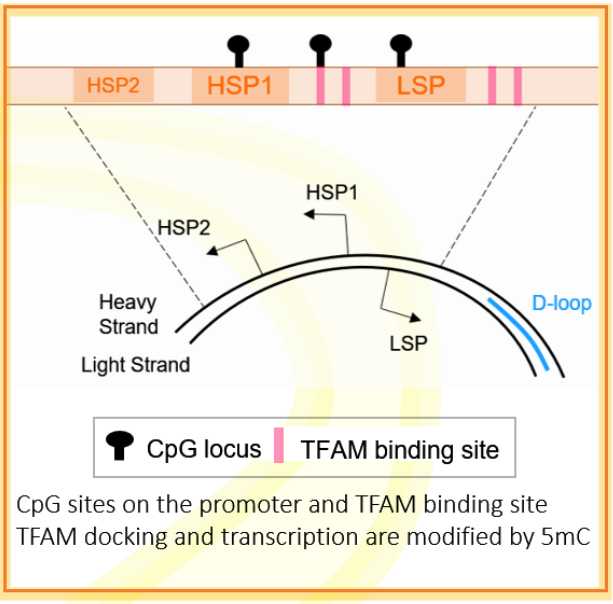
It has been suggested that, **TFAM** binding to the promoters LSP and HSP1 is partially regulated by 5mC

Dostal and Churchill 2019

Cytoprotection

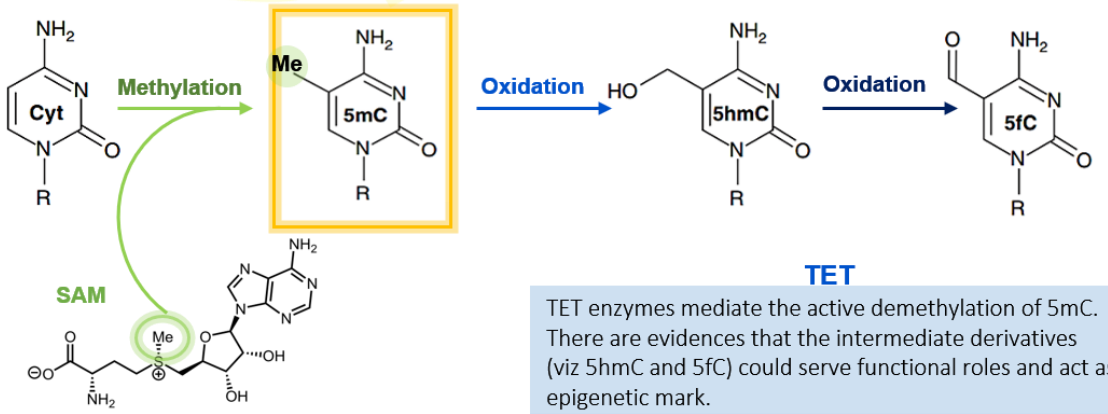
MDP

It has been suggested that, Mitochondrially-derived peptides (**MDP**) are partially regulated by D-loop methylation Breton et al 2019



CpG sites on the promoter and TFAM binding site TFAM docking and transcription are modified by 5mC

5mC is present in the mitochondria, ad its levels are altered by CVD and environmental exposure.



DNMT

DNA methyl transferase enzymes catalyse the addition of methyl group from the universal donor S-adenosylmethionine (SAM).

DNMT presence and activity has been confirmed inside the mitochondria.

DNMT activity and **SAM availability** can be modulated by diet (e.g. polyphenols)

TET

TET enzymes mediate the active demethylation of 5mC. There are evidences that the intermediate derivatives (viz 5hmC and 5fC) could serve functional roles and act as epigenetic mark.

TETs' presence has been confirmed inside the mitochondria.

5hmC

Low level of 5hmC
Gene expression regulation
5hmC has been recorded inside the mitochondria

5fC

Very low level of 5fC
Enriched at poised enhancers
DNA-protein interactions

Present inside mitochondria

Unknown

Figure 1.4 Methylation and demethylation of cytosine.

In the central part of this image, it is depicted the methylation and demethylation of Cytosine. First, the enzyme DNMT catalyses the methylation of cytosine to 5-methylcytosine (5mC) using a methyl group from the universal methyl-donor S-Adenosyl Methionine (SAM). 5mC can be oxidized to 5hmC, which is an epigenetic mark of its own. Finally, 5hmC can be further oxidized by TET to 5fC. Both 5mC and 5hmC have been reported within the mitochondria (Shock et al., 2011). DNMT, the enzyme responsible for active methylation, and TET, the enzyme responsible for active demethylation, have been reported to relocate inside the mitochondria (Shock et al., 2011; Saini et al., 2017; Bellizzi et al., 2013). This suggests that mtDNA methylation is a tightly regulated process with a biological function. In the image, the "presence inside the mitochondria" is indicated by the violet rectangles and the mitochondria icon.

Intriguingly, the activity of DNMT and the availability of SAM can be modulated by diet, as shown in the lower-left corner in green (Link et al., 2013; Jia et al., 2016). The biological function of 5mC in mtDNA is unknown. However some studies suggest that i) 5mC modifies the docking of Mitochondrial transcription factor A (TFAM) at promoters and, possibly, mtDNA transcription (Dostal & Churchill, 2019) and ii) methylation is associated with mitochondrial-derived peptides (MDPs) (Breton et al., 2019). Intriguingly, these MDPs are involved in cytoprotection, insulin sensitivity regulation and the stress response (Lee et al., 2015; Okada et al., 2017; Breton et al., 2019).

HSP1: Heavy-Strand Promoter1; HSP2: Heavy-Strand Promoter2; LSP; Light-Strand Promoter; TFAM: Mitochondrial transcription factor A; MDP: Mitochondrially-derived peptides; Purple background with mitochondria icon: means "present inside the mitochondria"; blackened mitochondria icon: it has not been confirmed inside the mitochondria.

End of figure legend.

1.2.3 DNA methylation and ageing

Epigenetics alterations have been proposed as one of the primary hallmarks of ageing (López-Otín et al., 2013). In addition, there is growing support for the idea that alterations in DNA methylation at specific loci may be useful as an age estimator (Bell et al., 2012).

The methylation status of panels of CpG positions may be used to estimate the "methylation age" of individuals (DNAm age), which captures not just chronological age but potentially biological age, and which predicts all-cause mortality in later life (Hannum et al., 2013; Marioni et al., 2015; Quach et al., 2017). Horvath identified a collection of 353 CpG positions, whose methylation status varied systematically with the age, irrespectively of sex and tissue type (Horvath, 2013). This pattern of CpG methylation is replicated across a spectrum of cell types in healthy individuals and this provides evidence that estimates of DNAm age are independent of the cell type and its life span. For example, estimates of DNA methylation obtained from monocytes that live a few weeks and neurons that can live for years equally reflect the chronological age of the individual, rather than of the cell type. Horvath suggested that methylation status at these CpG positions records the work of an "Epigenetic maintenance system", whose efficacy depends on genetic, environmental and stochastic factors (Horvath, 2013). Accordingly, DNAm age can be accelerated by perturbations that affect epigenetic stability, such as cancer and diseases (Horvath, 2013; Maierhofer et al., 2017; Perna et al., 2016; Y. Zheng et al., 2016) and slowed down in semi-supercentenarians and their offspring (Horvath et al., 2015) and in those with healthier lifestyles (Quach et al., 2017). Interestingly, Horvath et al also developed "DNAm PhenoAge" and "DNAm GrimAge", which are both associated with metabolic syndrome other than lifespan and healthspan (Levine et al., 2018; Lu, Quach, et al., 2019). Additionally, the same group developed a DNA methylation-based estimator of telomere length (DNAm TL), which can predict cardiovascular diseases incidence and it is associated with age, smoking history, physical fitness, and dietary variables (Lu, Seeboth, et al., 2019).
For all these reasons, epigenetics - and especially DNA methylation - is considered one of the best indicators of exposure and a predictor of the development of the resulting phenotype, disease, or ageing (Figure 1.1).
Furthermore, diet and lifestyle are major modifiers of the ageing process and metabolic risk (Bacalini et al., 2017; Danaei et al., 2009; Zhong et al., 2017). These factors have multiple mechanisms of action that are likely to include the induction of epigenetic modifications (Figure 1.4) (Bollati, Favero, et al., 2014; Caple et al., 2010; Fontana et al., 2010; Quach et al., 2017; Gensous et al., 2020; Sae-Lee et al., 2018).

1.2.4 DNA methylation and obesity

Obesity is associated with acceleration of the epigenetic clock both in liver tissue and blood (Horvath et al., 2014; Nevalainen et al., 2017; Rönn et al., 2015; van Dijk et al., 2015), which adds to the strong correlation between BMI and patterns of DNA methylation in the blood

(Marioni et al., 2015; Nevalainen et al., 2017; Perna et al., 2016; Quach et al., 2017; Wahl et al., 2017). Intriguingly, this methylation signature can be reversed by weight loss, physical activity and healthier diet (Milagro et al., 2011; Rönn et al., 2015; Gensous et al., 2020; Fraszczyk et al., 2020).

Recently, the development of techniques that allow extensive [e.g. Illumina HM 450K (Horvath, 2013) and 850K (Zaimi et al., 2018)] and precise [e.g. pyrosequencing (Tost & Gut, 2007)] quantification of patterns of methylation across the genome has enabled the thorough investigation of DNA methylation signatures. The obesity-driven DNA methylation pattern, its amelioration through diet and physical activity, and even the influence of maternal diet on DNA methylation status can be detected using blood cells (Martínez et al., 2014; Milagro et al., 2011; Van Dijk et al., 2015). Therefore, blood-based DNA methylation has emerged as a biomarker with predictive potential, with the ability to guide therapeutic interventions and to individualize treatments (Milagro et al., 2011; Martínez et al., 2014; Rönn et al., 2015; Van Dijk et al., 2015).

1.2.5 DNA methylation and CVD risk

Epigenetics – and especially DNA methylation – has emerged as an important biomarker as it captures the links between genetic predisposition, modifiable exposure and diseases phenotypes (Figure 1.1)(Zhong et al., 2016).

The associations between "environmental factors → DNA methylation → CVD outcome" have been proven for several CpG sites. For example, Ma et al demonstrated that CpG sites, whose methylation is associated with diet, are correlated with CVD risk and all-cause mortality (Ma et al., 2020). Kazmi et al found that differentially methylated regions in blood DNA are associated with high blood pressure, and these associations are different in different ethnic groups (Kazmi et al., 2020). Westerman et al demonstrated that DNA methylation markers can add information to the common CVD risk factors and help to stratify the population according to individual risk (Westerman et al., 2020; Westerman & Ordovás, 2020).

Overall, these findings support the ability of DNA methylation markers to capture the interplay between genetic and environmental exposure, which is critical for the development of CVD. This is important for several reasons: first, DNA methylation markers are potentially modifiable, and secondly, they may reveal the underlying molecular mechanisms (Westerman & Ordovás, 2020; Ma et al., 2020; Tremblay et al., 2017).

1.3 Mitochondria

1.3.1 Mitochondrial structure and function

Mitochondria are organelles localised in the cytoplasm of eukaryotic cells. The main function of the mitochondria is to produce ATP through oxidation of nutrients in a process called "oxidative phosphorylation" (OXPHOS), that couples substrate oxidation with ADP phosphorylation. Within the mitochondrial matrix, the oxidation of sugars - also called the Krebs' cycle or citric acid cycle - amino acids, and fatty acids generate the electron motive force, and ultimately ATP production (Osellame et al., 2012). The majority of proteins (~1500) involved in the mitochondrial bioenergetics are encoded in the nuclear DNA; however, 13 proteins fundamental for the electron transport chain (ETC) are encoded in the mitochondrial DNA (Andrews et al., 1999a; Palmfeldt & Bross, 2017; Pfanner et al., 2019). Human mitochondria contain a small circular DNA, called mitochondrial DNA (mtDNA), of ~16,569 bp (Andrews et al., 1999a) that encodes 22 tRNAs, 13 proteins, 2 rRNAs, several "mitochondrial-derived peptides" (MDP), and non-coding RNAs (Breton et al., 2019; Dong et al., 2020; Ro et al., 2013). The mitochondrially-encoded proteins are involved in ATP production. The mitochondrially-encoded tRNAs and rRNAs are essential for mtDNA translation (D'Souza & Minczuk, 2018). The MDPs encoded in recently-discovered open reading frames (ORFs) (Breton et al., 2019) appear to have distinct biological activities, such as cytoprotection and insulin sensitivity regulation, and they are associated with prenatal exposures (Okada et al., 2017; Lee et al., 2015; Breton et al., 2019).

1.3.2 Mitochondrial DNA

Mitochondrial DNA is a circular double strand-molecule made up by 16,569 base pairs (Figure 1.5 and Table 1.1)(Andrews et al., 1999a). Coherent with its bacterial origins, mtDNA is polyploid; each mitochondrion contains 2-10 copies of mtDNA (Wiesner et al., 1992), and is gene-dense, with no introns and little non-coding DNA. Additionally, mtDNA encodes its own transcription machinery and there are alterations in the universal genetic code (D'Souza & Minczuk, 2018). For example, within human mitochondria, the codons "UGA" and "AUA", which encode for tryptophan and methionine respectively, do not correspond with the universal code valid for nuclear DNA (nDNA) (NCBI, 2019).

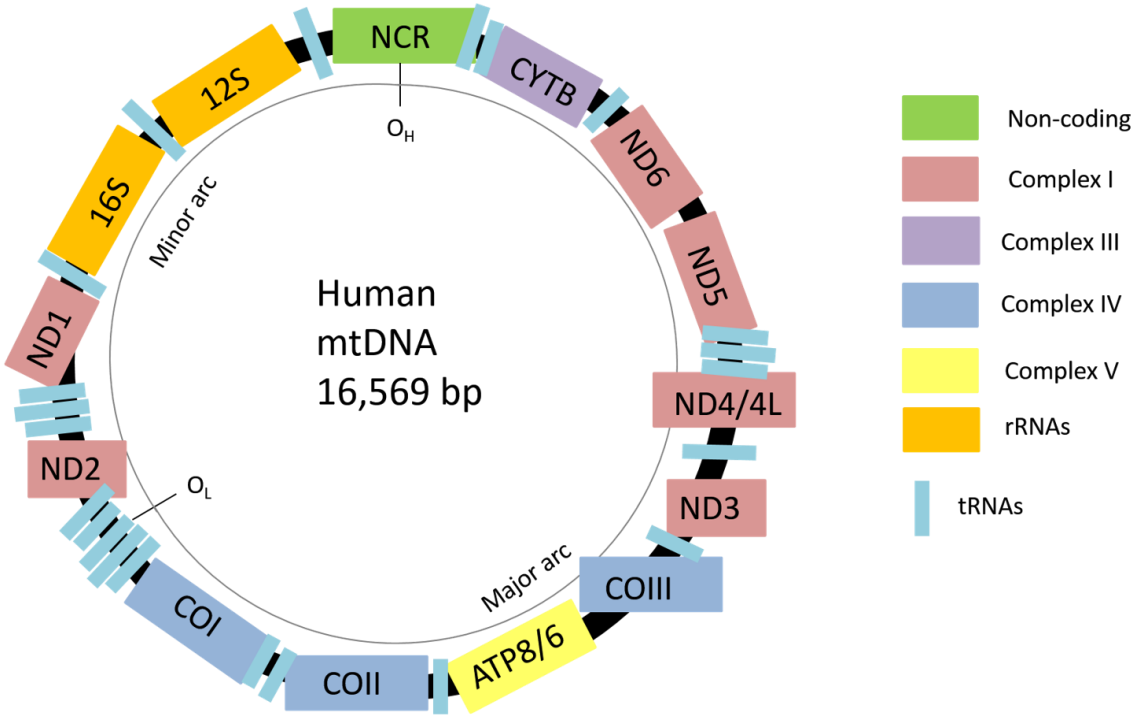


Figure 1.5 Mitochondrial DNA

Mitochondrial DNA map with gene annotation, the origin of replication for the heavy and light strand (O_H and O_L). The green area is also called D-loop. In this image, the Minor arc and Major arc are annotated.

Origin of replication for the heavy and light strand (O_H and O_L); Non-coding region (NCR) green colour; Complex III Cytochrome B (CYTB) purple colour; Complex I NADH dehydrogenase (ND) red colour; Complex IV Cytochrome C Oxidase (CO) blue colour; Complex V ATP-Synthase (ATP) yellow colour; ribosomal RNA (rRNA) orange colour; transfer RNA (tRNA) genes light blue bars.

Modified from (Nicholls & Minczuk, 2014)

Unlike nuclear DNA, mitochondria do not have histones. Instead, the mtDNA is compacted into nucleoids by the protein TFAM (Gustafsson et al., 2016; Dong et al., 2020). TFAM binds the mtDNA both with and without sequence specificity and has multiple functions including i) a structural role, ii) it coordinates mtDNA maintenance and gene expression (Kang et al., 2007; Dong et al., 2020), iii) it is the primary transcription initiation factor (Gustafsson et al., 2016), and iv) it is the regulator of the mtDNA copy number (Ekstrand et al., 2004). Recently, it has been suggested that the presence of 5-methylcytosine can alter TFAM binding *in vitro* and therefore regulate mtDNA recognition, compaction, and transcription (Dostal & Churchill, 2019). Interestingly, in the promoter regions of mtDNA, there are several CpG sites. For example, the "Light Strand Promoter" (*MT-LSP*) has one CpG site in its sequence:

"TTTCAAATTTTATCTTTTGGCGGTATGCACTTTTAACAGTCACCCCCCAACTAAC" , the "transcription factor binding site H" (*MT-TFH*) has two CpG site in its sequence: "ACCGCTGCTAACCCCATACCCCGAACCA", and the "Heavy Strand Promoter1" (*MT-HSP1*) has one CpG site in its sequence: "CGAACCAACCAAACCCCAAAGAC" (Figure 1.4) (Andrews et al., 1999b; Lott et al., 2013). Therefore, the presence of 5mC in these loci has the potential to regulate TFAM-dependent transcription and, indirectly, gene expression.

Feature	Nuclear DNA	Mitochondrial DNA
Size (in bp)	~3 x 10^9	16,569
Shape	Linear double helix	Circular double helix
Inheritance	Both parents	Maternal
DNA copies/ cell	2	~ 10 – 50,000 *
Number of genes	~ 20,000 to 25,000 protein-coding	13 protein-coding genes 24 non-protein-coding ORFs that codes for MDPs #
Gene density	~ 1 in 40,000 bp	1 in 450 bp
Introns	Found in almost any gene	Absent
% coding DNA	~3%	~93%
Histones	Associated with the DNA	TFAM †
CpG islands	24,000-27,000	None§
CpG density	1%	2.6% ‡
Methylation	Present (3-4% of all Cs (~70-80% of all CpGs))	Present (~ 1.5-5% of all Cs) (both CpG and non-CpG)
Hydroxymethylation	Present	Present ‖

Table 1.1 Comparison between nuclear DNA and mitochondrial DNA

This table was made using information from (Kazak et al., 2012; Lambertini & Byun, 2016; Mposhi et al., 2017; Wijst et al., 2017).

**(Clay Montier et al., 2009) † (Dostal & Churchill, 2019) ‡(Lambertini & Byun, 2016; McClelland & Ivarie, 1982) §(Mposhi et al., 2017; Shmookler Reis & Goldstein, 1983; Wijst et al., 2017), ‖(Bellizzi et al., 2013; Dzitoyeva et al., 2012a; Ghosh et al., 2016; Shock et al., 2011), # (Okada et al., 2017; Lee et al., 2015; Breton et al., 2019).*

1.3.2.1 Mitochondrial DNA replication

MtDNA replication is accomplished by a multi-protein replisome. So far the complete replication machinery and the mechanism of mtDNA replication have not been fully reconstructed *In vivo* (Fontana & Gahlon, 2020; Korhonen et al., 2003; Falkenberg, 2018; Uhler & Falkenberg, 2015). However, the minimal essential replication machinery sufficient for *in vitro* replication is composed of the mtDNA polymerase γ (Polγ), the Twinkle helicase that unwinds the double-helix, the mtDNA single-strand DNA binding proteins (mtSSB) that stabilize the single helix, and the DNA-dependent RNA polymerase (POLMRT), which provides the two RNA primers (Falkenberg, 2018; Fontana & Gahlon, 2020).
Despite uncertainties about replication mechanisms, it has been ascertained that mtDNA replication starts from the non-coding region (NCR) within the D-loop (Figure 1.5). In the NCR there are two promoters for two polycistronic transcripts, the H-strand promoter (HSP) and the L-strand promoter (LSP). Additionally, the origin of replication for the H strand (O_H) is located in the NCR, while the origin of replication for the L strand is located outside the NCR at position (nt5721-nt5798). Interestingly, some of the replication events started from O_H (nt441-nt110) end at the termination-associated sequence (TAS) (nt16157-nt16162), depicted in yellow in figure 1.NEW. This replication product is called 7S-DNA and remain bound to the parental L-strand to form the D-loop, in which the H-strand is displaced. The D-loop is therefore formed by a "triple strand" with the L-strand nucleotides bonded to the 7D-DNA and the H-strand on top. The D-loop structure represents an "open conformation" that has been hypothesized to regulate initiation of H-strand replication and transcription (Falkenberg, 2018; Nicholls & Minczuk, 2014; Podlesniy et al., 2019). Since the replication and the transcription machinery are not compatible, it has been hypothesized that the D-loop may act as a switch between replication and transcription (Podlesniy et al., 2019).

1.3.2.2 Mitochondrial DNA transcription

The mitochondrial genome encodes 13 proteins that are essential for the electron transport chain. MtDNA transcription is initiated at the two promoters HSP and LSP within the NCR (Figure 1.4, 1.5 and 1.NEW) and requires three proteins TFAM, POLMRT, and mitochondrial transcription factor B2 (TFAMB2). First TFAM, which is bound to the mtDNA within the promoter regions, recruits POLMRT and then TFB2M binds POLMRT to modify its structure and open up the promoter (D'Souza & Minczuk, 2018). At this point, the transcription elongation factor (TEFM) is used by POLMRT to generate the long polycistronic segment of RNA called the "primary transcript". These transcripts undergo endonucleolytic excision by the mtRNase P and polyadenylation by polyadenylic-Acid RNA polymerase (mtPAP). At this

point, mt-tRNA undergo post-transcriptional maturation including several nucleotide modificationsafter which it is assembled with proteins imported from the cytoplasm (Figure 1.NEW C). Translation of polyadenylated mt-mRNA is dependent on nuclear-encoded proteins including mitochondrial initiation factors (mtIF2 and mtIF3), mitochondrial elongation factors (EFTu, EFTs, and EFGM), and ribosome release factors (mtRF1, mtRF1a, and ICT1) that are responsible for transcription termination (Figure 1.NEW) (D'Souza & Minczuk, 2018).

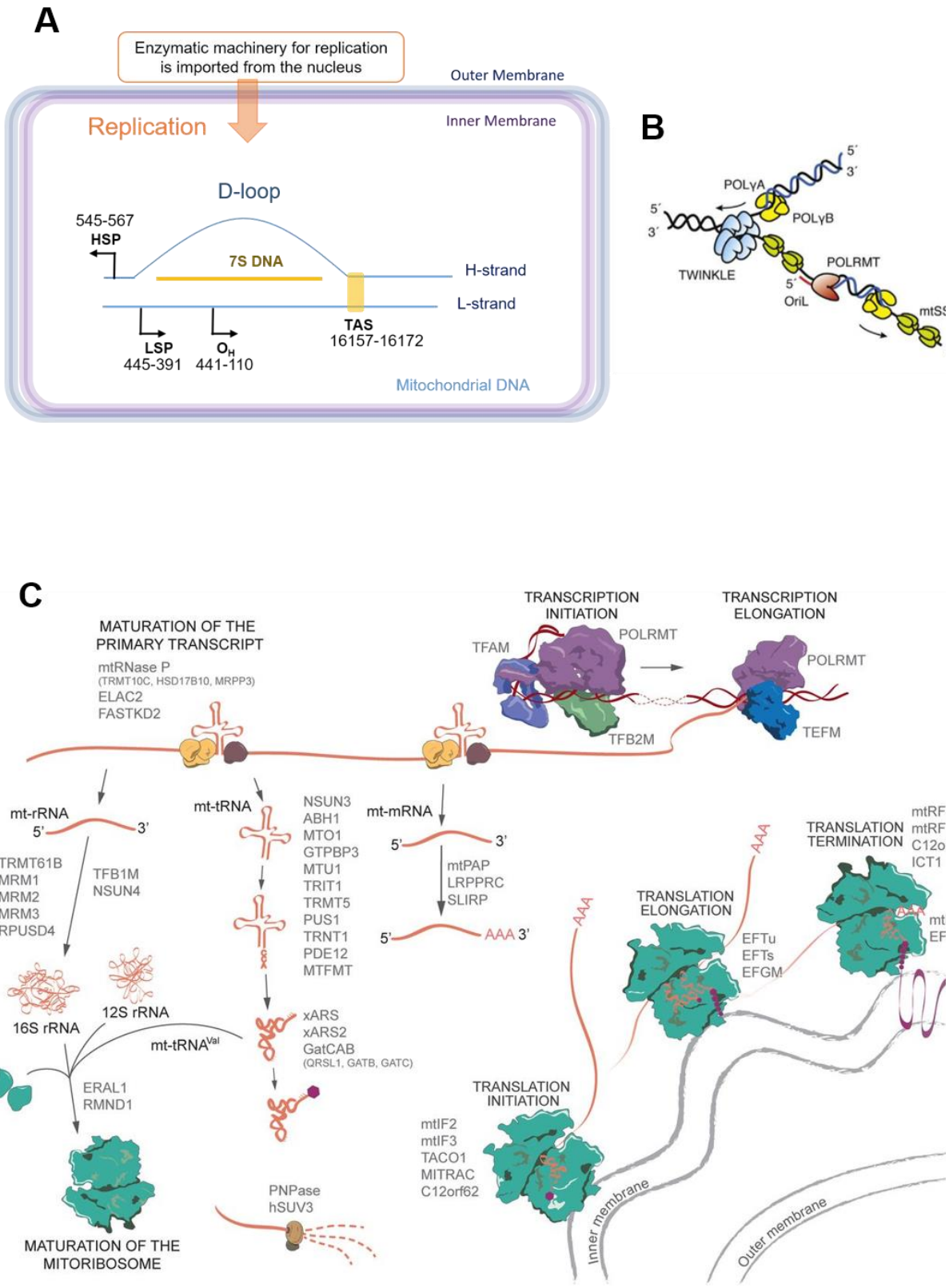

A
Enzymatic machinery for replication is imported from the nucleus
Outer Membrane
Inner Membrane
Replication
D-loop
545-567
HSP
7S DNA
H-strand
L-strand
TAS
16157-16172
LSP
445-391
O_H
441-110
Mitochondrial DNA
B
POLγA
POLγB
TWINKLE
POLRMT
OriL
C
MATURATION OF THE PRIMARY TRANSCRIPT
mtRNase P
(TRMT10C, HSD17B10, MRPP3)
ELAC2
FASTKD2
TRANSCRIPTION INITIATION
TFAM
POLRMT
TFB2M
TRANSCRIPTION ELONGATION
POLRMT
TEFM
mt-rRNA
TRMT61B
MRM1
MRM2
MRM3
RPUSD4
TFB1M
NSUN4
16S rRNA
12S rRNA
mt-tRNA^Val
ERAL1
RMND1
mt-tRNA
NSUN3
ABH1
MTO1
GTPBP3
MTU1
TRIT1
TRMT5
PUS1
TRNT1
PDE12
MTFMT
xARS
xARS2
GatCAB
(QRSL1, GATB, GATC)
PNPase
hSUV3
mt-mRNA
mtPAP
LRPPRC
SLIRP
AAA
TRANSLATION INITIATION
mtIF2
mtIF3
TACO1
MITRAC
C12orf62
TRANSLATION ELONGATION
EFTu
EFTs
EFGM
TRANSLATION TERMINATION
mtRF
mtRF
C12o
ICT1
Inner membrane
Outer membrane
MATURATION OF THE MITORIBOSOME

Figure 1.6 Mitochondrial DNA replication and transcription

A) mtDNA D-loop structure. This image represents the non-coding region (NCR) of the mtDNA. In the non-coding region, there are two promoters: the H-strand promoter (HSP) and the L-strand promoter (LSP). There is one origin of replication: H-strand origin of replication (O_H).

B) mtDNA replication fork. The minimal replication machinery requires Pol γ, Twinkle helicase (blue hexamer), POLRMT (red), and the mtSSB (green). The DNA polymerase γ (yellow) is composed of two subunits: the monomer POLγA and the dimer POLγB. The template DNA is illustrated in black, nascent DNA in blue and RNA primer in red at the Origin of replication.

Modified from (Uhler & Falkenberg, 2015).

C) mtDNA transcription. The minimum transcription machinery is composed of TFAM, POLRMT, and TFB2M. Then TEFM is required for the elongation step and allows POLRMT to generate the transcript.

The mtDNA is transcribed in two long polycistronic transcripts called "primary transcripts" that require maturation. The maturation of the primary transcript requires first an endonucleotytic cleavage performed by mtRNA P, then the polyadeniylation by mtPAP. At this point, mt-rRNA and mt-tRNA undergo chemical modifications, while the protein-coding RNA undergo translation.

Modified from (D'Souza & Minczuk, 2018)

1.3.3 Mitochondria in CVD and ageing

The lack of a protective "nuclear membrane", the absence of histones, and the localization of mtDNA in close proximity to the ROS-generating electron-transport chain are responsible for the higher mutation rate of mtDNA in comparison with nuclear DNA (López-Otín et al., 2013). Additionally, polymerase gamma (POLG) – the polymerase responsible for copying mitochondrial DNA - has reduced fidelity and may also contribute to the higher rate of mtDNA mutagenesis (Figure 1.3) (Song et al., 2005). Usually, defective mtDNA copies can be compensated for by other mtDNA molecules without causing mitochondrial dysfunction. However, this leads to the coexistence of different version of the mtDNA within the same cell (Greaves et al., 2014). Different mutations can arise and can be maintained in small proportions, creating a mosaic of different sequences called "heteroplasmy". Heteroplasmy

has been associated with diseases, and especially age-related diseases, as the mutations in mtDNA accumulate with time (Zsurka et al., 2018; Wallace & Chalkia, 2013; Greaves et al., 2014).

Coherently, dysfunctional mitochondria have been classified as a hallmark of ageing and CVD (López-Otín et al., 2013; Bray & Ballinger, 2017; Greaves et al., 2010; Hudson et al., 2014; Nooteboom et al., 2010; Seo et al., 2010; Yu et al., 2012). Since mitochondria orchestrate a plethora of processes including Ca2+ homeostasis, tightly regulated redox signalling, nDNA gene expression via nuclear–mitochondria crosstalk, and arbitration of cell survival and death (Yu et al., 2012; Shaughnessy et al., 2014; Diot et al., 2016), it is of paramount importance that sufficient mtDNA remains intact (~ 40% of intact mtDNA) (Stewart & Chinnery, 2015). As noted above, mtDNA is essential for cellular function because it encodes exclusively some of the proteins of the respiratory chain (Kazak et al., 2012). Consequently, it is probable that anything that affects adversely mitochondrial function [including correct mtDNA replication and expression], such as mtDNA mutations, deletions (Fukui & Moraes, 2009; Yu-Wai-Man et al., 2010), and, conceivably, methylation (Zhong et al., 2016), could create a respiratory chain deficiency.

Mitochondria are in the frontline of the cellular response to the environment. Environmental exposure affects mitochondrial especially by increasing ROS (Chew et al., 2020; Leni et al., 2020). This has repercussions not only for mitochondria-mediated mechanisms, such as orchestration cell death and survival but also for mtDNA, as this is not protected within a membrane (Byun et al., 2016). As a result, mitochondrial dysfunctions are well-established players in the development of CVD and CVD comorbidities and of risk factors such as diabetes and obesity (Figure 1.3) (Cheng & Almeida, 2014; Cheng et al., 2014; Osellame et al., 2012; Reczek & Chandel, 2015; Zheng et al., 2015; L. D. Zheng et al., 2016; Yu et al., 2012).

1.3.4 Mitochondria in platelets

Platelets are small (2-3 µm) anucleated cell with a lifespan of 7-10 days (Figure 1.7) (Melchinger et al., 2019; Harker et al., 2000; Leeksma & Cohen, 1955). Mitochondria in platelets are key regulators. In fact, lacking a nucleus, platelets' ability to participate in haemostasis is completely dependent on the strategic packaging of small RNA and organelles created inside the megakaryocytes, above all, mitochondria (Melchinger et al., 2019; L. Wang et al., 2017).

Mitochondria not only orchestrate platelet apoptosis (Leytin et al., 2009; Baaten et al., 2018; Wang et al., 2013), which is fundamental the turnover of dysfunctional platelets, but also

provide the ATP to sustain the morphological and functional changes in platelets during activation and aggregation (Figure 1.7, middle panel) (Iyer & Dayal, 2020; L. Wang et al., 2017). Interestingly, platelet mitochondrial dysfunctions have been associated with metabolic and neurodegenerative diseases, such as diabetes, CVD, Parkinson's, and Alzheimer's disease (L. Wang et al., 2017; Baccarelli & Byun, 2015; Bordeaux et al., 2010; Ježková et al., 2019) (Figure 1.7). For all these reasons, mitochondria in platelets, apart from being accessible, are also are potentially sensitive biomarkers.

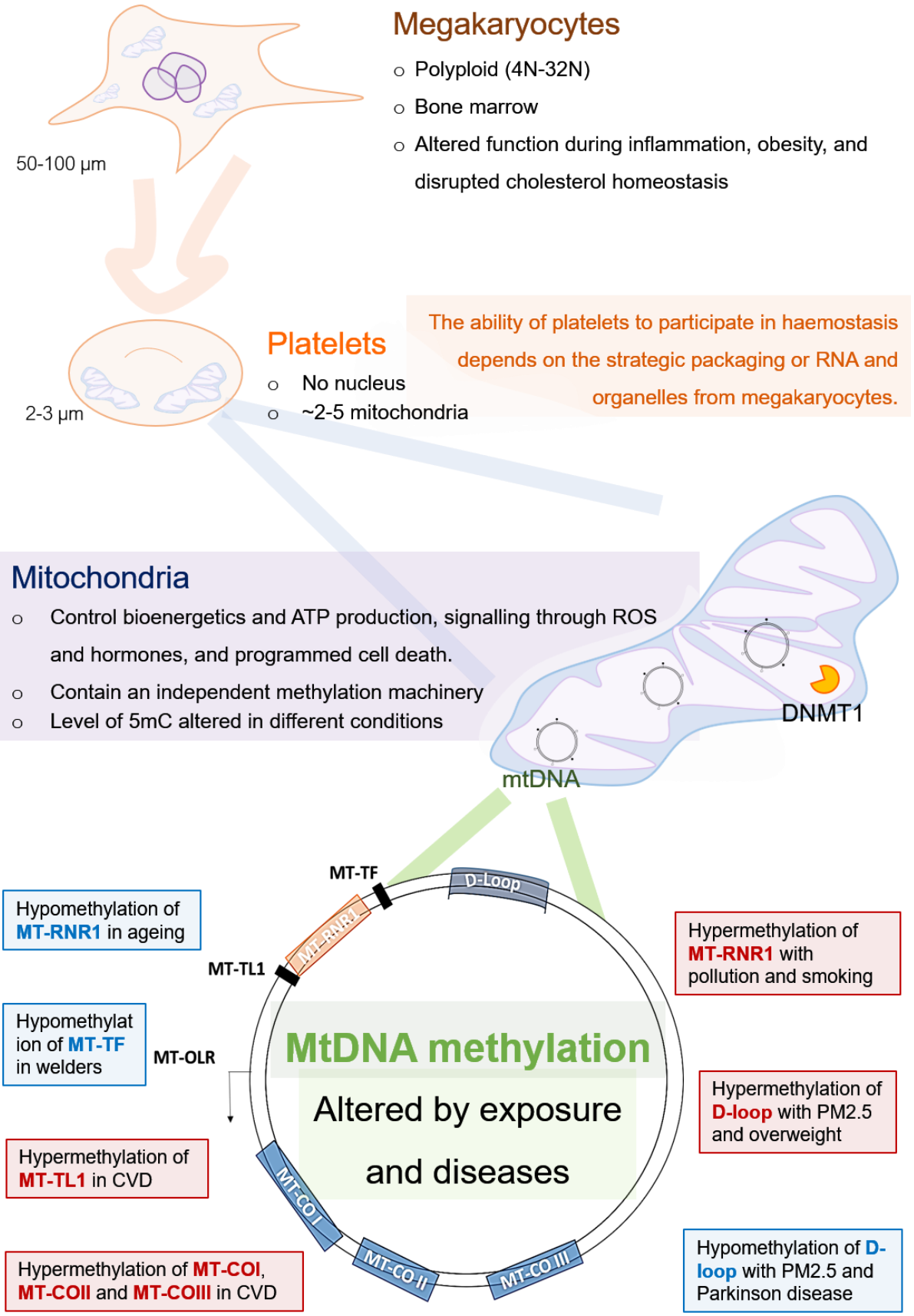

Megakaryocytes
Polyploid (4N-32N)
Bone marrow
Altered function during inflammation, obesity, and disrupted cholesterol homeostasis
50-100 µm
Platelets
No nucleus
~2-5 mitochondria
2-3 µm
The ability of platelets to participate in haemostasis depends on the strategic packaging or RNA and organelles from megakaryocytes.
Mitochondria
Control bioenergetics and ATP production, signalling through ROS and hormones, and programmed cell death.
Contain an independent methylation machinery
Level of 5mC altered in different conditions
DNMT1
mtDNA
MT-TF
D-Loop
MT-RNR1
MT-TL1
MT-OLR
MT-CO I
MT-CO II
MT-CO III
MtDNA methylation
Altered by exposure and diseases
Hypomethylation of MT-RNR1 in ageing
Hypomethylation of MT-TF in welders
Hypermethylation of MT-TL1 in CVD
Hypermethylation of MT-COI, MT-COII and MT-COIII in CVD
Hypermethylation of MT-RNR1 with pollution and smoking
Hypermethylation of D-loop with PM2.5 and overweight
Hypomethylation of D-loop with PM2.5 and Parkinson disease

Figure 1.7 Mitochondria in platelets and mtDNA methylation.

This image summarises the relevance of mitochondria in platelets. Platelets are an anucleated cell type (size 2-3 μm) deriving from megakaryocytes (size 50-100μm). Megakaryocytes undergo a maturation process called thrombopoiesis during which RNA, organelles and granules are assembled in the cytoplasm of megakaryocytes. Then, the terminal ends of the megakaryocytes cytoplasm, called "proplatelets processes", are fragmented into platelets and shed into the BM circulation (Patel et al., 2005). Therefore the mitochondria of platelets are assembled within the megakaryocytes and their ability to participate in haemostasis is dependent on correct megakaryocytes maturation.
Mitochondria in platelets regulate and support the morpho-functional changes that occur during platelet activation and aggregation (Garcia-Souza & Oliveira, 2014; L. Wang et al., 2017). Mitochondria in platelets are dysregulated in metabolic and neurodegenerative diseases (Melchinger et al., 2019; L. Wang et al., 2017; Baccarelli & Byun, 2015).
Human mtDNA methylation level is also altered in people with age-related conditions such as Parkinson and CVD (Baccarelli & Byun, 2015; Blanch et al., 2016). In this image, only studies that use BS-pyrosequencing have been included (complete list of references Table 1.2).
Platelets mtDNA was therefore investigated as an indicator of environmental exposure, lifestyle, and CVD risk in the SPHERE cohort.
Bottom Panel. Light blue rectangles: hypomethylation or reduction in methylation %; red rectangles: hypermethylation or increase in methylation %.
End of figure legend.

1.3.5 Mitochondria, mtDNA and risk of age-related diseases

Given the ability of mitochondria in platelets and mitochondrial DNA to serve as an indicator for exposure and cardiovascular diseases (Baccarelli & Byun, 2015; Lambertini & Byun, 2016; Janssen et al., 2015; Wallace et al., 2016; Vos et al., 2020), the study of mtDNA epigenetic patterns has the potential to provide a robust prognostic biomarker. Recent studies also suggest that mtDNA methylation is associated with clinically relevant outcomes such as newborn birth weight (Vos et al., 2020) and excessive adiposity in adults (Bordoni et al., 2019).

1.4 Mitochondrial epigenetics

1.4.1 MtDNA methylation

The presence of 5mC inside the mitochondria was first reported in the 70s (Nass, 1973; Vanyushin et al., 1971; Dou et al., 2019). The low amount of methylated cytosines, as well as the different methodologies used to quantify mtDNA methylation, led to a controversy over whether the presence of methylated cytosine is just a "random chemical modification" or it is part of a fully regulated mechanism with biological significance (Mechta et al., 2017; Liu et al., 2016).
The controversy about mtDNA methylation hinges around: i) the presence of methylation machinery within the mitochondria, ii) the biological significance of mtDNA methylation, and iii) how 5mC can regulate gene expression (Figure 1.8).

To date, the presence of DNMT, and its association with 5mC and with mtDNA gene expression, within the mitochondria has been confirmed by independent groups (Shock et al., 2011; Saini et al., 2017; Dou et al., 2019). In addition, the presence of the active demethylation enzymes TETs in mitochondria have been reported (Figure 1.4) (Dzitoyeva et al., 2012a; Chen et al., 2012). Remarkably, the methylation and oxidation of cytosine seem to be site-specific (Chen et al., 2012; Dzitoyeva et al., 2012b; Iacobazzi et al., 2013; Dou et al., 2019). Interestingly, in nDNA the reconfiguration of 5mC and 5hmC has been associated with ageing and exposure (Zampieri et al., 2015; Barrow et al., 2018); their presence inside the mtDNA seems to suggest a highly regulated epigenetic landscape.
Another aspect of the controversy is the biological significance of mtDNA methylation, which I have described in the paragraph 1.4.3, and the mechanism by which 5mC can regulate mtDNA transcription, which I have described in the paragraph 1.3.2 (Figure 1.4).

1.4.2 MtDNA methylation in disease

Altered mtDNA methylation is associated with a wide range of diseases (Baccarelli & Byun, 2015; Devall et al., 2016a) and can be used as an indicator to monitor relevant exposures and to predict the CVD outcome (Corsi et al., 2020) (Table 1.2).
Ageing is one of the factors that modulate mtDNA methylation patterns in both mice and humans (Dzitoyeva et al., 2012a; Mawlood et al., 2016; Rönn et al., 2008; Bacalini et al., 2017). Further, CVD and environmental exposures influence patterns of mtDNA methylation (Baccarelli & Byun, 2015; H.-M. Byun et al., 2016; Byun & Baccarelli, 2014; Janssen et al.,

2015; Lambertini & Byun, 2016; L. D. Zheng et al., 2016; Zheng et al., 2015). In particular, strong correlations have been reported between increased D-Loop methylation and insulin resistance (Zheng et al., 2015), between increased *MT-ND6* methylation and non-alcoholic fatty liver disease (Pirola et al., 2013), and between D-Loop methylation and diabetic retinopathy (Mishra & Kowluru, 2015a) (Table 1.2 and Figure 1.7). Therefore, mtDNA methylation has been proposed as a next-generation biomarker with prognostic potential for CVD (Baccarelli & Byun, 2015; Iacobazzi et al., 2013; L. D. Zheng et al., 2016; Corsi et al., 2020).

Disease/ condition	Source of mtDNA	Method	Differentially methylated locus	Level of methylation	Citation
Ageing	Human blood	bisulphite sequencing	MT-1215 and MT-1313 (12S rRNA)	Hypomethylation with ageing	(Mawlood et al., 2016)
Ageing and survival	Human blood, buffy coat	BC, methylation-sensitive probes, and RT-qPCR	MT-932 and MT-3078 (MT-RNR1 12S rRNA, MT-RNR2)	MT-932 hypermethylation with ageing	(D'Aquila et al., 2015)
Environmental pollution PM1	Human blood, buffy coat	BS-pyrosequencing	MT-TF/MT-RNR1 and D-loop	MT-TF hypermethylated with PM1	(Byun et al., 2013)
CVD	Human platelets	BS-pyrosequencing	MT-CO1, MT-CO2, MT-CO3, and MT-TL1	Hypermethylated	(Baccarelli & Byun, 2015)
Environmental pollution, PM2.5	Placenta	BS-pyrosequencing	MT-RNR1 and D-loop	Hypermethylation	(Janssen et al., 2015)
PM2.5	Human blood	BS-pyrosequencing	D-loop	Hypomethylation	(H.-M. Byun et al., 2016)
Smoking in pregnancy	Placenta	BS-pyrosequencing	MT-RNR1	Hypermethylated	(Janssen, Gyselaers, et al., 2017)
Thyroid hormones	Placenta	BS-pyrosequencing	D-loop and MT-RNR1	Hypomethylated	(Janssen, Byun, et al., 2017)
Non-alcoholic fatty liver	Human Liver biopsy	Methylation Specific-PCR	MT-ND6	Hypermethylated in non-alcoholic steatohepatitis (NASH)	(Pirola et al., 2013)

disease (NAFLD)					
Obesity	Blood (buffy coat)	Methylation Specific-PCR	D-loop	Hypermethylated in participants with obesity and insulin resistance	(Zheng et al., 2015)
obesity and pre-diabetes	Blood (buffy coat)	Methylation Specific-PCR	D-loop MT-ND6	Hypermethylated with higher HOMA-IR Hypermethylated with higher HOMA-IR and BMI	(L. D. Zheng et al., 2016)
Alzheimer Diseases	Entorhinal cortex (Human postmortem brain)	Pyrosequencing	D-loop MT-ND1	Hypermethylation in AD-related pathology Hypomethylation in AD-related pathology	(Blanch et al., 2016)
Parkinson's Diseases	Substantia Nigra (human postmortem brain)		D-loop	Hypomethylation in PD	
Welders	Blood	BS-pyrosequencing	D-loop and MT-TF	Hypomethylation in welders	(Xu et al., 2017)
Overweight	Buccal swab	BS-pyrosequencing	D-loop	Hypermethylation of D-loop	(Bordoni et al., 2019)
L-carnitine supplementation	Human platelets	BS-pyrosequencing	D-loop	Hypermethylation of D-loop	(Bordoni et al., 2020)
Future CVD event	Human platelets	BS-pyrosequencing	MT-CO1 nt6807, MT-CO3 nt9444, MT-TL1 nt3254	Hypermethylation in all three CpGs	(Corsi et al., 2020)
In utero exposure to air pollution and smoking	Placenta	BS-pyrosequencing	D-loop and LDLR2	Hypermethylation of D-loop with air pollution exposure	(Vos et al., 2020)

Table 1.2 Summary of papers that correlate risk factors, CVD and other diseases with mtDNA methylation in human.

In this table, only studies that used human tissues and single CpG resolution techniques have been included. The light blue background indicates hypomethylation while the light orange background indicates hypermethylation.

1.4.3 Biological significance of mtDNA methylation

Although there is evidence of a correlation between mtDNA methylation and gene expression (Breton et al., 2019; Mishra & Kowluru, 2015b; Patil et al., 2019; Dou et al., 2019), small changes in mtDNA methylation may not affect gene expression. Notwithstanding, small-magnitude changes, such as 1% methylation change, particularly if consistent across a population, may be a robust indicator of exposure and can predict a future outcome (Lu, Seeboth, et al., 2019; Breton et al., 2017b; Corsi et al., 2020; Levine et al., 2018; Lu, Quach, et al., 2019; Leenen et al., 2016a). This is especially true in a healthy population where changes in patterns of mtDNA methylation are not as pronounced as in populations with fully manifested CVD.

The difficulties in linking mtDNA methylation to gene expression lie in the specific features of mtDNA: i) mtDNA comes in many copies (it is said to be polyploid) and ii) mtDNA is transcribed in two single polycistronic transcripts (Table 1.1) (Dou et al., 2019; Kazak et al., 2012). Each polycistronic transcript it is subsequently cleaved into single mRNA and tRNA. How mitochondrial genes, that derive from the same polycistronic transcript, are regulated to have a variable expression it is still unknown (Mercer et al., 2011; Dou et al., 2019).

Given the fact that a single mitochondrion can contain up to 10 copies of mtDNA (Satoh & Kuroiwa, 1991; Wiesner et al., 1992) and that there are many mitochondria per cell, it is not surprising that mtDNA methylation level has not always been correlated with mtDNA expression, as the mitochondria can transcribe the proteins needed using only the “good” copies of mtDNA. Moreover, despite the presence of only three promoters and only two polycistronic transcripts, one from the light strand (LSP) and one from the heavy strand (HSP2), the amount of individuals rRNAs and tRNAs varies widely. This has been ascribed to post-transcriptional cleavage and processing (D’Souza & Minczuk, 2018; Mercer et al., 2011). Since the mechanisms of regulations of mtDNA transcription and translation remain be fully uncovered, it is difficult to speculate on the roles of 5mC and 5hmC in the regulation of these processes.

1.4.4 Current methodologies for mtDNA methylation analysis

Given the controversy surrounding mtDNA methylation, the methodology chosen to analyse mtDNA methylation, and especially its changes in healthy participants, it is of paramount importance.

A variety of platforms developed to study patterns of methylation in nuclear DNA (nDNA) have been adapted to study mtDNA methylation, including immunoprecipitation (Shock et al., 2011; Devall et al., 2017; Wolters et al., 2017; Ghosh et al., 2014, 2016), mass spectrometry (Infantino et al., 2011; Menga et al., 2017), and pyrosequencing (Liu et al., 2016; Baccarelli & Byun, 2015; Corsi et al., 2020; Bordoni et al., 2020; Xu et al., 2017). However the appropriateness of these methodologies for the study of mtDNA methylation has been challenged (Liu et al., 2016; Mechta et al., 2017; Owa et al., 2018).

The most important consideration when studying mtDNA methylation is to determine which methods can address the challenges associated with: i) NUMTS, ii) circular and supercoiled mtDNA structure, and iii) low level of mtDNA methylation (Figure 1.8) (Liu et al., 2016; Owa et al., 2018; Bintz et al., 2014; Lambertini & Byun, 2016; Byun & Barrow, 2015a).

1.4.4.1 Nuclear Pseudogenes

A potential source of error in mtDNA methylation analysis can arise from the inclusion of "nuclear sequences of mitochondrial origin" NUMTs or "nuclear-mitochondrial pseudogenes"(Devall et al., 2017; Hazkani-Covo et al., 2010). NUMTs are sequences of mtDNA that have translocated into the nucleus and been incorporated into nuclear DNA through non-homologous end-joining or repair of double-strand breaks (Hazkani-Covo et al., 2010). Insertion of NUMTs into nDNA is an evolutionary process that took place many times during evolution and it is still ongoing. It remains controversial whether i) these nuclear regions are highly methylated because they are not expressed and, therefore, have potential to interfere with assessment of mtDNA methylation (Hazkani-Covo et al., 2010), and ii) the exclusion of NUMTs from the analysis using bioinformatic methods provides accurate results (Jayaprakash et al., 2015; Mechta et al., 2017; Dou et al., 2019). In fact, high-throughput techniques that are used to clean-up methylation data and remove the influence of NUMTS rely on the accurate annotation of the NUMTs. However, the annotation of NUMTs varies in different versions of the human genome sequence and therefore it cannot be considered reliable (Jayaprakash et al., 2015). Although, pyrosequencing can reproducibly, and accurately, detect minor variants, such as NUMTs, (Bintz et al., 2014) the preliminary bisulfite conversion reduces the complexity of mtDNA and NUMTs. In fact, bisulfite

treatment would convert all the unmethylated cytosine into uracils making the mtDNA and NUMT sequences look more similar (Mechta et al., 2017).

Therefore, to analyse the mtDNA methylation unequivocally it is essential to purify DNA of mitochondrial origin only or to use primers that align only to the mtDNA, due to secondary structures (Shock et al., 2011; Byun & Barrow, 2015a; Byun & Baccarelli, 2014; Baccarelli & Byun, 2015).

Accordingly, in the studies reported in this thesis, mtDNA was always purified and the presence of nuclear DNA was tested for using PCR to exclude nuclear contamination (section 2.2.2.2). In the *in vitro* experiments, the genomic DNA of MEG-01 cells was initially isolated and subsequently, the mtDNA was purified using the protocol described in section 2.4.2 and figure 2.1 (Jayaprakash et al., 2015). While in the human study, platelets, a cell type without a nucleus, were used consequently DNA extracted from platelets consists of mtDNA only (figure 2.1). However, since platelet DNA might be contaminated by cell-free DNA of nuclear origin present on the surface of the platelets, an additional step to remove this DNA was also used to ensure the purity of platelet mtDNA (section 2.4) (Corsi et al., 2020; Baccarelli & Byun, 2015).

1.4.4.2 Circular DNA

MtDNA is a circular double-stranded molecule that acquires a coiled and supercoiled structure that provides resistance to bisulfite conversion (Liu et al., 2016; Owa et al., 2018; Mechta et al., 2017; Nicholls et al., 2018).

As discussed in section 2.5, bisulfite conversion is a very effective means of distinguishing unmethylated from methylated cytosines within DNA. However, bisulfite conversion relies on the conversion of unmethylated cytosine residues that are not base-paired, and therefore full access to each CpG site is crucial. Double-strand circular mtDNA reduces bisulfite conversion efficiency and false positive are recorded (Liu et al., 2016). A fundamental issue of mtDNA methylation measurement are the false positives that result from mtDNA topology and that are not distinguishable from methylated cytosines (Owa et al., 2018). To address this problem, a prelaminar denaturation step, to make mtDNA single-stranded, followed by digestion with restriction enzymes, to linearize the mtDNA, are required (Matsuda et al., 2018). Therefore, when analysing my samples, linearization of mtDNA with *BamHI* was always performed, as suggested by Liu et (Liu et al., 2016).

1.4.4.3 Low methylation levels of mtDNA

Methylation of mtDNA, and especially at the single CpG level, has been found to be very low (Byun et al., 2013; Lambertini & Byun, 2016; Xu et al., 2017; Shock et al., 2011). This is especially true in healthy participants (Corsi et al., 2020; Byun, 2019). Therefore to measure mtDNA methylation, it is imperative to use a technique with single-nucleotide resolution, low error rate, and that, through the use of internal quality control, allows for correction of artefacts dependent on mtDNA topology, that could give rise to false positives (Owa et al., 2018). Pyrosequencing has all of these qualities (Infantino et al., 2011; Liu et al., 2016; Owa et al., 2018). Therefore, in this thesis, bisulfite conversion (BC) followed by pyrosequencing was the approach used to characterise and to quantify mtDNA methylation in samples from both *in vitro* and *in vivo* studies.

Given the controversy surrounding mtDNA methylation, it is essential to remove all potential sources of false positives, by adapting the protocol to the specific characteristics of the mtDNA, and by choosing a technology that not only quantifies methylation but also detects incomplete bisulfite conversion (Owa et al., 2018; Liu et al., 2016).

Bisulfite conversion, followed by PCR for amplification, and pyrosequencing offers an elegant approach for the accurate quantification of mtDNA methylation at a single CpG site (Tost & Gut, 2007; Owa et al., 2018). This is essential for the analysis of low level mtDNA methylation at individual CpG sites. In fact, the majority of methylated cytosines in the mtDNA are suggested to be located outside the CpG sites (Iacobazzi et al., 2013; Patil et al., 2019; Morris et al., 2020).

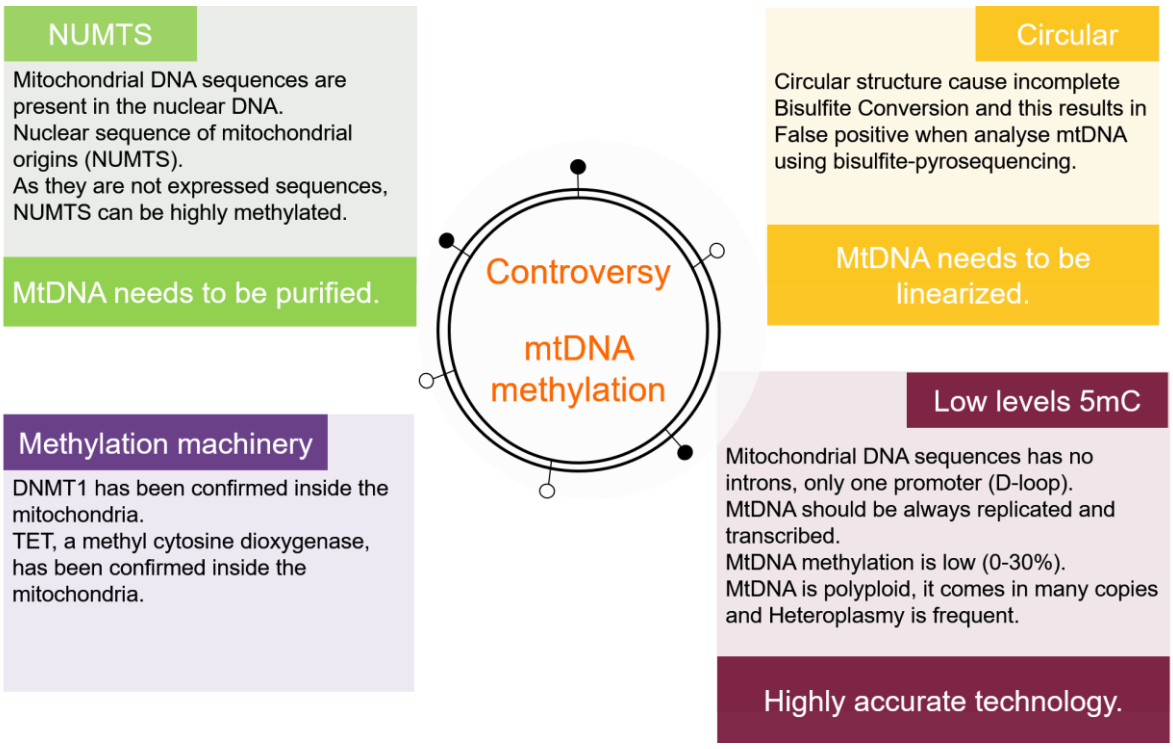


Figure 1.8 Controversy over mtDNA methylation

The controversy over the presence and the possibility to measure mtDNA methylation due to: i) NUMTs or "nuclear-mitochondrial pseudogenes", ii) Circular structure of mtDNA that interfere with bisulfite conversion and iii) low level of mtDNA methylation. As illustrated in the bottom left side of the figure, the "methylation machinery" occurs in mitochondria which address another aspect of the controversy in respect to the mechanisms of mtDNA methylation. The presence of the enzyme DNMT1, its interaction with the mtDNA, and the TET enzymes have been reported in mitochondria (Shock et al., 2011; Mishra & Kowluru, 2015b; Dzitoyeva et al., 2012b).
End of figure legend.

1.5 Diet and CVD risk

1.5.1 Overview of diet and CVD risk

Poor diet is a fundamental cause of cardiometabolic diseases, including CVD, and progress of these diseases can be modified through improving diet (Danaei et al., 2009; Estruch et al., 2018; Mozaffarian, 2016; Meier et al., 2019; Stanaway et al., 2018). In addition, dietary factors have profound effects on patterns of DNA methylation (Voelter-Mahlknecht, 2016; Mathers et al., 2010) and change the epigenetic patterns associated with obesity and CVD (Figure 1.9) (Milagro et al., 2011; Gensous et al., 2020). Dietary patterns low in whole grains, fruit, and vegetables have the strongest impact on CVD risk (Meier et al., 2019; Angelino et al., 2019). Several items of the Mediterranean diet have been suggested to contribute to its protective effects against CVD. Among them, there are polyphenols, present in fruit and vegetable, that have been found to be associated with lower CVD risk (Figure 1.9)(Aune et al., 2018; Kim & Je, 2017).

1.5.2 Mediterranean diet

The Mediterranean diet (MeDiet) is defined as a dietary pattern typical of olive tree growing countries of the Mediterranean basin, such as Italy and Greece, in the 1960s. Broadly, the MeDiet is a plant-based diet with a high intake of fruit, vegetables, nuts, legumes, and unprocessed cereals. The intake of meat, processed meat, and dairy products is low except for long-preservable cheeses. The main source of fat is olive oil, which is rich in monosaturated

fatty acids. Typically, the MeDiet includes moderate consumption of wine during meals (Trichopoulou et al., 2014). A number of observational and intervention studies support the protective role of the MeDiet against CVD and its association with longevity (Trichopoulou et al., 2014; Gensous et al., 2020; Estruch et al., 2018; de Lorgeril et al., 1999).

Intervention studies with the MeDiet have demonstrated protection against CVD, so providing evidence of causality and confirming the findings from observation studies in which higher adherence to MeDiet patterns are associated with lower CVD risk. For example, in 1999 De Lorgeril et al demonstrated that MeDiet interventions reduce the risk of recurrence of myocardial infarction in 243 participants (de Lorgeril et al., 1999). More recently, in a Spanish cohort of 7447 participants at high risk of CVD was randomized to a control diet (advice on a low-fat diet), a MeDiet supplemented with extra virgin olive oil, or a MeDiet supplemented with nuts. The incidence of major CVD event was lower in the groups assigned with a MeDiet, supplemented with either oil or nuts (Estruch et al., 2018).

Several mechanisms have been proposed to explain how the MeDiet reduces mortality and disease incidence,. For example, two groups have demonstrated that the MeDiet, along with lifestyle interventions, may affect patterns of DNA methylation and reduce epigenetic ageing (Gensous et al., 2020; Fitzgerald et al., 2020). Another mechanism by which the MeDiet may protect against CVD events is through anti-inflammatory effects on platelets. In fact, the MeDiet has been found to reduce platelet count and activation in healthy individuals (Bonaccio et al., 2014; Antonopoulou et al., 2006a; Gavriil et al., 2019). The MeDiet is associated with reduced platelet count, which is a biomarker of low-grade inflammation, in a healthy Italian population (Bonaccio et al., 2014). Importantly, this association appeared to be driven by the relatively high dietary fibre and antioxidant contents of the MeDiet (Bonaccio et al., 2014). Additionally, lipids typical of the MeDiet of Greece (mono- and polyunsaturated fatty acids), reduce platelet aggregation in both diabetic and healthy participants (Antonopoulou et al., 2006b). These associations between MeDiet and platelets activity and number is particularly interesting, as platelets do not have a nucleus and their aggregation is regulated by mitochondria. Accordingly, the only DNA molecule in platelets is represented by the mtDNA.

I hypothesised that, given the important contribution of dietary pattern to CVD risk, mtDNA methylation level in platelets may be associated with diet (Hypothesis 2, section 1.6.2).
In particular, there is growing evidence that polyphenols, a group of phytochemicals found in cocoa, wine, fruit and vegetables, can improve cardiometabolic risk (Milenkovic et al., 2014; Mozaffarian, 2016). Polyphenols have been reported to have an effect on i) risk of CVD

(Tangney & Rasmussen, 2013; Li et al., 2019), ii) patterns of DNA methylation (Subramaniam et al., 2014), iii) mitochondrial function and mitochondrial number (Cheng et al., 2014), and iv) platelet activation (Hubbard et al., 2004a) (Figure 1.9 and 1.10).
I hypothesise that, since polyphenols are DNMT inhibitors, they may alter mtDNA methylation level *in vitro* (Hypothesis 3, section 1.6.2).

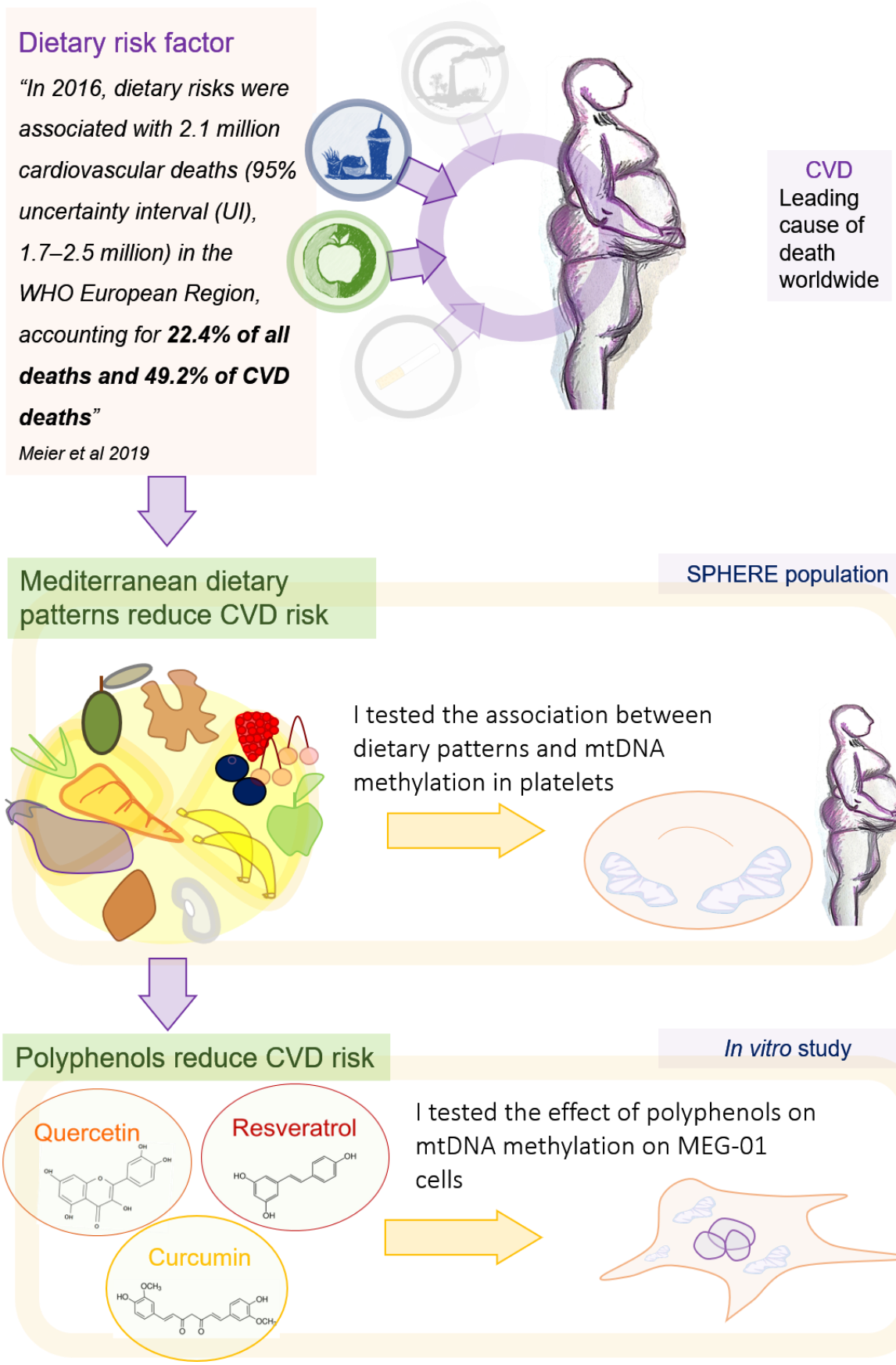


Figure 1.9 Effect of dietary patterns and nutrients on CVD risk

This image outlines the relationship between dietary risk factors, the Mediterranean diet, and polyphenols with CVD risk. Additionally, in this image, it is also summarised how I tested this relationship during my PhD.

In the upper part, the dietary risk factors are the leading drivers or CVD risk in the European population (Meier et al., 2019).

In the middle part, the focus is on Mediterranean dietary patters -rich in whole grains, fruit, and vegetables- that have been associated with reduced risk of CVD (Meier et al., 2019; Angelino et al., 2019).

In the lower part, three polyphenolic compounds – mostly present in fruit and vegetables- are depicted. Resveratrol, Quercetin, and Curcumin have been suggested to reduce CVD risk (Aune et al., 2018; Kim & Je, 2017).

During my PhD, I tested the association between a Mediterranean dietary pattern with the mtDNA methylation in platelets from healthy participants (middle right corner).

During my PhD, I also tested whether the three polyphenolic compounds Resveratrol, Curcumin, and Quercetin alter the mtDNA methylation level of megakaryocytes. This experiment has been conducted in vitro using a megakaryocytic cell line model called MEG-01 (bottom right corner).

End of figure legend.

1.5.3 Polyphenols

Polyphenols are secondary metabolites produced by plants as a defence against pathogens, predators and UV rays (Pandey & Rizvi, 2009a; Gambini et al., 2015). Resveratrol(5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol) is found in fruits such as grapes, blueberries and blackberries, and in derivates such as wine, and chocolate (Gambini et al., 2015) (Figure 1.10 A). Quercetin(2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is a flavonoid found in fruits and vegetables including onions, apples, and capers (Hubbard et al., 2004a; Carlos-Reyes et al., 2019; Seo et al., 2015) (Figure 1.10 B). Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is extracted from the rhizomes of *Curcuma longa* (Bagheri et al., 2020) (Figure 1.10 C). Hereinafter, I will use the term "Resveratrol" to refer to the molecule "5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol" and "resveratrol" for the collective mix of Resveratrol and Resveratrol metabolites (e.g trans-resveratrol-3-O-sulfate; (3) trans-resveratrol-4'-O-sulfate; (4) trans-resveratrol-3,4'-O-disulfate; (5) trans-resveratrol-3-O-glucuronide; (6) trans-resveratrol-4'-O-glucuronide; (7) dihydroresveratrol (DHR); (8)

3,4’-O-dihydroxy-trans-stilbene; and (9) lunularin). The same applies also for Quercetin and Curcumin (Springer & Moco, 2019).

Resveratrol, Quercetin, and Curcumin are relatively small lipophilic compounds that can cross the cell membrane and enter the nucleus and the mitochondria (De Paepe & Van Coster, 2017; Bagheri et al., 2020; Costa et al., 2016). Polyphenols have pleiotropic activities and interact with a variety of signalling molecules in different compartments of the cell (Gupta et al., 2012). Although the potential associations of polyphenols and lower risk of cardiovascular diseases, cancer, and dementia have been widely described, the mechanisms still remain unclear (Gupta et al., 2012; Bagheri et al., 2020; Serban et al., 2016; Costa et al., 2016).

Additionally, the issue of the human *in vivo* active dose and bioavailability of Resveratrol, Quercetin, and Curcumin remains of paramount importance (Gambini et al., 2015; Priyadarsini, 2014; Xia et al., 2017; Sundaram et al., 2019; Serban et al., 2016). In fact, in vitro studies use high doses difficult to achieve through normal diet.

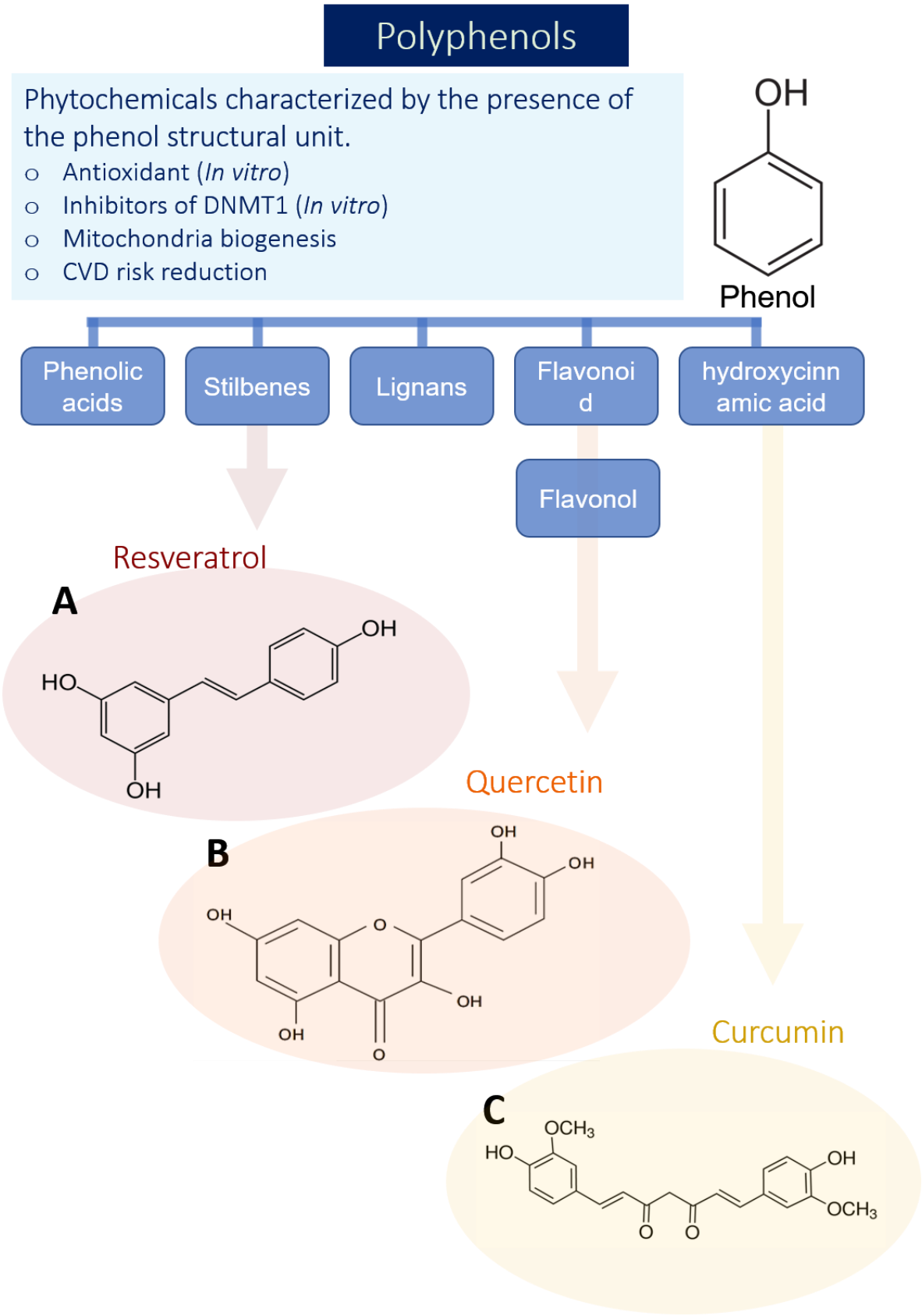


Figure 1.10 Resveratrol, Quercetin, and Curcumin chemical structure.

This image summarises the classification of polyphenols and specifies the chemical structure of Resveratrol, with the red background, Quercetin with the orange background, and Curcumin with the yellow background.

***A**) Resveratrol, or 5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol, is a stilbenoid. It has two phenolic rings connected by double styrene (Gambini et al., 2015). In the image, the trans-isomer, the most predominant and stable form, is represented.*

***B**) Quercetin, or 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one, is a flavonol. Quercetin has the two phenolic rings plus a number of hydroxyl groups, which confer the molecule its antioxidant activity (Costa et al., 2016).*

***C**) Curcumin, or (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a hydrophobic molecule with high affinity for DMSO and ethanol. It is also called "diferuoloyl methane" as it has two ferulic acid residues on both sides, connected by a methylene bridge (Bagheri et al., 2020; Priyadarsini, 2014).*

End of figure legend.

1.5.4 Polyphenols and CVD risk

Polyphenols-rich food have been found to reduce cardiometabolic risk factors in several intervention studies (Tangney & Rasmussen, 2013; Aune et al., 2018; Novotny et al., 2015; Gavriil et al., 2019; Santhakumar et al., 2015; Carnevale et al., 2012; Gresele et al., 2008; Hubbard et al., 2004a). This is especially relevant to adults with overweight and obesity who are at higher risk of CVD (McKay et al., 2018). However, neither the appropriate dose and duration of treatment that maximise the proposed effects of polyphenols in reducing cardiometabolic risk, nor the *in vivo* mechanism for any effects, have been fully elucidated (Cory et al., 2018). Additionally, Resveratrol and Quercetin have been suggested to have hormetic actions, or rather protecting cells at low doses while being toxic at higher doses (Xia et al., 2017; Costa et al., 2016; Calabrese et al., 2010).

The observed improvements in CVD risk may be secondary to improvements in some obesity-related metabolic factors such as body weight, blood glucose, insulin resistance, blood pressure, inflammation, oxidative stress, and lipid accumulation (Serban et al., 2016; Seo et al., 2015; Larson et al., 2012; McKay et al., 2018; Akbari et al., 2019; Zhou et al., 2019; Zhao et al., 2017a; Seo et al., 2015; Cory et al., 2018; Aguirre et al., 2014). *In vitro* studies demonstrate a potent antioxidant function of Resveratrol (Xia et al., 2017), Quercetin (Costa et al., 2016), and Curcumin (Larasati et al., 2018). Despite the *in vitro* evidence that Resveratrol, Quercetin, and Curcumin are direct ROS scavengers (Costa et

al., 2016), their *in vivo* antioxidant activity has been proposed to be the result of other pathways, such as activating the Nrf2-ARE pathway or by modulating autophagy (Costa et al., 2016).

In the case of Resveratrol, the antioxidant activity has been suggested to follow DNA methylation changes (Xia et al., 2017; Naoi et al., 2019). Quercetin and Curcumin have been found to act as both antioxidant or pro-oxidant agents depending on the concentration and cell type (Naoi et al., 2019; Larasati et al., 2018; Costa et al., 2016). For example, Larasati et al demonstrated that treatment of leukaemia derived cells, including MEG-01, for 24 hours with 50 μM Curcumin resulted in an almost doubling of ROS concentration (Larasati et al., 2018).

Polyphenols have also been shown to suppress lipid accumulation and obesity-related inflammation *in vitro* and in human participants (Seo et al., 2015; Zhao et al., 2017b). Quercetin has been found to lower blood pressure in people with metabolic syndrome, in smokers (Serban et al., 2016), and in those with stage 1 hypertension (Larson et al., 2012). Curcumin reduces blood glucose concentration in diabetic participants (Aggarwal, 2010) and improves their lipid profile (Gupta et al., 2012). Resveratrol in intervention studies was reported to decrease in BMI and waist circumference both in participants with metabolic syndrome (Zhou et al., 2019; Méndez-del Villar et al., 2014) and participants with non-alcoholic fatty liver disease (Faghihzadeh et al., 2014).

All considered, the evidence from human studies remains fragmentary and the mechanisms of polyphenols' beneficial action have not been discovered.

1.5.5 Polyphenols and DNA methylation

Dietary methyl donors are essential for the mitochondrial methylation processes (e.g. protein), mtDNA stability (Ormazabal et al., 2015) and mtDNA methylation (Infantino et al., 2011; Menga et al., 2017; Saini et al., 2017). On the other hand, dietary inhibitors of DNMT, such as polyphenols, may play a role in the prevention of CVD (Figure 1.11)(Pandey & Rizvi, 2009b; Rangel-Huerta et al., 2015; Widmer et al., 2013). The polyphenolic compounds Resveratrol, Quercetin, and Curcumin have already shown some efficacy in human in short-term studies but their mechanism of action and their effects on CVD pathogenesis remain largely unknown.

Recently, it has been proposed that these bioactive compounds can act at the epigenetic level by inhibiting DNMT enzymes (Figure 1.11) (Link et al., 2013; Sae-Lee et al., 2018). Two mechanisms have been suggested. First, some polyphenols are readily methylated by SAM,

which is converted to S-adenyl-homocysteine (SAH), and this reduces the amount SAM available for other methylation events in the cell, especially DNA methylation. In fact, methyl-transferases are sensitive to the ratio of SAM-SAH and this ratio can modify their activity. Additionally, some polyphenols possess a high binding affinity to the catalytic pocket of DNMT and, therefore, inhibit its action directly (Fang et al., 2007; Mirza et al., 2013).

Finally, polyphenols seem to have a pervasive effect on platelet activity, inasmuch they can reduce platelet activation and aggregation (Chuengsamarn et al., 2014; Hubbard et al., 2004b; Pawlaczyk et al., 2011; Vita, 2005). This cell type is relevant to my research question, as platelets do not have nDNA but only mtDNA.

1.5.6 Diet and mtDNA methylation

Diet and mitochondrial folate metabolism are two factors that influence CpG methylation (Anderson et al., 2012; Cuyàs et al., 2017). In fact, diet regulates the availability of cofactors for, and inhibitors of, DNMT. For example, folate is an important methyl donor in the one-carbon metabolism pathway which is essential for the methylation of nDNA (Figure 1.11) (Mathers et al., 2010; McKay et al., 2011; Mentch et al., 2015). When folate supply is low, the competition for limited methyl groups may result in hypo-methylation of nDNA (Pufulete et al., 2005). For these reasons folate metabolism can regulate epigenetics indirectly in mitochondria (Jang et al., 2005; Kim et al., 2009; Obeid, 2013; Salbaum & Kappen, 2012) (Figure 1.11). As DNMTs are sensitive to SAM:SAH ratio and can be paused by their absence, folate deficiency and more generally dietary deficiencies, especially at prenatal stages, cause long-lasting epigenetic marks (Crider et al., 2012; McKay et al., 2011).

The relationship between disrupted one-carbon metabolism and mtDNA hypermethylation in oocytes has been assessed in pigs with polycystic ovarian syndrome (PCOS) (Jia et al., 2016). This condition presents with high circulating homocysteine concentrations and polycystic ovaries and, intriguingly, it is correlated with a number of metabolic aberrations such as dyslipidaemia, hypertension, insulin resistance and type II diabetes (Jia et al., 2016). In this model, hyperhomocysteinemia is caused by upregulation of DNMT1, which methylates DNA, betaine-homocysteine methyl-transferase (BHMT), which methylates homocysteine to produce methionine, and Glycine-N methyl-transferase (GNMT), which transforms SAM into its demethylated counterpart, SAH (Figure 1.11). In parallel, higher mtDNA methylation is accompanied by lower expression of mitochondrially encoded proteins and this results in a shortage of ATP (Jia et al., 2016). This evidence supports the idea that SAM availability and presence of DNMT inhibitors have implications for mtDNA methylation patterns and for the

expression of mitochondrially-encoded genes (Figure 1.11). In addition, overexpression of the SAM mitochondrial carrier (SAMC), which transports SAM into the mitochondria, results in mtDNA hypermethylation and the consequent impairment of oxidative phosphorylation (Menga et al., 2017).

On the other hand, an association between a pathological decrease in SAM and hypomethylation of mtDNA has been reported in Down Syndrome patients (Infantino et al., 2011). This association confirms the importance of SAM for mtDNA methylation.

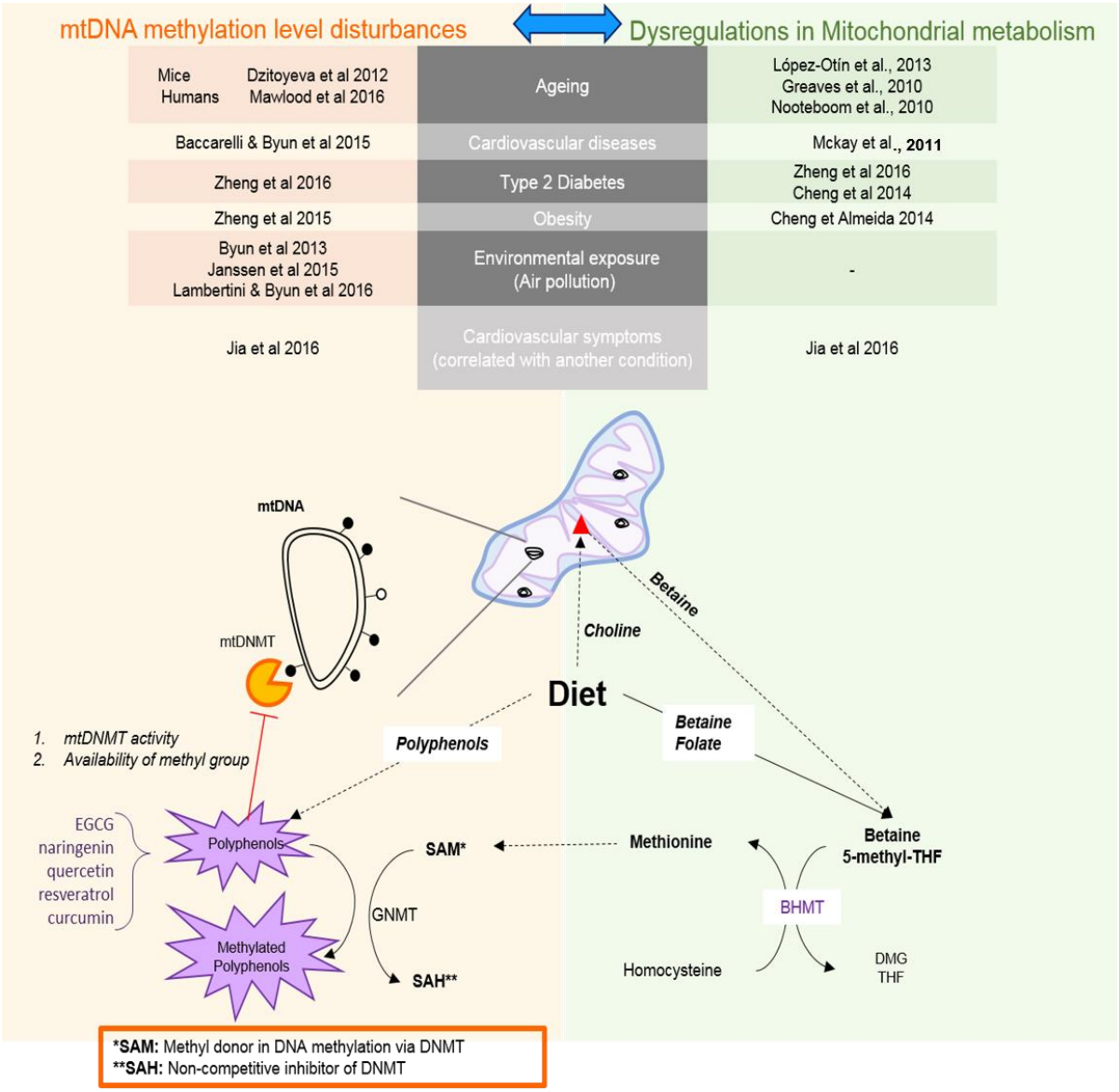


Figure 1.11 Folate cycle and one-carbon transfers reaction in the presence of methyl donors and polyphenols.

This image is divided into two colours: the green area, on the right, covers the "Dysregulation in Mitochondrial metabolism" and includes the description of the one-carbon metabolism. The orange area, on the left, covers the "mtDNA methylation

disturbances" and describe the mechanism by which polyphenols can prevent mtDNA methylation. Mitochondrial one-carbon metabolism is connected to the mtDNA methylation via the production of an essential methyl donor: methionine. The level of SAM (the methyl donor in many methylation reactions) is dependent on the presence of dietary methyl donors, including choline, betaine and folate, and on dietary methyl-acceptors such as polyphenols. Therefore, the availability of SAM is dependent on both methyl donors and polyphenols, both of which are derived from the diet; and so, diet is positioned at the centre. In the upper part, the table summarises the main papers that linked the dysregulation of mitochondrial metabolism and mtDNA methylation disturbances to ageing, CVD, type 2 diabetes, obesity and environmental exposure.

THF: tetrahydrofolate; DMG: dimethylglycine; DNMT: DNA-methyl-transferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocyteine; BHMT: betaine-homocysteine methyl-transferase (violet); GNMT: Glycine-N methyl-transferase (violet).

Modified from: (Crider et al., 2012; Lee et al., 2005a; Obeid, 2013; Salbaum & Kappen, 2012). End of figure legend.

1.5.7 Potential for prevention of CVD through modulation of mitochondrial DNA methylation by diet

The therapeutic potential of modulating mitochondrial epigenetics emerged with the discovery that the anticonvulsant and mood stabilizer valproate alters mtDNA methylation (Chen et al., 2012; Gu et al., 2012). Additionally, when the large yellow croaker (*Larimichthys crocea*) – a marine fish found in the northwest Pacific ocean - was fed olive and perilla oils there were increases in *MT-RNR1* methylation and decreases in methylation of *MT-ND4L* and *MT-TR* (Liao et al., 2015). Further, in a subsequent study, these fishes were fed with three different diets containing a low, moderate or high amount of lipids and the mtDNA methylation pattern, transcription and mtDNA copy number were evaluated. In the case of low or moderate intake of lipids, the mtDNA copy number was increased and the mtDNA hypomethylated which increased transcription. In contrast, with the high lipid intake the mtDNA was highly methylated in the D-Loop. The authors concluded that, in response to dietary supply and energy demand, the mtDNA methylation pattern can be adjusted to meet the ATP requirements (Liao et al., 2016). Similarly, fructose has been found to alter mtDNA methylation status and its expression in rat hepatocytes (Yamazaki et al., 2016) and high

glucose in retinal endothelial cells increases mtDNA methylation (Mishra & Kowluru, 2015b).

However, to the best of my knowledge, there are no published research studies on the effect of modulating mitochondrial epigenetics through diet in humans.

1.6 Hypotheses, Aims & Objectives

1.6.1 Summary

My research aims to test whether mtDNA methylation can be used as a predictive biomarker of future CVD incidence and whether the pattern of mtDNA methylation correlates with diet. To achieve this aim, a population with 200 adults with overweight and obesity, but otherwise healthy, were selected at Baseline. These individuals were followed for up to 5 years and the incidence of mild and severe CVD was recorded. I quantified methylation of specific loci within mtDNA from platelets at Baseline and used these data to create a model to predict CVD outcome at Follow-up (Hypothesis 1). Since CVD risk is attributable to behavioural factors, such as diet (Meier et al., 2019), the association between the Mediterranean diet and mtDNA methylation level at Baseline was also investigated (Hypothesis 2).

Finally, given the similarities between the nuclear DNMT and the mtDNMT (Shock et al., 2011), it seems likely that the mtDNA methylation pattern might be affected by natural DNMT inhibitors, such as polyphenols (Infantino et al., 2011; Menga et al., 2017; Saini et al., 2017). Therefore, I tested the effect of the polyphenolic compounds Resveratrol, Quercetin, and Curcumin, *in vitro*, on patterns of mtDNA methylation in megakaryocytes (Hypothesis 3).

1.6.2 Hypotheses

Hypothesis 1 MtDNA methylation in platelets, at Baseline, can be used to predict the future CVD outcome in a population of adults with overweight and obesity but otherwise healthy (Figure 1.2 and 1.3).

Hypothesis 2 MtDNA methylation in platelets at Baseline is associated with lifestyle factors and, notably, diet (Figure 1.9).

Hypothesis 3 Treatment of the platelet precursor cell, MEG-01, with the polyphenolic compounds Resveratrol, Quercetin, and Curcumin, *in vitro*, alters patterns of mtDNA methylation (Figure 1.9).

1.6.3 Aims

- To test the hypotheses stated above by investigating:
- The correlation between mtDNA methylation at specific CpG sites at Baseline with the future risk of CVD (Hypothesis 1).
- The association between patterns of mtDNA methylation at Baseline with adherence to the Mediterranean Diet (Hypothesis 2).
- The effects of treatment with specific polyphenols on patterns of mtDNA methylation in the megakaryocyte, MEG-01 cell 0(Hypothesis 3).

1.6.4 Objectives

- To use an existing prospective cohort study to select a group of 200 adults with overweight and obesity, but otherwise healthy (Baseline), of whom 116 remained healthy, while 84 developed CVD in the following 5 years (Follow-up).
- To quantify mtDNA methylation at specific CpG sites in platelets at Baseline.
- To investigate relationships between mtDNA methylation at Baseline with the CVD outcome at Follow-up.
- To assess adherence to the Mediterranean diet (MeDiet Score) at Baseline for the cohort of adults with overweight and obesity.
- To investigate the association between the mtDNA methylation at Baseline with the MeDiet Score at Baseline.
- To set up an *in vitro* culture of MEG-01 cells.
- To test the effect of polyphenolic treatment (Resveratrol, Quercetin, and Curcumin) on mtDNA methylation levels in MEG-01 cells.

Chapter 2: Methods

2.1 Samples overview

The samples used to analyse mtDNA methylation as a predictor of CVD risk and in response to diet are platelets, and their precursor megakaryocytes. Platelets have been collected from human participants (see section 2.2) from the SPHERE study, while megakaryocytes have been cultured *in vitro* using MEG-01 cell line (see section 2.3). Figure 2.1 depicts an overview of the samples' type [e.g. human platelets vs MEG-01 cell culture], of how these have been collected [e.g. the platelets come from the SPHERE study], how the mtDNA has been extracted from these samples, and how the mtDNA has been analysed.
In this chapter, I will first describe the origins of the samples (see section 2.2 and 2.3), and then the methodologies used to investigate mtDNA methylation (see section 2.4, 2.5, and 2.6). Finally, the methods for the analysis of dietary pattern (MeDiet) are described in section 2.8.

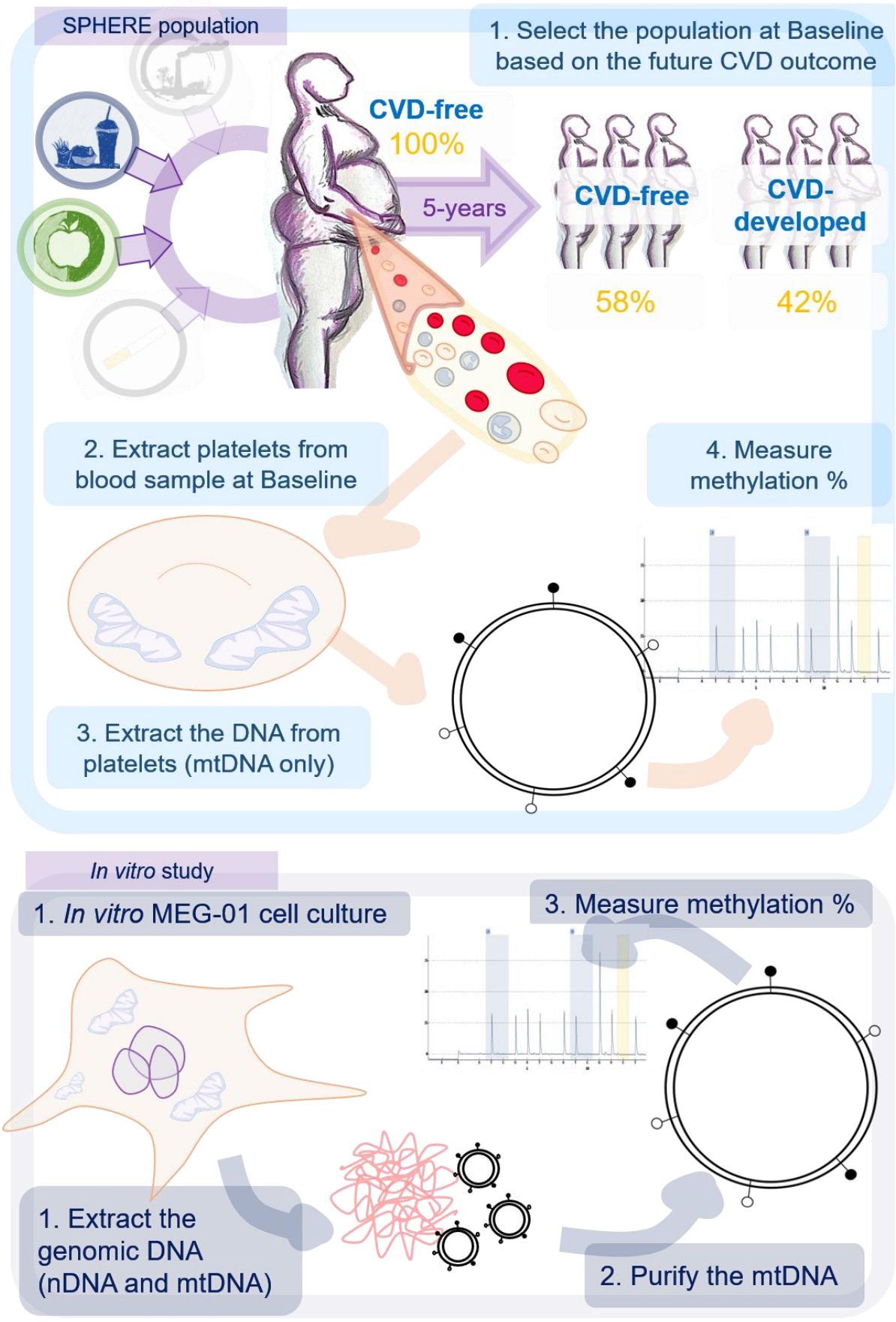


Figure 2.1 Summary of the samples

This image summarises the samples used to investigate the question "does mtDNA methylation change with increased risk of developing CVD and in response to diet?". There are two sample types: platelets and MEG-01 cells.

The top panel provides an overview of the use of platelets for mtDNA methylation analysis. The plasma samples had been collected within the "SPHERE study" at Baseline (n=200 participants CVD-free). The 200 participants were selected from the SPHERE population using a nested case-control design. The nested case-control design was chosen so that it is possible to compare the outcomes (CVD during Follow-up) of two groups of participants that were apparently indistinguishable at Baseline.

Platelet-rich plasma was extracted from blood collected at Baseline, and DNA was extracted from the platelets. Since there is no nucleus in the platelets, it was mtDNA. Finally, the mtDNA methylation percentage was measured using BS-pyrosequencing.

The bottom panel outlines the use of MEG-01 cells for mtDNA methylation analysis. First, MEG-01 cells were cultured in vitro. Then, genomic DNA was extracted from MEG-01 cells. Since MEG-01 cells have both multiple nuclei and mitochondria, the extracted DNA is a mixture of nDNA and mtDNA. Therefore, mtDNA was isolated with a specific protocol and, subsequently, methylation at specific loci was measured.

The nucleus is represented in violet; the nDNA is represented in pink.

End of figure legend.

2.2 Selection of participants from the SPHERE (Susceptibility to Particle Health Effects, miRNAs and Exosomes) study.

2.2.1 The SPHERE Study

The major aims of my PhD were to determine whether patterns of platelet mtDNA methylation at Baseline predict risk of CVD (Chapter 5) and to investigate possible associations with habitual diet (Chapter 4). This required access to a prospective cohort study in which participants were extensively phenotyped at Baseline, including the collection of dietary and anthropometric data (e.g. BMI, age, weight, blood pressure, fasting blood glucose) and archiving of blood samples, and for whom detailed information on later CVD outcomes was available. For these reasons, The Susceptibility to particle health effects, miRNA and exosomes (SPHERE) Study was utilised (Bollati, Iodice, et al., 2014). The

SPHERE Study was set up to investigate the molecular mechanisms that link diet and other environmental exposures with disease development, in this case, CVD. The study design, research aims and measurements were approved by the local Institutional Review Board ('Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico' review board). During the study coordinated by Professor Valentina Bollati in Milan, 2,000 susceptible individuals, including adults with overweight (BMI between 25 and 30 kg/cm²) and obesity (BMI ≥ 30 kg/cm²) were recruited in Lombardy (Italy) between 2010 and 2015 and followed up for 5 to 8 years. At recruitment, the participants were free of chronic diseases, and had no previous diagnosis of cancer, stroke, or heart disease (Bollati, Iodice, et al., 2014). DNA samples, lifestyle and diet questionnaire were collected at Baseline (Figure 2.2).

In 2013, the average age of the participants was 51.9 ± 13.6, female participants represented 73.6% of the population, and the 95.8% were of white ethnicity. The health outcomes were recorded from the hospitalization data from the Italian National Health Service (Bollati, Iodice, et al., 2014). This population was suitable for our study inasmuch as it is representative to the general population, in which more than 50% of the individuals have overweight or obesity (CRUK, 2016), it is homogeneous in terms of CVD risk biomarkers (Table 5.1 and Table 4.1), and the participants were selected so that a those who would remain CVD-free at Follow-up and those who will develop CVD at Follow-up were sex- and BMI- matched. As a results, we selected 200 participants at Baseline, of which 42% developed CVD within 5 years and 58% remain CVD-free. The presence of comorbidities such as respiratory and kidney disorders was minimized in the group of CVD-developed at Follow-up. In fact, the total number of participants who developed respiratory diseases - primary or secondary diagnosis- as comorbidities of CVD is n=17.

The SPHERE* cohort

*Susceptibility to particle health effects, miRNA and exosomes (SPHERE)

Question:

Examine molecular mechanisms underlying the effects of PM exposure in relation to health outcomes (e.g. CVD)

Primary outcome: cardiometabolic and respiratory health effect

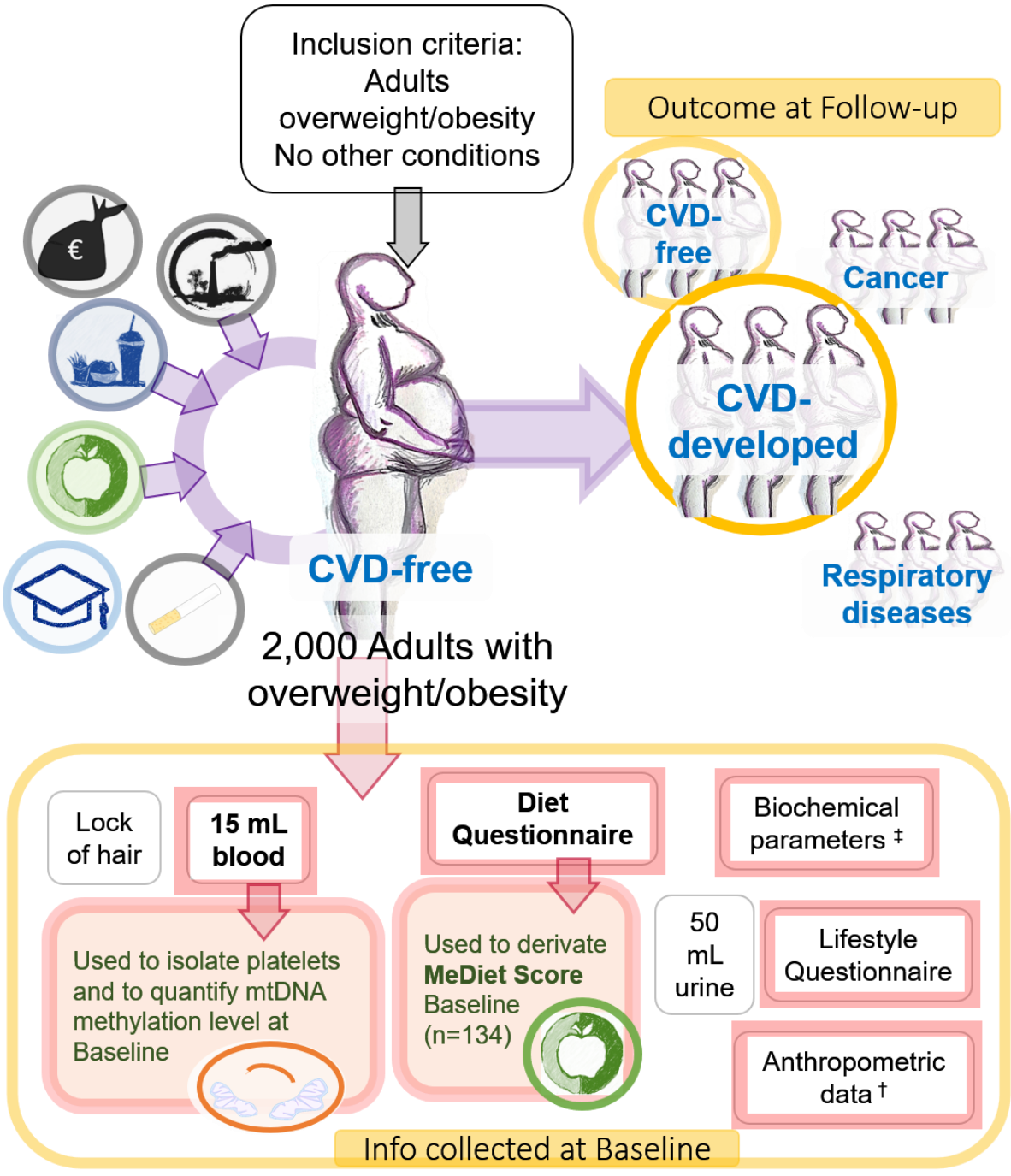


Figure 2.2 Description of the SPHERE cohort

This image summarises the main question of the SPHERE study (upper section) and how the participants have been recruited (lower section). In the centre (black square) there are the inclusion criteria, in the bottom section there is the range of information that has been collected for each participant at Baseline (yellow rectangle), and the right side the outcome that has been recorded for the SPHERE study.

At Baseline, 2,000 participants with overweight or obesity were recruited. The participants did not have a previous diagnosis of Alzheimer's disease, Parkinson's disease, bipolar disorder, heart disease or stroke, multiple sclerosis, or cancer (Bollati, Iodice, et al., 2014). Information about the participants' background, education, lifestyle, diet, air pollution exposure, and smoking habit were collected at Baseline. Additionally, a full blood analysis, urine analysis, and anthropometric measurements (e.g. BMI, waist-to-hip ratio, age, and sex) were collected at Baseline. These 2,000 adults were followed for 5-8 years and the development of CVD, pulmonary diseases, cancer and other diseases have been recorded.

For my project, 200 participants were selected using a nested case-control study design. The participants were stratified according to the diseases outcome: CVD-free at Follow-up or CVD-developed at Follow-up. The participants were sex- and BMI- matched and the blood samples from the first visit (Baseline) was analysed for mtDNA methylation level.

PM: Particulate Matter

The red rectangles highlight the information about the participants that have been used during the analysis.

[†]Anthropometric parameters include: age, sex, blood pressure, body weight, height, waist circumference, waist-to-hip ratio, spirometry, and electrocardiogram (ECG).

[‡]Biochemical parameters include Hemogram, Fibrinogen, C-reactive protein, Total cholesterol, HDL, LDL, Triglyceride, Serum creatinine, AST, ALT, Gamma-Glutamyltransferase, fasting blood glucose, Homocysteine, TSH, Glycated haemoglobin, Postprandial glycaemia, Insulin level, 2-hour pos- glucose insulin level, platelets count, erythrocytes count, Urinary pH, Uric acid.

2.2.1.1 Nested case-control design

The nested case-control design was chosen so that it is possible to compare the outcomes (CVD during Follow-up) of two groups of participants that were apparently indistinguishable at Baseline. In this study, I investigated the use of mtDNA methylation as a predictive biomarker in a healthy population (Chapter 5) and for an association with diet at Baseline (Chapter 4). Given the fact that mtDNA methylation is typically very low and that this was a healthy population, the expected differences between CVD-free and CVD-developed at Follow-up in mtDNA methylation were predicted to be small. Likewise for the differences in mtDNA methylation associated with diet (n=134). Therefore, it was extremely important to reduce all the sources of possible confounders to have enough power to detect small differences in mtDNA methylation. Accordingly, the selection of a sub-set of 200 participants from the 2000 SPHERE participants, was performed as follow:

i) The participants did not develop other diseases, including cancer, respiratory, and kidney diseases during the period of follow-up.

ii) The participants should be distributed between the CVD-developed (42%) and CVD-free (58%) at Follow-up. Incidence of 42% of CVD is higher than by chance, as it around 28% for the men and 20% of the women in the UK between 55 to 74 years old (Scholes et al., 2018). In this way, it is possible to better highlight the small changes in mtDNA methylation biomarker in the healthy population at Baseline.

iii) The participants were sex and BMI-matched at Baseline.

Figure 2.3 summarises the selection of the subset of participants that used in this project. At Baseline n=200 CVD-free participants were recruited and their anthropometric and cardiometabolic risks factors were recorded, plasma samples were collected, and the food frequency questionnaire was completed by n=134 participants. At Follow-up, after 5 years, n=84 of these 200 participants had developed CVD, while n=116 remained CVD-free.

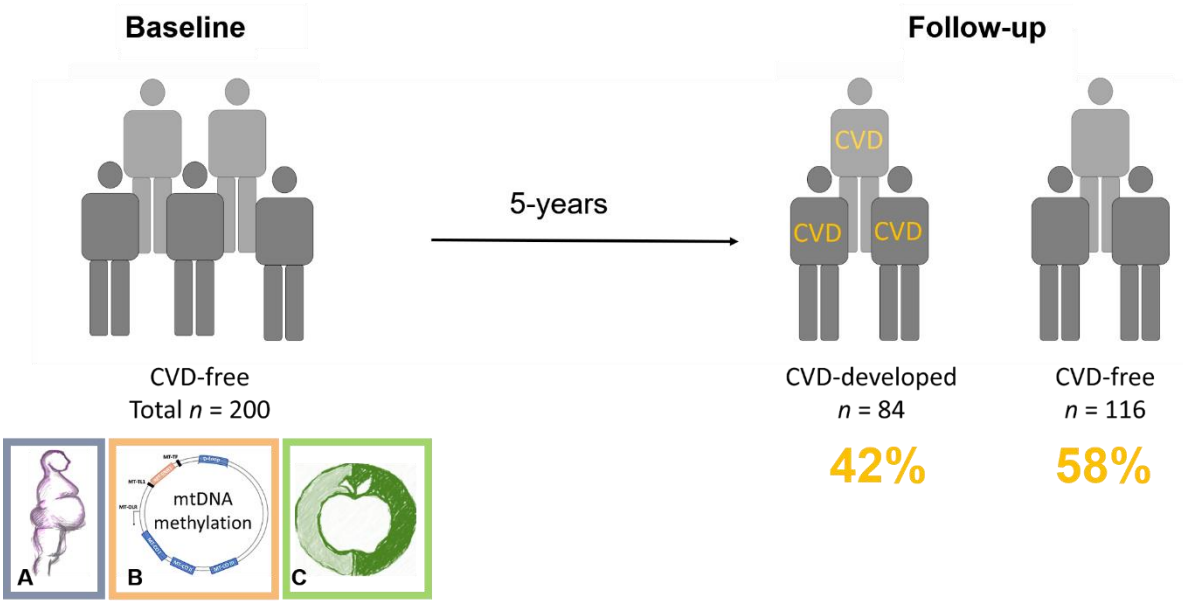


Figure 2.3 Nested case-control study design and selection of participants.

*On the left side of the image, at Baseline, the population of adults with overweight and obesity (n=200) is CVD-free. Within 5-years follow-up, on the right-hand side, 42% of the participants (n=84) developed mild or severe CVD events, while 58% of the participants remained CVD-free (n=116). Being a nested case-control study, the participants have been chosen so that the CVD-free and CVD-developed at Follow-up groups of participants are sex-matched and BMI-matched. Additionally, information about CVD risk factors (**A**) and food frequency questionnaire (**C**) were collected at Baseline by the collaborators. I analysed the patterns of mtDNA methylation in platelets collected at Baseline (**B**).*

2.3 MEG-01 cell culture

The MEG-01 cell line (Sigma-Aldrich; 94012401) that originated from human megakaryoblastic leukaemia, was purchased from Sigma-Aldrich (Ogura et al., 1985). This cell line was Accredited by the European Collection of authenticated Cell cultures (ECACC) operated by Public Health England and all the karyotypes are characterized by the Philadelphia chromosome (Ogura et al., 1985). The MEG-01 cell line was cultured in RPMI 1640 (Sigma-Aldrich; R0883 or R8758) plus 2mM L-Glutamine solution (Sigma-Aldrich; G7513), 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich; F9665), and 10 mL/L of Penicillin-Streptomycin 10 mg/mL (Sigma-Aldrich; P4333-100ML). The cells were grown in T25, T75, or T150 flask (Starlab; CC7682-4825, CC7682-4875, CC7682-4815 respectively) and kept in the incubator at 37˚C with 5% CO_2.

2.4 Extraction of mtDNA

2.4.1 Extraction of mtDNA from platelets

This section describes the protocol that was used to extract mtDNA from platelets. This protocol has also been described in previous publications from our research group (Corsi et al., 2020; Byun, 2019).

2.4.1.1 Isolation of platelets from plasma and mtDNA extraction

Plasma samples from 200 participants were used to isolate platelet mtDNA as described previously (Baccarelli & Byun, 2015). Blood collected in EDTA tubes was processed to obtain plasma (Bollati, Iodice, et al., 2014) and 200 µl plasma was centrifuged at 1400 x*g* to obtained the platelet-rich pellet. Since cell-free DNA of nuclear origin may be attached to the platelet surfaces, this was eliminated by treatment with DNaseI (30U, Roche: 4716728001) (Table 2.1).

Step	**Amount**	**Incubation**
Treatment	3 µl DNaseI (30U)	37˚ C for 3 hours or Room temperature overnight
	1.2 µl 10x buffer	
	7.8 µl water	
Inactivation	10 minutes incubation at 75 °C	

Table 2.1 DNaseI treatment of cells

DNaseI (Roche: 4716728001)

DNaseI was heat-inactivated by 10 minutes incubation at 75 °C. The EZ DNA Methylation Direct kit (Zymo Research; D5021) was used for extraction and bisulfite conversion of mtDNA simultaneously. First the platelet pellet was digested using proteinase K. To 12 µl of platelets sample were added 13 µl M-Digestion Buffer (2X) and 1 µl Proteinase K. The digestion took place at 50˚C for 2 hours. Then the digested sample was centrifuged for 5 minutes at 10,000 x g and the supernatant used for the following steps. To maximise bisulfite conversion efficiency (Liu et al., 2016), mtDNA was linearised with *Bam*HI (Thermo Fisher; ER0052) for 1 hour at 37 ˚C. The 20 µl of linearised mtDNA was bisulfite converted using

130 µl of CT Conversion Reagent solution. The tube was placed in a thermal cycler to undergo 98°C incubation for 8 minutes [denaturation step], and 64°C incubation for 3.5 hours [bisulfite conversion]. The samples was placed on the Zymo-Spin™ IC Column containing 600 µl of M-Binding Buffer, then centrifuged at >10,000 x g for 30 seconds. The flow-through was discarded. Then 100 µl of M-Wash Buffer were added to the column and centrifuged again. 200 µl of M-Desulphonation Buffer were added to the column and incubated at room temperature (20-30°C) for 15-20 minutes. The fully bisulfite converted mtDNA was eluted in 20 µl water and stored at -80^{o}C until analysis.
Bisulfite-PCR reactions were performed using 1 µl of bisulfite-converted mtDNA, 9 µl water, 12 µl Hot-Start GoTaq® DNA Polymerase (Promega; M5123), 1 µl forward primer (10 pmole) and 1 µl reverse biotin-labelled primer (10 pmole) (see section 2.5 for details on PCR and agarose gel).

2.4.2 MEG-01 mtDNA methylation analysis

This section describes the protocol that was used to extract mtDNA from MEG-01 cells and to investigate methylation at specific mtDNA loci.

2.4.2.1 Isolation of genomic DNA

Genomic DNA, composed of both nuclear and mitochondrial DNA, was isolated using the DNeasy Blood & Tissue Kit (Qiagen; 69504) according to the manufacturer instructions. The cells in PBS/medium were centrifuged at 3631 rpm (1400 xg) for 15 mins. To remove cell-free DNA from the cell surfaces, DNaseI (Roche: 4716728001) treatment was performed (Table 2.1). Subsequently, to lyse the cells, 200 µl of the guanidine salt detergent "Buffer AL" was added the sample, the mixture was vortexed immediately for 20 sec and then incubated at 56°C for 10 minutes. The sample was then washed with 200 µl 100% absolute ethanol (Fisher BioReagents™, BP2818-100) and transferred to the DNeasy Mini spin column. While the DNA was still attached to the column filter, it was washed first with 500 µl of "Buffer AW1" and then with 500 µl of "Buffer AW2". Finally, the genomic DNA was eluted in 50 µl of elution buffer "Buffer AE".

2.4.2.2 Purification of mtDNA

To study mtDNA methylation, it is essential to purify the mtDNA and to ensure that it does not contain NUMTs of nDNA origin (Lambertini & Byun, 2016; Owa et al., 2018;

Jayaprakash et al., 2015) (Figure 2.1 and 2.4). To do so the circular nature of mtDNA can be used to digest the nuclear DNA with Plasmid-Safe™ ATP-Dependent DNase (Epicentre; E3110K). This will digest only the linear nDNA while the circular mtDNA remains intact. Subsequently washing the DNA using paramagnetic beads Agencourt AMPure XP (Beckman Coulter; A63881). The digestion reaction and the subsequent washing were repeated 8 times, as described by (Jayaprakash et al., 2015).

After mtDNA had been purified, it was bisulfite converted using EZ DNA Methylation-Gold kit (Zymo Research; D5005). Finally, PCR was used to assess the presence of nuclear DNA using primers for the nuclear gene *PPARCG1A* (gene ID: NM_013261*)* (see section 2.5.2.2 and Figure 2.6).

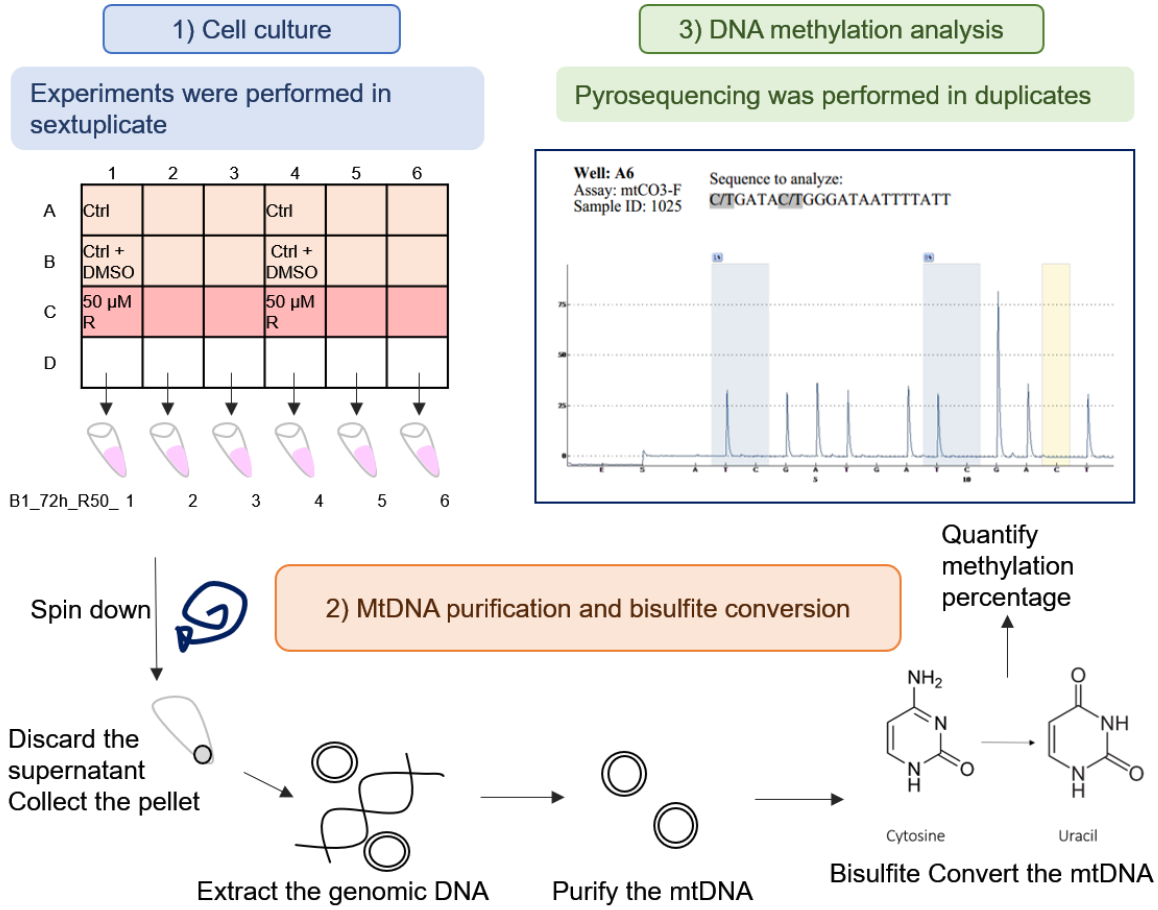


Figure 2.4 Purification of mtDNA from MEG-01 cells.

In this conceptual diagram the stages of cell culture and treatment (1), genomic DNA extraction, mtDNA purification (2), and methylation analysis (3) are reported.

2.5 Characterisation and quantification of mtDNA methylation

The major aim of my project was to investigate whether patterns of mtDNA methylation predict CVD risk. Therefore, during my project, I focused on the analysis of CpG sites whose methylation have been found to be higher in participants with CVD (Baccarelli & Byun, 2015) and in response to environmental factors associated with CVD development (Byun & Barrow, 2015a; Byun et al., 2013, 2016). In addition, I have devised a further primer set to investigate methylation of the L-strand replication origin *MT-OLR* (Table 2.2).

2.5.1 Bisulfite conversion

Bisulfite conversion is a chemical method used to distinguish methylated cytosine (5mC) from unmethylated-cytosine within a known sequence, that was devised initially by Frommer et al 1992 and by Clark et al 1994 (Frommer et al., 1992; Hayatsu, 2008).
To be fully accessible to sodium bisulfite and to ensure optimal conditions for conversion of cytosine to uracil is required a linearised, single-strand DNA molecule (Owa et al., 2018) (Figure 2.5). To maximize the BC efficiency when using mtDNA, the circular mtDNA molecule was digested with *BamHI* (NEB; R0136 or Thermo Fisher; ER0052) and therefore linearised (Liu et al., 2016; Owa et al., 2018). Bisulfite conversion was performed using the EZ-DNA methylation Direct Kit (Zymo Research; D5021) for platelets mtDNA (see section 2.4.1), while mtDNA from MEG-01 cells was bisulfite converted using EZ DNA Methylation-Gold kit (Zymo Research; D5005) (section 2.5.1.1).
During BC, mtDNA undergoes not only chemical modifications, with cytosines converted to uracils, but also physical modifications, with large double-strand DNA molecules, fragmented into single-stranded mtDNA pieces. For these reasons, the BC mtDNA was aliquoted in two tubes, one preserved at -80 °C and one at -20 °C for direct use in PCR.

2.5.1.1 Bisulfite conversion of mtDNA from MEG-01 cells

Purified mtDNA from MEG-01 cells (section 2.4.2.2) was linearised using *BamHI* (Thermo Fisher; ER0052). 17 µl of the sample were mixed with 1 µl *BamHI* enzyme and 2 µl 1x Buffer and incubated at 37˚C for 1 hour. At this point, the kit EZ DNA Methylation-Gold kit (Zymo Research; D5005) was used to BC the linearised mtDNA. First, 130 µl of the CT conversion Reagent was added to 20 µl of linearised mtDNA. The sample was pipetted up and down and then incubated at 98 °C for 10 minutes for heat denaturation and then at 64 °C for 2hours and 50 minutes for bisulfite conversion. Then, the BC mtDNA was bound to the

filter of the Zymo-Spin IC Column that had been washed with 600 µl of M-Binding Buffer. The column was subsequently washed with 100 µl of M-Washing Buffer, and then the BC mtDNA was desulphonated with 200 µl of M-Desulphonation Buffer for 20 minutes at RT. After desulphonation (Figure 2.5) the fully BC mtDNA was ready to be washed twice with 200 µl of M-Wash Buffer and eluted with 20 µl of M-Elution Buffer.

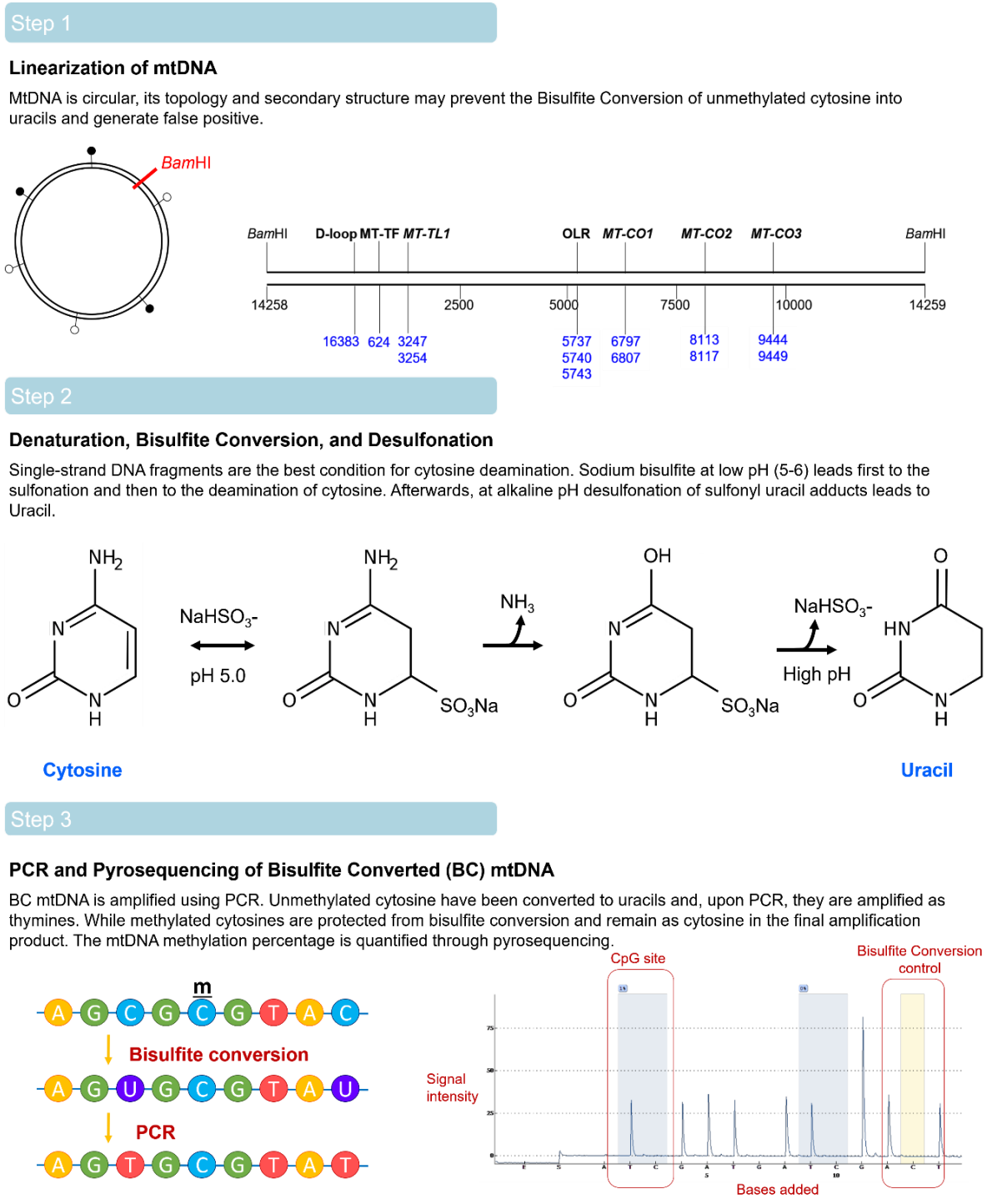


Figure 2.5 Bisulfite Conversion to analyse mtDNA methylation

Step 1) *The circular mtDNA was linearised using BamHI.* ***Step 2)*** *at low pH, Sodium Bisulfite first sulfonates and then deaminates unmethylated cytosines. By increasing the pH to an alkaline level the sulfonyl uracil is desulfonated to uracil. Although deamination of 5-mC has been reported upon treatment with Sodium Bisulfite, this happens very slowly and there is no risk of false-negative as long as the treatment with $NaSO_3$- lasts only a few hours (Hayatsu, 2008). Therefore, the protocol used for my samples involved a deamination step of about 3 hours and the treatment of mtDNA with $NaSO_3$ lasted only 20 mins.*

Step 3) *The modified mtDNA was amplified using PCR. The unmethylated cytosines had been converted to uracils and, upon PCR, they were replicated as thymines. This product was sequenced using pyrosequencing, and by comparing the original sequence to the BC one, the methylation status of the DNA was deducted.*

2.5.2 PCR

A comprehensive list of the primers used for mtDNA methylation analysis is provided in Table 2.2. The primers used to amplify the BC mtDNA sequence were highly selective as demonstrated by either testing on Rh0 cells or using bioinformatic methods (Byun et al., 2013). Additionally, the CpG sites of interest were close to the sequencing primer to reduce the chances of having an SNP that would produce aberrant peaks in the pyrogram (Tost & Gut, 2007; Bintz et al., 2014). For example, for *MT-CO1*, the sequencing primer was located only three nucleotides before the CpG site of interest.

The primers used to interrogate CpG sites within *MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-TL1*, the mitochondrial D-loop, and *MT-TF* were selected from the literature (Baccarelli & Byun, 2015; Byun et al., 2013). The Light-strand origin of replication "*MT-OLR"* primer was designed based on the function of the region, as there is a promoter, and on the structure of the region, which contains 5 CpG sites, two of which are within a "CCGG" sequence. In fact, the "CCGG" region is the recognition site for two methylation-sensitive restriction enzymes *HpaII* and *MspI* (Saini et al., 2017). This may lead to higher methylation to protect the mtDNA from restriction enzymes, but this is not fully supported by data (Liu et al., 2016).

The optimal amount of DNA to undergo BC is 200-500 ng. However, due to the much smaller amount of mtDNA available for BC, it is difficult to assess its quantity and quality directly. Hence, the BC mtDNA was used for PCR and then each amplificated sample was visualised on an agarose gel.

Bisulfite-PCR reactions were performed using 1 µl of bisulfite-converted mtDNA, 9 µl nuclease-fee water (Qiagen;129114), 12 µl Hot-Start GoTaq® DNA Polymerase (Promega; M5123), 1 µl forward primer (10 pmole) and 1 µl reverse biotin-labelled primer (10 pmole). The samples were arranged in 96-wells PCR plates (Starlab; E1403-1200) together with the positive (a known sample of mtDNA) and negative (sample with no DNA) controls, the plate was sealed and placed in the "Sensoquest lab cycler" (Göttingen, Germany) thermocycler for commencing the programme as described in table 2.3.

Assay	**Primer sequence**	**Measured CpG**	**Nucleotide (nt#) ***	**Product size (bp)**	**Annealing temperature (°C)**
MT-CO1	Forward: TATTAATTGGTTTTTTAGGGTTTAT Reverse-biotin labelled: CAACAAATCATTTCATATTACTTCC Sequencing primer: TATTTATAGTAGGAAT Sequencing entry: AGAC/tGTAGATATAC/tGAGTATATTTTATTTT	CpG1 CpG2	6797 6807	177	52
MT-CO2	Forward: TTTATGAGTTGTTTTTATATTAGGTTTAAA Reverse-biotin labelled: ACTCCACAAATTTCAAAACATTAAC Sequencing primer: TAAAAATAGATGTAAT Sequencing entry: TTTC/tGGAC/tGTTTAAATTAAA	CpG1 CpG2	8113 8117	123	52
MT-CO3	Forward: TATATTATTTGTTTAAAAAGGTTTT Reverse-biotin labelled: AATAAAAAACTCAAAAAAATCCTAC Sequencing primer: TATATTATTTGTTTAAAAAGGTTTT Sequencing entry: CGATAYGGGATAATTTTATT	CpG1 CpG2	9444 9449	95	52
MT-TL1	Forward: TAGGGTTTGTTAAGATGGTAGAGTT Reverse-biotin labelled: ACAATAAAAAATAAAAAATTAACCATAAAT Sequencing primer: TAGGGTTTGTTAAGATGGTAGAGTT Sequencing entry: TAGGGTTTGTTAAGATGGTAGAGTT	CpG1 CpG2	3247 3254	117	52
D-loop	Forward: TGTGTAGATATTTAATTGTTATTA Reverse-biotin labelled: CAAATCTATCACCCTATTAACCAC Sequencing primer: TAATTAATTAATATATTT Sequencing entry: TAGTAAATATGTTC/tGTTT	CpG1 CpG2 CpG3	16383 16399 16407	254	52
MT-TF	Forward: TAAAGTAATATATTGAAAATGTTTAGA Reverse-biotin labelled: TACTTAATACTTATCCCTTTTAAT Sequencing primer: TATTGAAAATGTTTA Sequencing entry: GAC/tGGGTTTATATTA	CpG1	624	168	52
MT-OLR	Forward: AATTGGTTTTAATTTATTTTTTT Reverse-biotin labelled: AACCTCTTTTTACCAACTCC Sequencing primer: AATTGGTTTTAATTTATTTTTTT Sequencing entry: C/tGTC/tGTC/tGGGAAAAAAGGT	CpG1 CpG2 CpG3 CpG4 CpG5	5737 5740 5743 5755 5767	126	52

Table 2.2 PCR primers and Pyrosequencing assays used to analyse mtDNA methylation.

This table lists the Forward, Reverse, and Sequencing primers for the genes *MT-CO1, MT-CO2, MT-CO3, MT-TL1*, D-loop, *MT-TF*, and *MT-OLR*.

Temperature	time	Step	Number of cycles
94˚C	2 min	Initialization	X 1
94˚C	40 sec	Denaturation	X 50
54 ˚C	40 sec	Annealing	
72 ˚C	40 sec	Elongation	
72 ˚C	5 min	Final extension	X 1

Table 2.3 Thermocycler programme for PCR.

Thermocycler programme for the amplification of bisulfite converted (BC) mtDNA genes and BC nDNA gene *PPARCG1A* (section 2.5.2.2). Polymerase Chain reaction (PCR) consists of consecutive cycles in which the double-strand (ds) DNA is first denatured to single-strand (ss) DNA, then the primers hybridize to their complementary template sequence. Primers are extended by polymerase enzyme to amplify the target region.

2.5.2.1 Preparation and use of an Agarose gel

The agarose gel was used to check the quality of the PCR product and to confirm that the correct sequence had been amplified by comparing it against a positive control. To verify the specificity of the PCR amplification, the absence of product in the negative controls was checked for every PCR reaction.

First stock solution of 50X Sodium Borate was prepared as follow: 20 gr NaOH powder and 112.5 g of Boric Acid were mixed with distilled water to a total of 1L. The pH was measured and, if necessary, adjusted to pH 8.0. The agarose gel was made by melting 2 g of agarose powder (Sigma-Aldrich; A9539-500G) in 100 mL of 1% Sodium Borate buffer to create a 2% agarose gel. 2 µl of PCR product was loaded, which already contains the die (Hot-Start GoTaq® DNA Polymerase in a Ready-to-Use Master Mix, colour green), in the well and run at 200 V for 15 minutes. To confirm the size of the bands 1 µl of 100 bp DNA Ladder was used (NEB; N3231L).

2.5.2.2 Detection of Nuclear DNA contamination

After mtDNA bisulfite conversion, nDNA contamination was assessed using PCR primers designed for a nDNA gene (Baccarelli & Byun, 2015; Jayaprakash et al., 2015). The *PPARCG1A* gene primers (PPARGC1A-F: TTTTTTGTGTAKTTTGTTGTTTTAA and PPARGC1A-R: TTACAAAAAATTTTAATTATTATATAACCA) were used. PCR was performed according to the description above (section 2.5). The PCR products were visualized on a 2% agarose gel (Figure 2.6) according to section 2.5.2.1.

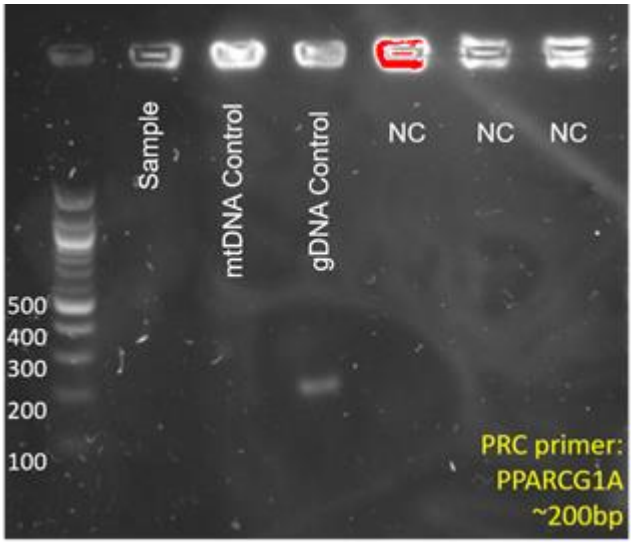


Figure 2.6 The absence of nuclear contamination in purified mtDNA

This image shows the PCR results for PPARCG1A using mtDNA isolated from genomic DNA (see section 2.4.2.1). This method was also used to verify the purity of mtDNA extracted from platelets (see section 2.4.1).

In this case, the "Sample" is mtDNA isolated from the genomic DNA using the Mseek method as described by (Byun & Barrow, 2015a)(section 2.4.2.2). The PCR primer PPARCG1A has an amplification product of ~200bp as confirmed by the positive control genomic DNA (gDNA) Control. Additionally, the mtDNA Control, which is pure mtDNA, does not present any bands similar to the negative controls (NC).

2.5.3 Pyrosequencing

Pyrosequencing is a reproducible and accurate sequencing-by-synthesis method that allows researchers to monitor quantitatively the incorporation of nucleotides into a growing DNA strand (Tost & Gut, 2007).

Pyrosequencing involves the primer-directed sequencing of a known template by the subsequent addition of nucleotides (Figure 2.5). Each nucleotide is conjugated with a pyrophosphate (PPi) molecule that is released and converted into light (Figure 2.7). Pyrosequencing technology is based on a reaction cascade system orchestrated by four main enzymes:

i) DNA polymerase, which catalyses the elongation of the DNA template using the correct deoxynucleotide pyrophosphate.
ii) ATP sulfurylase, which generates ATP from PPi.
iii) Luciferase, which oxidizes D-luciferin into oxyluciferin the light-emitting compound.
iv) Apyrase, which degrades unincorporated nucleotides at each sequential step.

The amount of light generated is proportional to the number of nucleotides that are incorporated into the synthesized DNA strand. After the release of each nucleotide, the light is recorded as a peak and the height of the peak gives quantitative information about how many nucleotides have been incorporated and allows researchers to annotate the correct sequence.

The dispensation order is based on the known sequence and, if the nucleotide is not incorporated, no light is released and this is recorded as no peak in the pyrogram. This "no-signal" position can be used as a built-in control for single nucleotide polymorphisms (SNP), the heteroplasmic region of the mtDNA, and C/T control to check the bisulfite conversion efficiency (see Chapter 2.2.3.2) (Tost & Gut, 2007; Bintz et al., 2014).

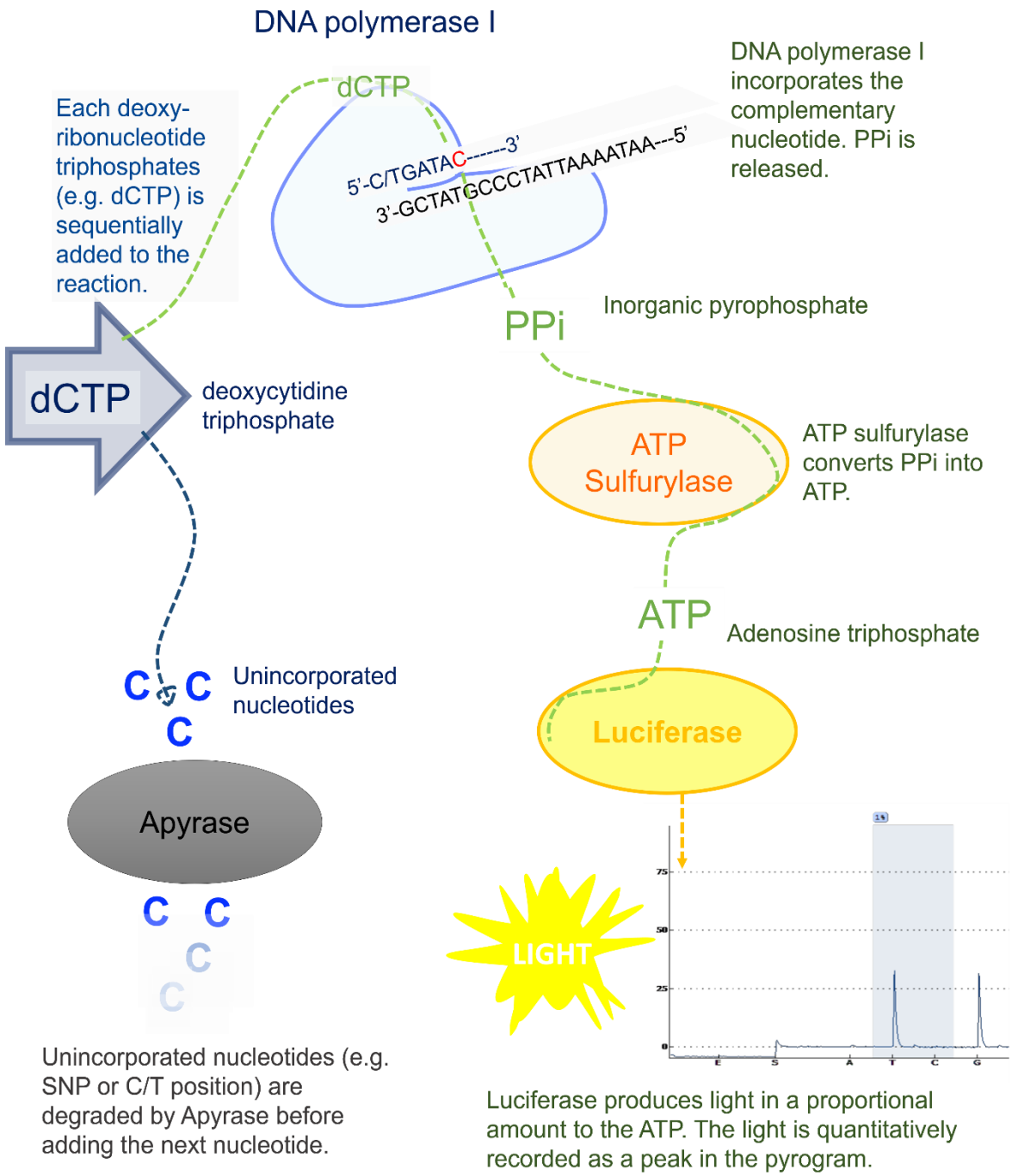


Figure 2.7 Pyrosequencing technology

The enzymatic cascade of pyrosequencing technology. At first, one single deoxynucleotide triphosphate is added; in this image, it is the deoxycytidine triphosphate (dCTP) on the top left. If dCTP is complementary to the DNA template, the DNA polymerase will incorporate it in the nascent strand (5'to 3'). This results in the release of inorganic pyrophosphate (PPi) which is converted into ATP by ATP sulfurylase. Luciferase uses ATP to oxidize D-Luficerin, which results in the production of oxyluciferin. Oxyluciferin is in an

electronically-excited state and releases a photon of light (λ=560nm) when it goes back to ground-state. The photon, proportional to the amount of ATP, is detected by a charge-coupled device (CCD) camera (Tost & Gut, 2007).

2.5.3.1 Pyrosequencing protocol

Pyrosequencing was performed using a PyroMark Q96 ID (Qiagen) instrument and PyroMark Gold Q96 Reagents (Qiagen; 972804). For pyrosequencing, the use of a sequencing primer is needed. The sequencing primer is a single-stranded nucleic acid that is used by the DNA polymerases to add the nucleotides to synthesize the complementary strand. The sequencing primer can be the same as the PCR forward primer or a different one. For example, the sequencing primer was identical to the PCR forward primer for the genes *MT-CO3*, *MT-TL1*, and *MT-OLR* (Table 2.2). The PCR Reverse primer must be biotinylated, which means that a small biotin molecule is conjugated to the oligonucleotide. In this way, the single-stranded DNA molecule that it is synthesized from the biotinylated primer can be captured using Streptavidin. The single-stranded DNA template conjugated to biotin is isolated and used for the Pyrosequencing assay (Royo et al., 2007). All the primers were ordered from the company "Integrated DNA Technologies" (IDT). 96-well PCR plates (Starlab; E1403-1200) were used as a vessel for pyrosequencing reaction. Per each well, 10 μl of PCR product was mixed with Binding buffer Solution (Table 2.4) to immobilize the single-strand DNA template on the Streptavidin-Sepharose beads for the following purification and washing step on the Vacuum prep station (supplied with the PyroMark Q96). After being washed, the ssDNA bound to the beads is released in the PyroMark Q96 Plate Low (Qiagen; 979002) with the Annealing Buffer containing the sequencing primers. The plate low is then placed in the heating block inside the PyroMark Q96 instrument and secured with the plate-holding frame. In parallel, the "reagent cartridge" or "dispensation cartridge" (Qiagen; 979004) containing the nucleotides, each in one separate compartment, the enzymes, and the substrate, is placed in the dispensing unit of the PyroMark Q96 instrument.

Binding Buffer solution per well

1x Binding Buffer (Qiagen; 979006)	40 μl
Streptavidin-Sepharose beads (GE Healthcare; 17-5113-01)	2 μl
PCR product	10 μl

Water (Qiagen; 129114)	28 µl

Annealing Buffer Solution per well

Annealing Buffer (Qiagen; 979009)	38.4 µl
Sequencing primer (1pM)	1.6 µl

Table 2.4 Quantities of each solution added to each well of the 96-well plate for pyrosequencing

The Binding Buffer solution was added to the single-strand DNA template in the PCR plate. Then the DNA, captured by the Streptavidin-Sepharose beads was washed in alkaline conditions and then released in the Annealing buffer solution with the sequencing primers. The Sequencing primers for *MT-CO3, MT-TL1,* and *MT-OLR* assays were the same as the forward primers. For the assay of *MT-CO1, MT-CO2,* D-loop, and *MT-TF* the sequencing primer differed from the forward primer.

Pyrosequencing allowed each mtDNA position to be interrogated rigorously and and facilitated detection of low levels of variants present in the mixture (Bintz et al., 2014). This allowed the detection of heteroplasmic sites, SNP, and possibly NUMTS contamination, to direct the interpretation of the data (Bintz et al., 2014).

2.5.3.2 Pyrosequencing assays

Pyrosequencing assays were devised to maximise the accuracy of mtDNA methylation detection. Specifically, the primers and the sequencing assay were devised with internal quality controls to verify the bisulfite conversion efficiency (Owa et al., 2018; Liu et al., 2016; Baccarelli & Byun, 2015; Byun & Barrow, 2015a; Tost & Gut, 2007).

For example, the *MT-OLR* sequence that anneals with the *MT-OLR* forward primer contains eight cytosines that are converted into uracil during bisulfite conversion. Therefore, the primer was designed to anneal to the fully bisulfite converted sequence. Equally, the *MT-OLR* reverse primer contains three thymines that anneal with three uracils in the bisulfite converted *MT-OLR* sequence. These cytosines are called “conversion dependent annealing nucleotides” and their presence in the primers increases the specificity of the BC PCR product (Owa et al., 2018).

Further, not only are the primers specific for the fully BC sequence but also the *MT-OLR* sequence that is analysed contains two cytosines from non-CpG sites, also called CpH sites,

that serve as quality control during pyrosequencing for bisulfite conversion efficiency. The full list of primers is reported in Table 2.2.

Pyrosequencing was used to characterise and quantify DNA methylation within seven regions of the mtDNA: mitochondrially-encoded cytochrome-C-oxidases I, II, and III (*MT-CO1*, *MT-CO2*, and *MT-CO3*), mitochondrially-encoded tRNA leucine 1 (*MT-TL1*) and tRNA phenylalanine (*MT-TF*), D-loop, and mitochondrially-encoded light-strand origin-of-replication (*MT-OLR*). DNA methylation was measured at two CpG sites within *MT-CO1* [nucleotide (nt) positions 6797 and 6807], *MT-CO2* (nt8113 and nt8117), *MT-CO3* (nt9444 and nt9449) and *MT-TL1* (nt3247 and nt3254), at three CpG sites within D-loop (nt16383, nt16399, and nt16407) and *MT-TF* (nt624), and at five CpG sites within *MT-OLR* (nt5737, nt5740, nt5743, nt5755, and nt5767) (Figure 2.5 and Table 2.2). The mtDNA sequence and the nucleotide positions were based on the NCBI Reference Sequence: NC_012920.1. Amplified mtDNA PCR products were then used for the pyrosequencing reaction (PyroMark Q96 ID, QIAGEN) as described in section 2.5.3.1 (Baccarelli & Byun, 2015; Byun et al., 2013; Janssen et al., 2015; Lambertini & Byun, 2016; Byun & Barrow, 2015a; Corsi et al., 2020).

2.5.3.3 Pyrosequencing control

To establish the consistency and sensitivity of the pyrosequencing assay, "pyrosequencing control oligos" were run for each plate. Briefly, two custom-designed oligos were ordered; one with the presence of a "G" which would appear as a "C" in the complementary sequence synthesized during pyrosequencing and one oligo with an "A", which it is sequenced as a "T" during pyrosequencing. Since these controls do not require PCR or selection for single-strand molecules, the pyrosequencing controls were placed directly into the PyroMark Q96 Plate Low (Qiagen; 979002). In each well were added 1 µl of Oligo C -or Oligo T- (10 pmol/µl), 0.5 µl of the Sequencing Primer (pmol/µl) and 10.5 µl of Annealing Buffer. The sequencing reaction was performed using this sequencing entry C/TGTAT as described in Table 2.5. Figure 2.8 illustrates the pyrograms of 0% and 100% methylation controls.

100% methylation 0% methylation	Sequencing Primer: 5' AACGTTTGTTCCCGT 3' Oligo C: 5' TTGCGATACGACGGGAACAAACGTTG 3' Oligo T: 5' TTGCGATACAACGGGAACAAACGTTG 3' Sequencing entry: c/TgTAT

Table 2.5 Sequences of the control oligos used for pyrosequencing

This table reports the sequence and primers for assessing the consistency and sensitivity of the assay.

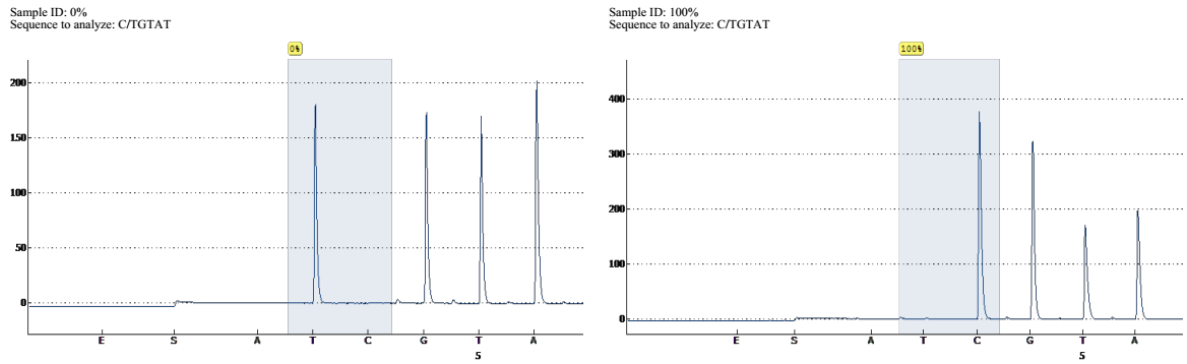


Figure 2.8 Pyrosequencing control oligos

This image represents the outputs from pyrosequencing run on the oligo "T", or 0% methylation, and the oligo "C", or 100% methylation assay. This test was performed to check the sensitivity and consistency of measuring 0% and 100% methylation.

Additionally, pyrosequencing positive controls, pyrosequencing negative control (annealing buffer and primers), and PCR negative control were added to the pyrosequencing plate.

2.2.3.4 Parameters used to interpret and quantify mtDNA methylation by pyrosequencing

Quantification of mtDNA methylation was performed using pyrosequencing. The quality of the results was evaluated by the PyroMark Q96 ID (Qiagen) software using the i) bisulfite treatment control, ii) the peak height, and the iii) Stringency levels.

i) The bisulfite treatment control was an internal control that evaluated the conversion efficiency of cytosines in the analysed sequence that are not followed by a guanine. The Bisulfite Treatment Control is expressed as a parameter in the "Analysis set up" that states the highest acceptable % of unconverted sequence to achieve the "passed" or "check" quality.

ii) Peak height threshold states the minimum signal value to be assigned to the "passed" or "check" quality.

iii) Stringency levels refer to how variable the peak height is at a specific position. The stringency level compares the pattern deviation and the sum deviation with theoretical values. These levels help identify SNPs.

The default values suggested by the PyroID96 Software user manual (described in Table 2.6) were used to distinguish methylation from background noise.

Unsuccessful Bisulfite Treatment	
Allowed percentage for passed quality	5.0%
Allowed percentage for check quality	7.0%
Peak Height Threshold	
Required peak height threshold for passed quality	7
Required peak height threshold for check quality	3
Stringency Levels	
Pattern variation in variable positions	Normal
Sum deviation in variable positions	Normal
Parameters	
A-peak reduction factor	0.90

Table 2.6 Pyrosequencing analysis parameters.

2.6 Quantification of total 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), and 5-formylcytosine (5fC) in platelets

I attempted to quantify the total amount of (global) 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), and 5-formylcytosine (5fC) in platelets using an ELISA-based method. This is of importance because it would give information on the whole epigenetic landscape of the cell by providing measurements of not only 5mC but also of the intermediates in its active demethylation (viz 5hmC, and 5fC).

Since bisulfite conversion does not allow researchers to distinguish between 5mC and 5hmC (Zampieri et al., 2015), specific antibodies were selected as a method of choice for this experiment.

Additionally, the majority of methylated cytosine in the mtDNA are suggested to be located outside the CpG sites (Iacobazzi et al., 2013; Patil et al., 2019; Morris et al., 2020).

First, the platelet pellet was treated with DNaseI (30U, ROCHE) to eliminate cell-free nuclear DNA. Then the platelet mtDNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen; 69504) (see section 2.4.1). The presence of 5mC in the whole mtDNA was assessed using MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) from

(Epigentek; P-1030). Additionally, I attempted to quantify the presence of 5hmC in the whole mtDNA, using MethylFlash Global DNA Hydroxymethylation ELISA Easy Kit (Epigentek; P-1032), and the presence of 5fC, using MethylFlash™ 5-Formylcytosine (5-fC)DNA Quantification Kit (Colorimetric) (Epigentek; P-1041). These kits were used following the manufacturer's instructions as described elsewhere (Barrow et al., 2018). In order to quantify the global 5mC content, a standard curve is generated using a 5mC positive control provided by the manufacturer. For 5mC, the 0.1%, 0.2%, 0.5%, 1%, 2% and 5% Positive controls (PC) were used. For 5hmC, 0.02%, 0.04%, 0.1%, 0.2%, 0.4%, and 1% 5hmC positive control were used. The negative controls (NC) contain un-methylated DNA. All samples were analysed in duplicate.

Briefly, the mtDNA was diluted to a final concentration of 20 ng in 8 µl volume. First, the mtDNA was bound to the assay wells that were specifically treated to have a high DNA affinity. This was done by mixing the mtDNA samples, the PC, and NC to 100 µl of the binding solution and add it to the well. The ELISA plate was then incubated at 37°C for 60 minutes. After the incubation, the binding solution was removed from the wells and the plate was washed with 150 µl of wash buffer three times. At this point, the 5mC antibody was added to the well and incubated at room temperature for 50 minutes to detect the methylated DNA fraction. After the incubation, the wells were washed five times with 150 µl of wash buffer. Finally, the colour developer solution was added to the wells and the absorbance generated was measured using a spectrophotometer. The developer solution was left in the wells for 5 minutes and the reaction was terminated with 100 ul of stop solution. The absorbance was measured at 450nm within 15 minutes and analysed using SkanIt software 4.1 (Thermo Fischer).

Unfortunately, the mtDNA amount that was extracted from 200µl plasma was not sufficient to obtain reliable ELISA results (data not shown).

2.7 Summary of the methods chosen to overcome mtDNA methylation controversy

Given the controversy surrounding mtDNA methylation, the methodology chosen to analyse mtDNA methylation, and especially its changes in healthy participants, is of paramount importance. Ascribed in Chapter 1, the most important consideration when studying mtDNA methylation is to determine which method that addresses the issues associated with: i) NUMTS, ii) circular and supercoiled mtDNA structure, and iii) low level of mtDNA methylation (Liu et al., 2016; Owa et al., 2018; Bintz et al., 2014; Lambertini & Byun, 2016;

Byun & Barrow, 2015a). Figure 2.9 summarises the steps taken in this project to address the controversy surrounding mtDNA methylation.

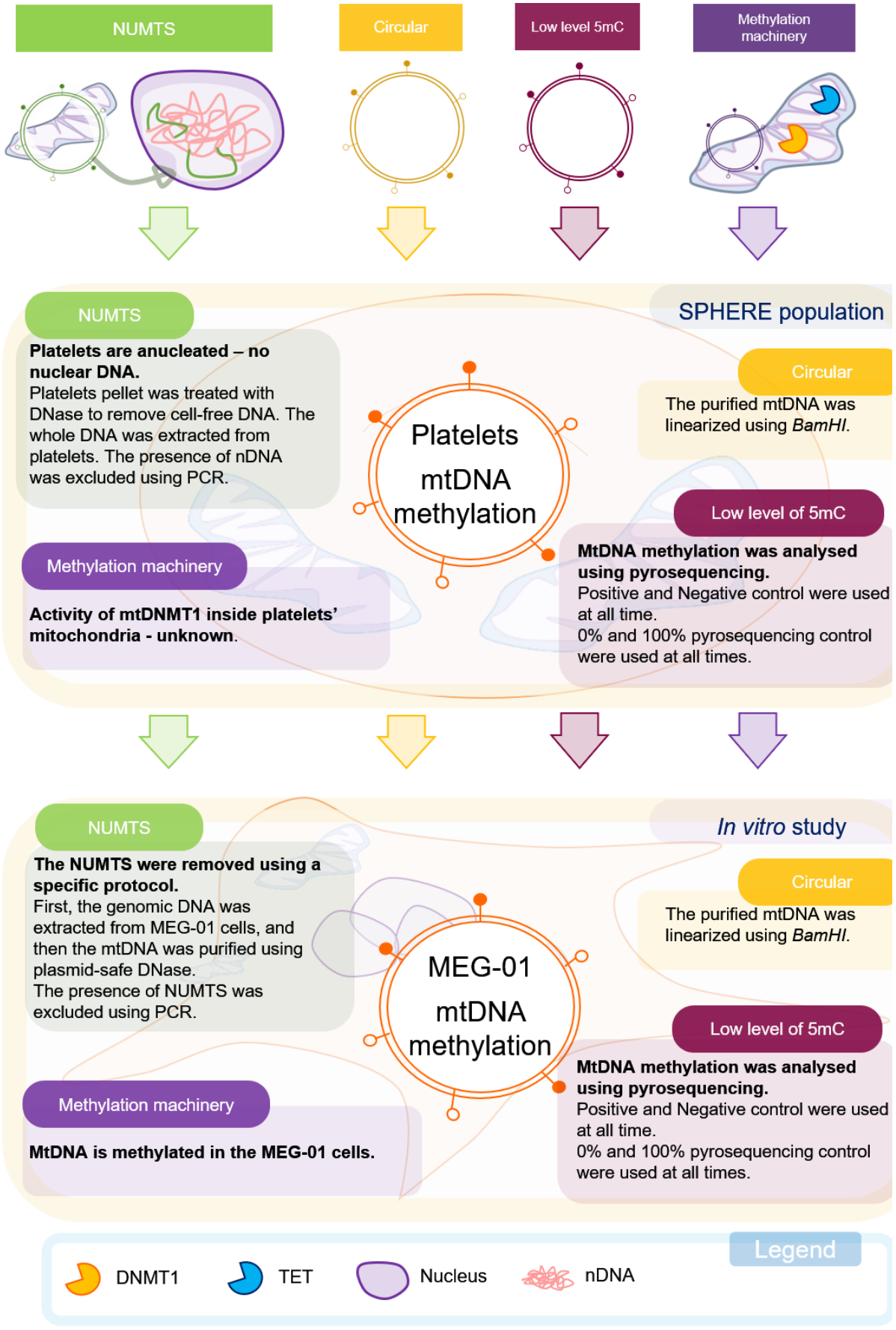

NUMTS
Circular
Low level 5mC
Methylation machinery
SPHERE population
NUMTS
Platelets are anucleated – no nuclear DNA.
Platelets pellet was treated with DNase to remove cell-free DNA. The whole DNA was extracted from platelets. The presence of nDNA was excluded using PCR.
Platelets mtDNA methylation
Circular
The purified mtDNA was linearized using BamHI.
Low level of 5mC
MtDNA methylation was analysed using pyrosequencing.
Positive and Negative control were used at all time.
0% and 100% pyrosequencing control were used at all times.
Methylation machinery
Activity of mtDNMT1 inside platelets' mitochondria - unknown.
In vitro study
NUMTS
The NUMTS were removed using a specific protocol.
First, the genomic DNA was extracted from MEG-01 cells, and then the mtDNA was purified using plasmid-safe DNase.
The presence of NUMTS was excluded using PCR.
MEG-01 mtDNA methylation
Circular
The purified mtDNA was linearized using BamHI.
Low level of 5mC
MtDNA methylation was analysed using pyrosequencing.
Positive and Negative control were used at all time.
0% and 100% pyrosequencing control were used at all times.
Methylation machinery
MtDNA is methylated in the MEG-01 cells.
Legend
DNMT1
TET
Nucleus
nDNA

Figure 2.9 Summary of the controversy surrounding mtDNA methylation and the adjustments adopted to analyse mtDNA methylation from platelets and MEG-01 cells.

The upper section summarises the issues to be addressed when attempting accurate measurement of mtDNA methylation i.e. the presence of NUMTs, the circular structure of mtDNA, the low level of 5mC, and the presence of the methylation machinery in the mitochondria.
The middle section illustrates the adjustment that I included in the protocol used to analyse mtDNA methylation in platelets. Whilst the platelets do not have a nucleus, they may be contaminated by DNA of nuclear origin that is present in plasma and which sticks on the platelet surface. Digestion with DNase was used to remove cell-free DNA.
Similarly, the lower section summarises the protocol adjustments used to analyse MEG-01 cell mtDNA methylation.

2.8 Assessment of dietary intake in the SPHERE study

A Food Frequency Questionnaire (FFQ), which reports monthly intakes of food items relevant to the Mediterranean Diet, was completed by 134 SPHERE participants out 200 (Figure 2.2 and 2.3). A translation of the original food frequency questionnaire used in the SPHERE study, devised by "Osservatorio Nazionale-Grana Padano" in 2005 and validated by (Festi et al., 2009), is reported in Table 2.7. A total of 134 participants completed the FFQ, of whom 70 were CVD-free at Follow-up (52%) and 64 developed CVD during Follow-up (48%).

2.8.2.1 Food Frequency Questionnaire

SPHERE Study food frequency questionnaire

Variable name	Variable classification	Variable name	Variable classification
Egg Stuffed Pasta	Weekly/Monthly diet	Butter	Weekly/Monthly diet
Pasta, Rice	Weekly/Monthly diet	Mayonnaise	Weekly/Monthly diet
Soup With Pasta	Weekly/Monthly diet	Beef White Or Red	Weekly/Monthly diet
Bread	Weekly/Monthly diet	Chicken, Turkey, Rabbit	Weekly/Monthly diet
Polenta	Weekly/Monthly diet	Pork	Weekly/Monthly diet
Pizza	Weekly/Monthly diet	Raw Ham	Weekly/Monthly diet
Bread Whole	Weekly/Monthly diet	Baked Ham	Weekly/Monthly diet

Crackers, Rusks, Bread Sticks	Weekly/Monthly diet	Bologna, Sausage	Weekly/Monthly diet
Cookies, Brioches	Weekly/Monthly diet	Salami, “Coppa”	Weekly/Monthly diet
Cornflakes	Weekly/Monthly diet	Whole Milk, Whole Yogurt	Weekly/Monthly diet
Snacks	Weekly/Monthly diet	Semi-Skimmed Milk, Low-Fat Yogurt	Weekly/Monthly diet
Pizza-Snack, White Pizza Bun	Weekly/Monthly diet	Green Cheese	Weekly/Monthly diet
Beans, Lentils, Chickpeas, Broad Beans, Soy	Weekly/Monthly diet	Ripened Cheese	Weekly/Monthly diet
Fresh Cooked Peas	Weekly/Monthly diet	Parmesan Cheese	Weekly/Monthly diet
Potatoes	Weekly/Monthly diet	Grated Parmesan Cheese	Weekly/Monthly diet
Carrots	Weekly/Monthly diet	Eggs	Weekly/Monthly diet
Raw Tomatoes	Weekly/Monthly diet	Fresh Or Frozen Fish	Weekly/Monthly diet
Tomato Sauce; Tomato Puree	Weekly/Monthly diet	Tuna, Other Fish In Oil	Weekly/Monthly diet
Salad Leaf	Weekly/Monthly diet	Chocolate	Weekly/Monthly diet
Cauliflower, Sprouts, Cabbage, Broccoli	Weekly/Monthly diet	Crisp	Weekly/Monthly diet
Spinach, Beets, Chard, Herbs And Catalonia	Weekly/Monthly diet	Ice cream	Weekly/Monthly diet
Peppers	Weekly/Monthly diet	Sweet Sparkling Drinks	Weekly/Monthly diet
Zucchini, Green Beans, Eggplant, Artichokes, Cucumbers	Weekly/Monthly diet	Fruit Juice (All Types)	Weekly/Monthly diet
Citrus	Weekly/Monthly diet	Honey	Weekly/Monthly diet
Apples	Weekly/Monthly diet	Wine (Glasses)	Weekly/Monthly diet
Apricot, Bananas, Pears, Peaches, Plums, Grapes	Weekly/Monthly diet	Beer (Jugs)	Weekly/Monthly diet
Nuts	Weekly/Monthly diet	Hard Liquor	Weekly/Monthly diet
Melon, Watermelon	Weekly/Monthly diet	Coffee (Small Cups)	Weekly/Monthly diet
Olive Oil	Weekly/Monthly diet	The (Cups)	Weekly/Monthly diet
Seed Oil	Weekly/Monthly diet		

Table 2.7 FFQ

Diet questionnaire used in the SPHERE study (Bollati, Iodice, et al., 2014) and devised by (Festi et al., 2009)

2.8.2.2. Mediterranean Diet Score

Adherence to the MeDiet was assessed by calculation of the "Mediterranean Diet Score" using single food items from the FFQ reported in Table 2.7. The "14-item Mediterranean Diet Adherence Screener (MEDAS)" developed originally by (Martínez-González et al., 2012) and updated by (Papadaki et al., 2018) was used to estimate the degree of adherence of each SPHERE Study participant to the Mediterranean diet (MeDiet) (Table 2.8). For example, for the question 1 "Do you use olive oil as main culinary fat?" of the MeDiet Score, the information of how much butter, margarine, seed oil and olive oil is consumed weekly/monthly in the FFQ is used to calculate the appropriate answer.

14-item Mediterranean Diet Adherence Screener (MEDAS)

Questions	Criteria for 1 point
1. Do you use olive oil as main culinary fat?	Yes
2. How much olive oil do you consume in a given day (including oil used for frying, salads, out-of-house meals, etc.)?	≥4 tbsp
3. How many vegetable servings do you consume per day? (1 serving: 200 g [consider side dishes as half a serving])	≥2 (≥1 portion raw or as a salad)
4. How many fruit units (including natural fruit juices) do you consume per day?	≥3
5. How many servings of red meat, hamburger, or meat products (ham, sausage, etc.) do you consume per day? (1 serving: 100–150 g)	<1
6. How many servings of butter, margarine, or cream do you consume per day? (1 serving: 12 g)	<1
7. How many sweet or carbonated beverages do you drink per day?	<1
8. How much wine do you drink per week?	≥7 glasses
9. How many servings of legumes do you consume per week? (1 serving: 150 g)	≥3
10. How many servings of fish or shellfish do you consume per week? (1 serving 100–150 g of fish or 4–5 units or 200 g of shellfish)	≥3
11. How many times per week do you consume commercial sweets or pastries (not homemade), such as cakes, cookies, biscuits, or custard?	<3
12. How many servings of nuts (including peanuts) do you consume per week? (1 serving 30 g)	≥3
13. Do you preferentially consume chicken, turkey, or rabbit meat instead of veal, pork, hamburger, or sausage?	Yes
14. How many times per week do you consume vegetables, pasta, rice, or other dishes seasoned with sofrito (sauce made with tomato and onion, leek, or garlic and simmered with olive oil)?	≥2

Table 2.8 MeDiet

Mediterranean diet score used to calculate the adherence of each participant to the Mediterranean diet (Papadaki et al., 2018).

Chapter 3: Investigating changes in patterns of mtDNA methylation in megakaryocytes in response to nutrients

3.1 Introduction

Dietary factors contribute to the CVD risk. The strongest impact on CVD risk is caused by dietary patterns low in whole grains, fruit, and vegetables (Meier et al., 2019; Angelino et al., 2019) (see section 1.5, Figure 1.1 and Figure 1.9). One of the possible positive effects of this dietary pattern is its antithrombotic effect (Violi et al., 2020). Interestingly, fruits and vegetables are rich in polyphenols, which have been associated with reduced CVD risk (Aune et al., 2018; Kim & Je, 2017) (see section 1.5.3 and Figure 1.10).

3.1.1 Polyphenols and platelet activity

Platelets are of primary importance in CVD (Figure 1.3) (Willoughby et al., 2002). Thrombotic complications are particularly high in adults with obesity (Hotoleanu, 2020). In fact, visceral adiposity is associated with significant and largely preventable venous and arterial thrombosis (Darvall et al., 2007; Hotoleanu, 2020). This is confirmed by the massive use of antiplatelet therapy to prevent CVD (Perk et al., 2012; Rothwell et al., 2018). Interestingly, all three polyphenols Resveratrol, Quercetin, and Curcumin have been found to mitigate the hyperaggregability of platelets *in vitro* (Yang et al., 2011; Gambini et al., 2015; Kim & Park, 2019; Vallance et al., 2019) and in humans (Hubbard et al., 2004a; Vallance et al., 2019). The mechanisms of platelets inhibition are still poorly defined, but the evidence suggests a direct effect of Resveratrol and Quercetin on platelets (Figure 4.1) (Shen et al., 2007; Lannan et al., 2016). Quercetin has been found to inhibit different aspects of thrombosis in humans. For example, calcium mobilisation, the exposure of phosphatidylserine on platelets, α-granule secretion, and collagen-induced and AA-induced activation are all reduced by the presence of Quercetin, suggesting that Quercetin may inhibit aggregation by multiple mechanisms (Vallance et al., 2019; Mosawy et al., 2013).

3.1.2 Polyphenols and mitochondria

Polyphenols are able to penetrate the mitochondrion and to regulate its activity (Naoi et al., 2019; Wood dos Santos et al., 2018). Resveratrol, Quercetin, and Curcumin promote mitogenesis (Aguirre et al., 2014; Zhou et al., 2019; Naoi et al., 2019) and have strong anti-apoptotic activity by direct regulation of the apoptotic pathway in the mitochondria (Naoi et al., 2019).

Additionally, Resveratrol and Curcumin have been suggested to inhibit mitochondrial lipid peroxidation in rat brain mitochondria (Aggarwal, 2010; Bagheri et al., 2020; Waseem & Parvez, 2016), and also in human platelets (Gambini et al., 2015). Interestingly polyphenols seemed to modulate the activity of electron transport complexes. Resveratrol has been found to increase significantly and specifically the activity of complex I of Electron Transport Chain (ETC) but not the enzymatic activity of the other complexes II and IV (Ferretta et al., 2014; Gueguen et al., 2015). Only one study reports that incubation with 100 μM Resveratrol for 48 hours, can increase the activity of complex II and IV. The author also warned that the response of the cell to Resveratrol seemed to be not uniform and highly dependent on the cell type (De Paepe & Van Coster, 2017). Treatment with a combination of 5 μM Curcumin and 50 μM Quercetin increased the expression of complex IV (Sandoval-Acuña et al., 2014; Bagheri et al., 2020; Waseem & Parvez, 2016), while Curcumin alone has been found to help the cells retain the complexes function in rats with aluminium-induced cytotoxicity (Bagheri et al., 2020).

3.1.2 Polyphenols and DNA methylation

Several possible mechanisms of action have been described for polyphenols. Among them, there is DNA methylation remodelling (Carlos-Reyes et al., 2019). DNA methylation is performed by DNA methyltransferases (DNMT), a class of enzyme that uses SAM as a methyl donor to methylate CpG sites on the DNA (Liu et al., 2009). Resveratrol has been found to remodel the DNA methylation pattern in several *in vitro* studies. This happens in a cell-specific and gene-specific manner. For example, in breast cancer cells, Resveratrol treatment increased DNA methylation in genes with oncogenic and pro-metastatic functions such as *MALM2*. This effectively reduced MALM2 expression and the downstream NOTCH signalling (Lubecka et al., 2016).

Quercetin was found to downregulate the DNA methylation level in a time- and dose-dependent manner *in vitro* (Sundaram et al., 2019). This depends on the ability of Quercetin to inhibit both DNMT, responsible for the DNA methylation and Histone deacetylase (HDAC), responsible for histone regulation (Sundaram et al., 2019). Quercetin has been found to reduce the activity of DNMT and cause DNA hypomethylation, both by interacting directly with DNMT and indirectly by increasing the concentration of SAH (Carlos-Reyes et al., 2019; Lee et al., 2005b).

Curcumin has been found to reduce global DNA methylation by 20-50% at 3.0 μM in MV4-11 cell line incubated for 72 hours (Liu et al., 2009). Additionally, Link et al found that curcumin-induced DNA methylation changes occurred in a time-dependent manner and in a gene- and cell-specific manner in colon cancer cells lines (Link et al., 2013). While, Maugeri et al found that 25 μM Curcumin treatment for 6 hours reduced high-glucose induced ROS and DNMT activity but not on DNA methylation level (Maugeri et al., 2018).

3.1.4 Mitochondrial DNA methylation

Since the discovery of the presence of DNA-methyl-transferase 1 (DNMT 1) inside the mitochondria (Saini et al., 2017; Shock et al., 2011) it has been hypothesized that, in mammalian mitochondria, DNA methylation is due to the presence of an independent DNA methylation machinery (Saini et al., 2017; Shock et al., 2011). In addition, there is evidence for epigenetic regulation of mtDNA expression and replication (Bellizzi et al., 2013; Feng et al., 2012).

3.2 Hypothesis

Given the ability of Resveratrol, Quercetin, and Curcumin to modulate DNA methylation, the hypothesis that polyphenols are able to alter the DNA methylation in the mitochondria was tested. Considering the effects of polyphenols on thrombosis and platelets aggregation - and due to the difficulties in culturing platelets-; the platelet's precursor cells, MEG-01 cells, were used to test the effects of polyphenolic treatment on mtDNA methylation (Hypothesis 3, see section 1.6.2).

3.3 Methods

3.3.1 MEG-01 cell culture

MEG-01 cell culture for the maintenance of the cell is described in section 2.3.

For the cell viability experiments, the cells were plated in either transparent 96-well plates (Starlab; CC7682-7596) or white 96-well plates with clear bottom (SLS; 3610). The opaque white polyester plates were specifically designed for fluorescence detection. Both types of plate were tissue-culture treated (e.g. with hydrophilic polyester surfaces that increase cell attachment) and individually wrapped. Additionally, a standard curve to extrapolating the cell concentration was made plating untreated cells in concentrations ranging from $1x10^3$ to $5x10^4$ cells/well in 96-well plates in 100 μl medium. For the DNA collection, the cells were plated

at the concentration of ~2x10^5 cells/well in 24-well plate (Starlab; CC7682-7524) with 1 mL of cell medium.

3.3.2 Treatment of cells with polyphenols

Resveratrol 99% (Sigma-Aldrich; R5010-100MG), Quercetin (Sigma-Aldrich; 1592409-500MG), and Curcumin, analytical standard (Sigma-Aldrich; 08511-10MG) were dissolved in dimethyl sulfoxide (DMSO), 99.7% (Sigma-Aldrich, D2650-5X5ML) at 100 µM, 200 µM, 1 mM, 2 mM, and 5 mM stock respectively. Then the stock was stored at -20°C. The MEG-01 were treated with polyphenols at the following concentrations: 10, 50, and 100 µM for Resveratrol and Quercetin, and 20, 50 and 100 µM for Curcumin. These concentrations were selected using the proliferation assay and viability assay.

The right amount of polyphenol stocks were dissolved in a final volume of 10 µl of DMSO and added to 90 µl of cells plus medium. Given the fact that DMSO is toxic to the cell, a 10 µl DMSO control (1:10) was added to the cells to evaluate the DMSO effects on the cell viability. Control cells without polyphenols or DMSO treatment were grown with each set of treatment for the same duration of the experiment. To determine the effect of Resveratrol, Quercetin, and Curcumin on cell viability and proliferation the cells were treated for 0 hours (h), 24 h, 48 h and 72 hours. The cell medium with the relevant polyphenols' dose was not changed during the duration of the treatment. For each treatment, three biological replicates were used.

3.3.2.1 WST-1 cell proliferation assays

WST-1 (4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate is a colourimetric assay used to measure the metabolic activity of MEG-01 (Sigma-Aldrich; 5015944001). WST-1 is cleaved to formazan by mitochondrial dehydrogenase in viable cells, which was measured with a spectrophotometer and correlates with the cell number. The absorbance was measured at 450 nm for the sample and 680 nm for the background with multi-well ELISA reader and the SKANIT software (version 4.1). The absorbance for each sample was calculated as follow: absorbance 450 nm- absorbance 680 nm.

As soon as WST-1 is added to the cells it starts to be metabolised by cellular mitochondrial dehydrogenase. Therefore, the absorbance was measured after 0.5, 1, 2, and 4 hours of WST-1 incubation at 37 °C. The absorbance measurements for ~5x10^4 cells/ well was already stabilised after 0.5 hours of incubation with WST-1. This was tested as follow.

The MEG-01 cells were seeded 24 h prior to the experiment in a 96-well plate (Starlab; CC7682-7596) at a density of ~$5x10^4$ cells/ well in a total volume of 90 µl. The day of the treatment (day 0) the cells were treated with polyphenols at the final concentration of 10, 50, and 100 µM for Resveratrol and Quercetin, and 20, 50, 100 µM for Curcumin. Each polyphenol concentration was dissolved in a final volume of 10 µl of DMSO. As a control 10 µl of DMSO were added to $5x10^4$ cells in 90 µl of medium.

Additionally, to assess the reaction of the polyphenols with medium and with the WST-1 reagent, 10, 50, and 100 µM for Resveratrol and Quercetin, and 20, 50, 100 µM for Curcumin dissolved into DMSO were added to 90 µl of medium alone.

3.3.2.2 Luciferase based viability assay

Since Quercetin and Curcumin interfere with the WST-1 assay (Chignell et al., 1994), a viability assay was conducted using CellTiter-Glo® 2.0 Assay (Promega; G9241) according to the manufacturer instructions. First, the assay was tested on medium plus polyphenols and its cross-reactivity with the polyphenols Resveratrol, Quercetin, and Curcumin was excluded (data not shown). This test was used to confirm that the assay was truly recording the presence of cells in the medium and not noise.

This assay was performed in triplicates. The cells were plated in 96-well plates with clear bottom (SLS; 3610) at the concentration of ~$2x10^4$ cells/well. A number of controls were set to ensure the accuracy of the measurements:

i) the "control", with the same amount of cells,
ii) ~$2x10^4$ cells/well, but no treatment,
iii) the "medium control" in which there is just the medium with no cells,
iv) "polyphenols+medium control" in which there are is the medium and polyphenols at different doses,
v) and "DMSO control", in which there is the ~$2x10^4$ cells/well treated with 10 µl of DMSO.

These controls were present in each plate and for each incubation time, 0, 24, 48, and 72 hours. Additionally, a standard curve has been produced using untreated cells at different concentrations $1x10^3$, $1x10^4$, $2x10^4$ and $5x10^4$ cells/well. The "DMSO control" (~$2x10^4$ cells/well treated with 10 µl of DMSO) has been used to evaluate the effects of the polyphenolic treatment. For the viability assay four times point were used, 0, 24, 48, and 72

hours and five different concentration of polyphenols. Namely, 20, 50, 100, 200, and 500 μM of Quercetin and Curcumin.

For each time and doses, there are triplicates sample. For each time point, the average of DMSO control luminescence has been set as 100% and the average of luminescence in the treated samples (e.g. 10 μM Quercetin) has been divided by the average of DMSO Control and then multiplied by 100.

3.3.3 Study design

After assessing the sub-toxic dose of Resveratrol, Quercetin, and Curcumin, the experiment to test the effect of polyphenols on mtDNA methylation was set up according to figure 2.4 (section 2.4.2). The cells were plated at the concentration of ~$2x10^5$ cells/well in 1mL of the growth medium, in a 24-well plate. This experiment was performed in sextuplicate.

The cells were incubated with 50 and 100 μM of Resveratrol for 72hours. The treatment with 50 and 100 μM Resveratrol was conducted using two different batches with their own Control and "DMSO control", batch 2 and batch 1 respectively. Each concentration of Resveratrol has been compared with its own control, viz batch 1 (B1) DMSO control with the B1 100 μM of Resveratrol treated samples, and batch 2 (B2) DMSO control with the B2 50 μM of Resveratrol treated samples. The cells treated with 10 and 50 μM of Quercetin, and 50 and 100 μM Curcumin have been incubated for 24 hours and compared to the same Control and DMSO control with the same incubation time.

3.3.4 Statistical analysis

The statistical analysis has been performed using excel (version 2016) for the cell proliferation and luminescence assays, while for the methylation data R version 3.6.1 (2019-07-05) was used. A general linear model was used to determine the effect of polyphenols on mtDNA methylation. To screen for batch effect a general linear model using R was set up using the following code: "lm(formula = meth_poly$CO2_CpG1_mean ~ treatment + treatment*dose + incubation + treatment*dose*incubation + batch + treatment*batch + treatment*dose*batch, data = meth_poly)".

3.3.5 Quality control

3.3.5.1 Pyrosequencing

For pyrosequencing description and protocol see section 2.5.3. The assay used to test the effect of polyphenols on mtDNA methylation are listed in table 2.2 (see section 2.5.2).

3.3.5.3 Methylation data processing

The methylation patterns of each PCR product was analysed in duplicates (Pyrosequencing Technical replicates). The similarity of these technical replicates was used to assess the precision of mtDNA methylation % determination. The patterns between pyrosequencing run1 and pyrosequencing run2 and between the biological replicates were analysed to check for outliers. This analysis was performed independently for each polyphenolic treatment.

A standard deviation (SD) bigger than 4% between technical replicates was considered a red flag. In the CpG sites for which only one run was available the SD between biological replicates was calculated. A SD bigger than 4% was considered a red flag and the outlier removed. A total of 17 values were considered outliers and were removed from the database.

The Spearman rank correlation coefficient between technical replicates was 0.96 (P<0.001) (Figure 3.1). The correlation coefficient and the coefficient of variation were calculated using R version 3.6.1 (2019-07-05).

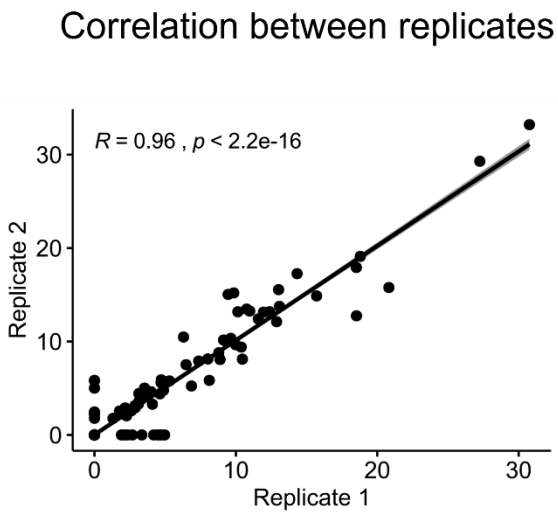


Figure 3.1 Correlation coefficient between technical replicates.

This scatter plot has been done using the available n=959 values in the run1 and n=859 values available from the run2. This analysis includes the 17 CpG sites distributed on the genes: MT-CO1, MT-CO2, MT-CO3, D-loop, MT-TL1, MT-TF, and MT-OLR. This correlation has been performed using the data after the outliers' removal.

Additionally to further establish the sensitivity and consistency of the analysis, for each plate, a 0% and 100% methylated control oligonucleotide were analysed (Byun & Barrow, 2015b). These oligonucleotides are generated by the synthesis of sequence and they have

respectively, a T or a C at the interrogated CpG site (See chapter 2.5.3.3 Pyrosequencing control). All the plates had optimal oligo results with the “C” oligo always measured ad 0% and the “T” oligo always measured as 100% methylation.

3.4 Results

3.4.1 Effects of polyphenols on cell viability

3.4.1.1 Cell proliferation assay with WST-1

First, a preliminary experiment with WST-1 and the polyphenols Resveratrol and Quercetin was performed to define the best incubation with WST-1 (Figure 3.2). In this experiment, three different doses of Resveratrol and Quercetin were used, namely 10, 50, and 100 μM. Only one incubation time with polyphenols, 24 hours, was tested and the measurements after WST-1 incubation were taken at 0.5, 1, 2, and 4 hours. The standard deviations increase with time of WST-1 incubation time, therefore the 0.5 hour incubation time was decided to be used for further experiments.

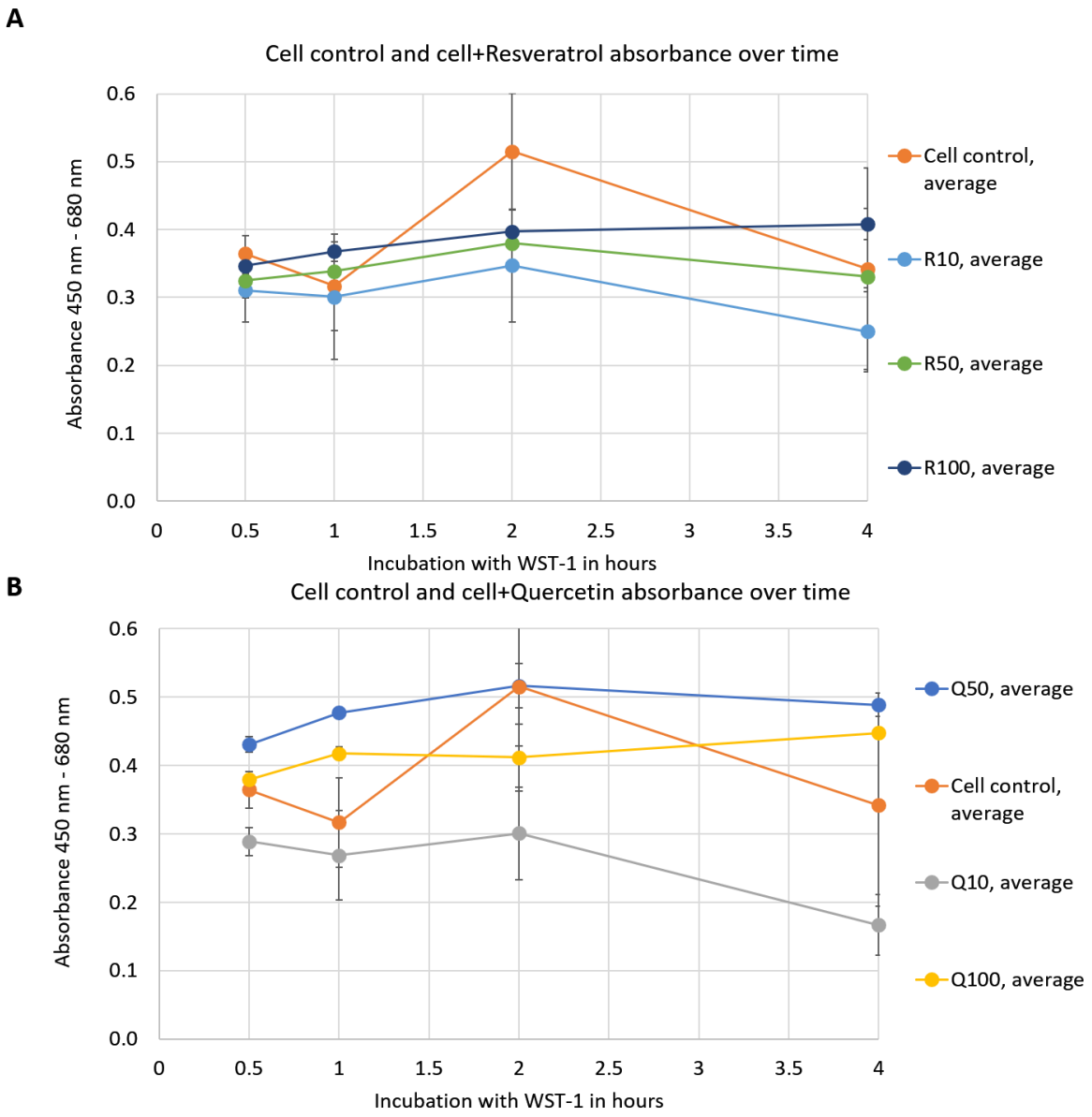


Figure 3.2 Estimation of the best WST-1 incubation time.

A) Resveratrol. R10: Resveratrol 10 µM; R50: Resveratrol 50 µM; R100: Resveratrol 100 µM. B) Quercetin. Q10: Quercetin 10 µM; Q50: Quercetin 50 µM; Q100: Quercetin 100 µM. Each dot represents the average of the triplicates for the treatment. The standard deviation is shown as vertical lines.

The cell control and treatment samples were all seeded at ~3x10^4 cells/well and incubation with polyphenols for 24 hours. R10: Resveratrol 10 µM; R50: Resveratrol 50 µM; R100: Resveratrol 100 µM; Q10: Quercetin 10 µM; Q50: Quercetin 50 µM; Q100: Quercetin 100 µM.

Additionally, WST-1 proliferation assay was found to react with medium plus polyphenols and generate signals in the absence of cells. This happens in a dose-dependent manner for Quercetin and Curcumin (Figure 3.3). This may be due to the reported absorbance of Curcumin between 408-500 nm and Quercetin at 365 nm (Nelson et al., 2017; C. Alva-Ensastegui et al., 2018)

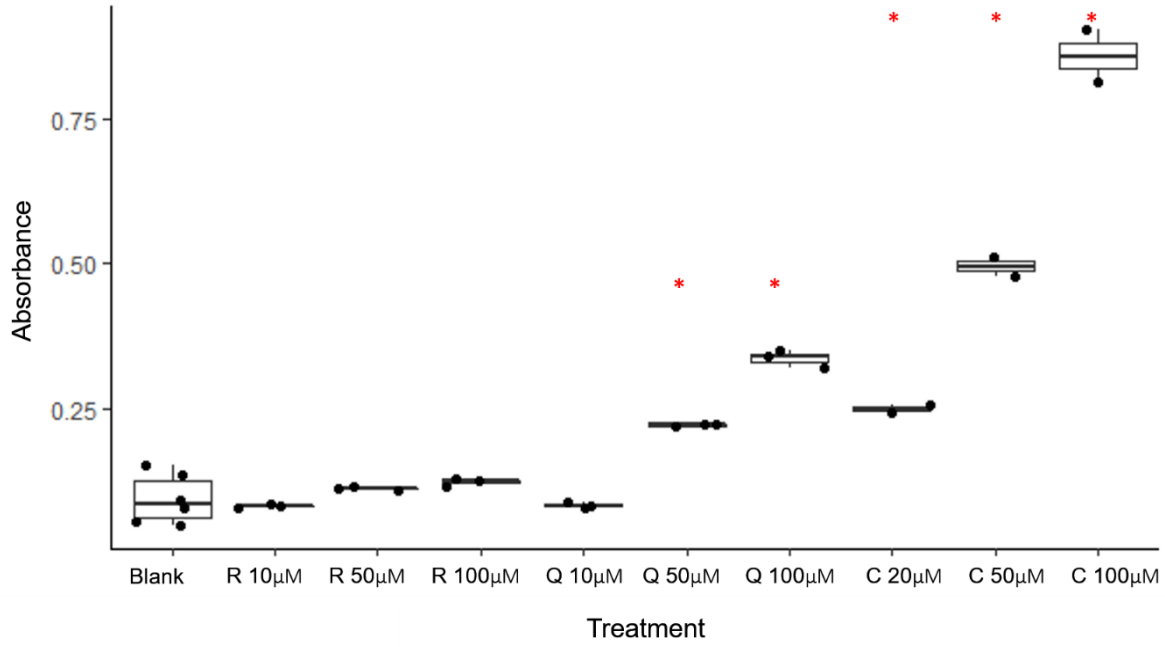


Figure 3.3 Absorbance of polyphenols.

Here are reported the results from the 0.5 hours WST-1 incubation of medium treated with the polyphenols at different concentrations. In the medium, there were no cells. On the Y-axis there is the Absorbance and on the X-axis the concentration of each polyphenol is reported. The red asterisks indicate a P value < 0.01.

R: Resveratrol; Q: Quercetin; C: Curcumin.

Therefore the WST-1 proliferation assay was used to determine the sub-toxic concentration of only Resveratrol. The absorbance measurements were taken in triplicates and the average for each treatment and time point was calculated. The percentage of cells for each treatment and incubation was calculated by dividing the absorbance of the sample for the absorbance of the DMSO Control at the same time point and by multiplying for 100. For each time point, the average of DMSO Control has been set as 100% and the average of absorbance in the treated samples (e.g. Resveratrol 10µM) has been divided by the average of DMSO Control

and then multiplied by 100 (Figure 3.4). None of the incubation time and Resveratrol's doses demonstrated to kill more than 50% of the cells, therefore for the further experiments the longest incubation time and doses were selected: 72 hours incubation with 50 and 100 µM of Resveratrol.

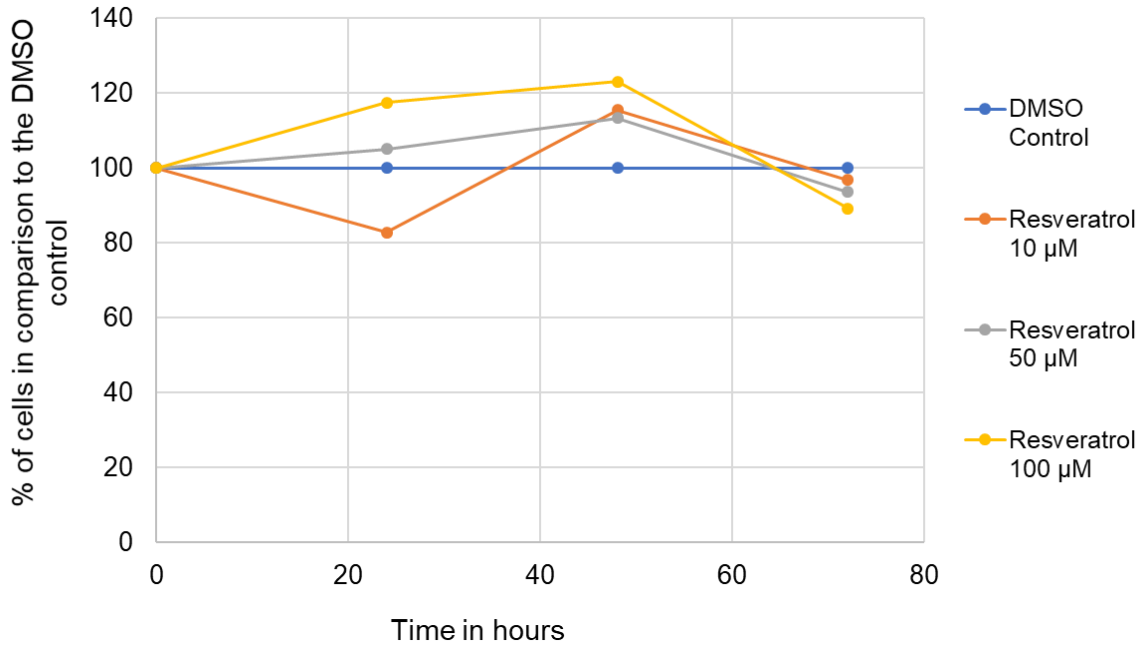


Figure 3.4 Proliferation assay for Resveratrol.

In this graph, the X-axis reports the time in hours and the Y-axis the percentage (%) of cells for each treatment and time point compared to the DMSO control.
The blue line represents the % of cells present in the DMSO Control that has been set as 100% for each time point. While the Resveratrol samples absorbance has been divided by the DMSO Control absorbance (for the same time point) and then multiplied by 100. At the last time point, the 72 hours incubation, all of the treatment resulted sub-toxic as more than 50% of the cells survived.

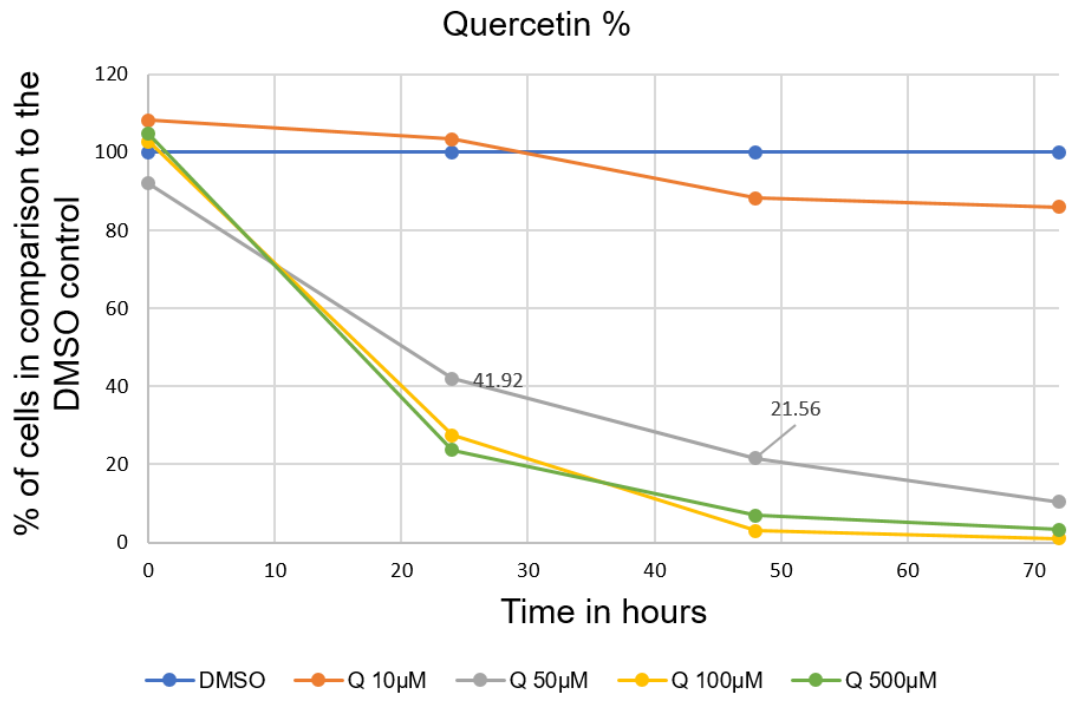
Quercetin and Curcumin sub-toxic doses were analysed using a different cell viability assay.

3.4.1.2 Cell viability assay with Celltiterglo 2.0 Assay

To determine the sub-toxic concentration of Quercetin and Curcumin a cell viability assay was conducted using a Luminescent assay, Celltiterglo 2.0, which does not react with these polyphenols (data not shown). Cells were plated on white 96-well plates that are opaque white polyester plates specifically designed to reduce luminescence interference between

samples (SLS; 3610). The luminescence was measure at four times points, namely 0, 24, 48, and 72 hours. For each time point five different concentrations of Quercetin and Curcumin were tested, namely 10, 20, 100, 200, and 500 µM. The experiment was performed in triplicates and the average of the three was used. The results for Quercetin 200 µM had high standard deviation and were excluded from the analysis. Figure 3.5 shows the percentage of cells after treatment with the different doses of Quercetin and Curcumin. The Incubation time was set as 24 hours and the sub-toxic doses for Quercetin were 10 and 50 µM, while for Curcumin 50 and 100 µM were selected (Figure 3.5).

A


Quercetin %
% of cells in comparison to the DMSO control
120
100
80
60
40
20
0
41.92
21.56
0
10
20
30
40
50
60
70
Time in hours
DMSO
Q 10µM
Q 50µM
Q 100µM
Q 500µM

B

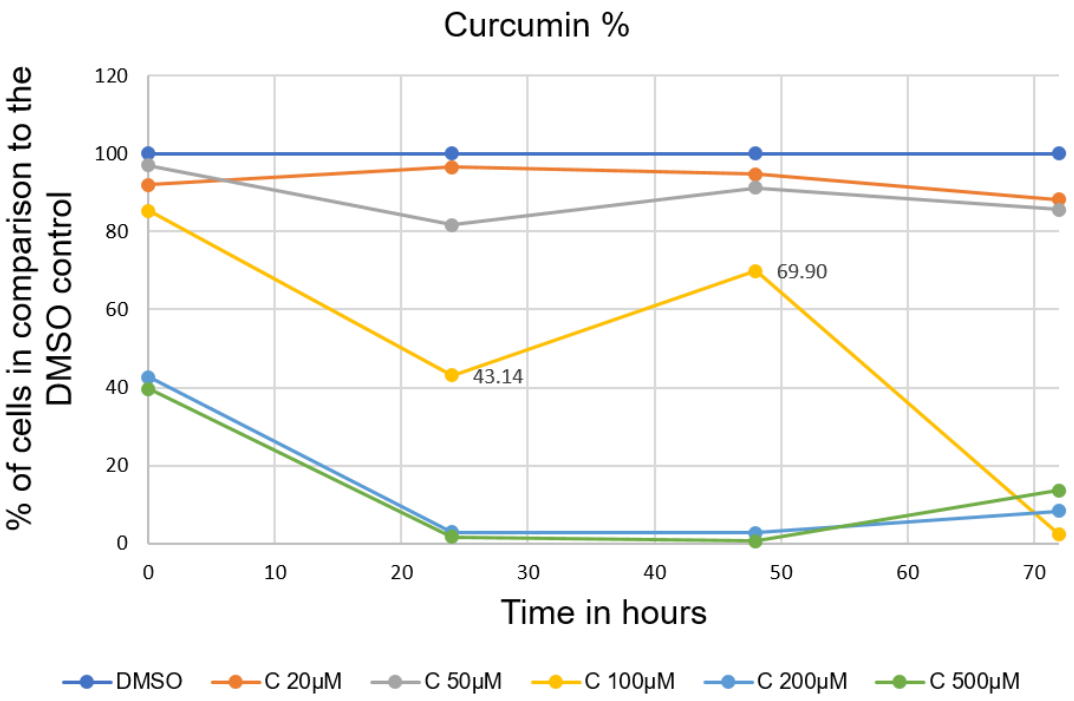

Curcumin %
% of cells in comparison to the DMSO control
120
100
80
60
40
20
0
69.90
43.14
0
10
20
30
40
50
60
70
Time in hours
DMSO
C 20µM
C 50µM
C 100µM
C 200µM
C 500µM

Figure 3.5 Cell viability assay for Quercetin and Curcumin

In this graph the X axis reports the incubation time in hours and the Y % of cell for each treatment and time point compared to the DMSO control.
The blue line represent the % of cells present in the DMSO control that has been set as 100% for each time point. While the Quercetin and Curcumin samples luminescence has been divided by the DMSO control luminescence (for the same time point) and then multiplied by 100. Soon after the addition of the polyphenols treatment the cell percentage starts to decrease due to the effect of the polyphenols. The samples for the treatment 200 µM Quercetin had a high standard deviation and were not included in the final analysis. A) Quercetin. For Quercetin the sub-toxic doses selected were 10 and 50 µM for 24 hours. B) Curcumin. For Curcumin the sub-toxic doses selected were 50 and 100 µM for 24 hours. Q: Quercetin; C: Curcumin.

3.4.2 Effects of polyphenols on mtDNA methylation from MEG-01 cells

3.4.2.1 Resveratrol induces mtDNA hypermethylation at *MT-CO2* CpG1

MEG-01 cells have been treated with 50 and 100 µM of Resveratrol (both concentration dissolved in a final amount of10 µl of DMSO) for 72 hours, and each treatment have been compared to its DMSO control treated with 10 µl of DMSO alone. After 72 hours treatment, mitochondrial DNA methylation was measured by BS-pyrosequencing. The mean % methylation of *MT-CO1* nt6798 CpG1 is 0% (SD=0) in all the treatment groups. The effect of Resveratrol on *MT-CO1*, *MT-CO2*, and *MT-CO3* CpG sites is reported in figure 3.6. The effect of Resveratrol on *MT-TL1*, D-loop, and *MT-TF* CpG sites is reported in figure 3.7. The effect of Resveratrol on *MT-OLR* CpG sites is reported in figure 3.8.

The effect of Resveratrol on mtDNA methylation level has been found to be different on different CpG sites. The effect of Resveratrol has been evaluated using ANOVA and the Tukey test. In the figures the mtDNA methylation percentage are reported for the controls, untreated cells, for the DMSO control, cells treated with 10 µl of DMSO alone, the 50 µM Resveratrol dissolved in 10µl of DMSO, and 100 µM Resveratrol dissolved in 10 µl of DMSO. All these cells have been incubated for 72 hours. The cells treated with Resveratrol come from two different batches. The only CpG site whose methylation was affected by Resveratrol treatment is *MT-CO2* nt8113 CpG1, whose methylation significantly increased with 50 µM Resveratrol incubation (P=0.0016).

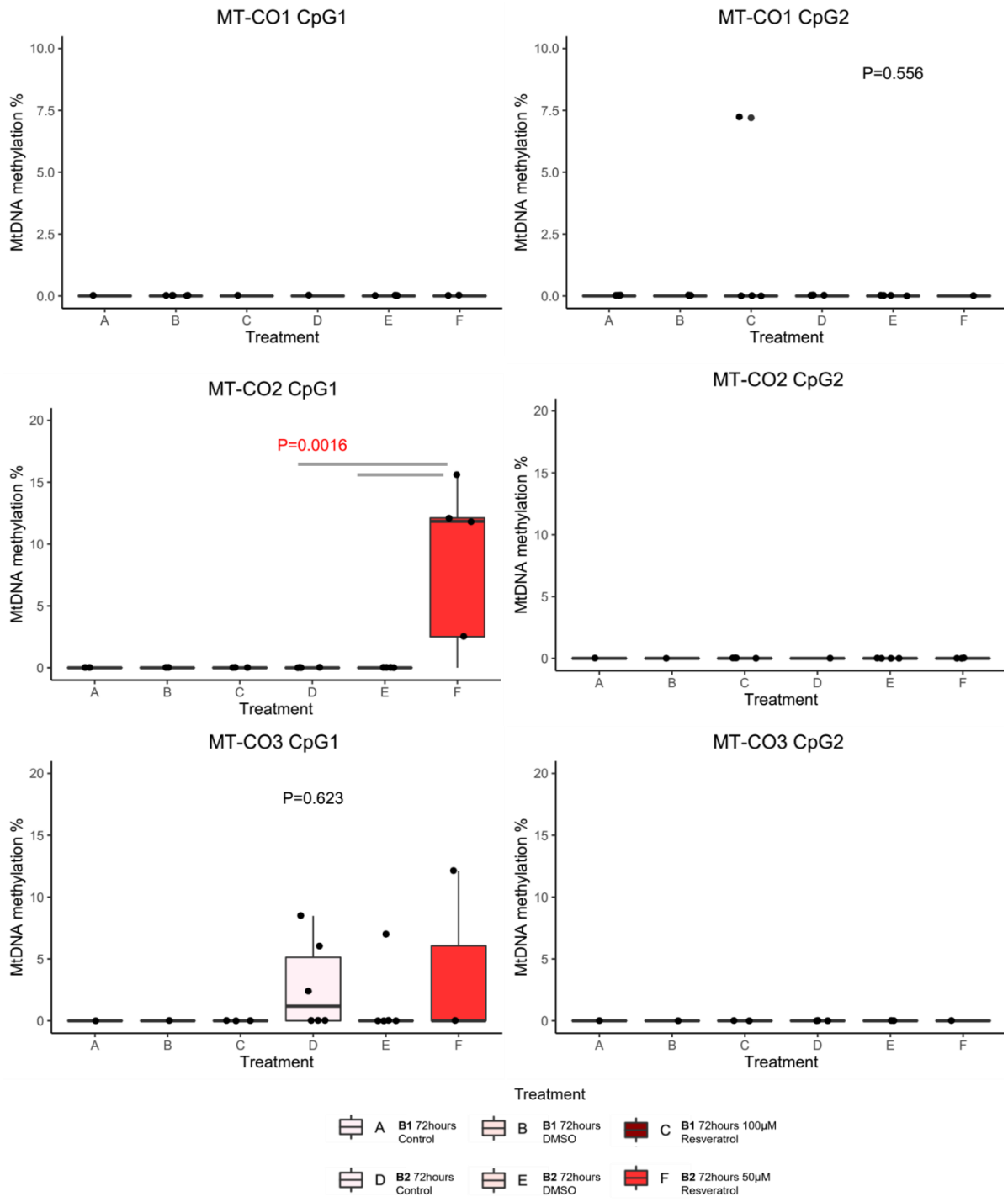


Figure 3.6 MtDNA methylation percentage at the genes *MT-CO1*, *MT-CO2*, and *MT-CO3*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. The significant P values are reported in red for the position MT-CO2 CpG2. The non-significant P values are reported in black, as for MT-CO1 CpG2

and MT-CO3 CpG1. These P values have been calculated with ANOVA between the treatment groups. MT-CO1 CpG1, MT-CO2 CpG2 and MT-CO3 CpG2 positions show 0% mtDNA methylation in all the samples, therefore there is no difference between the treatment groups and batch. B1: Batch 1; B2: Batch 2; 72hours: 72 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 50µM Resveratrol: cell treated with 50 µM of Resveratrol dissolved into 10 µl DMSO; 100µM Resveratrol: cell treated with 100 µM of Resveratrol dissolved into 10 µl DMSO.

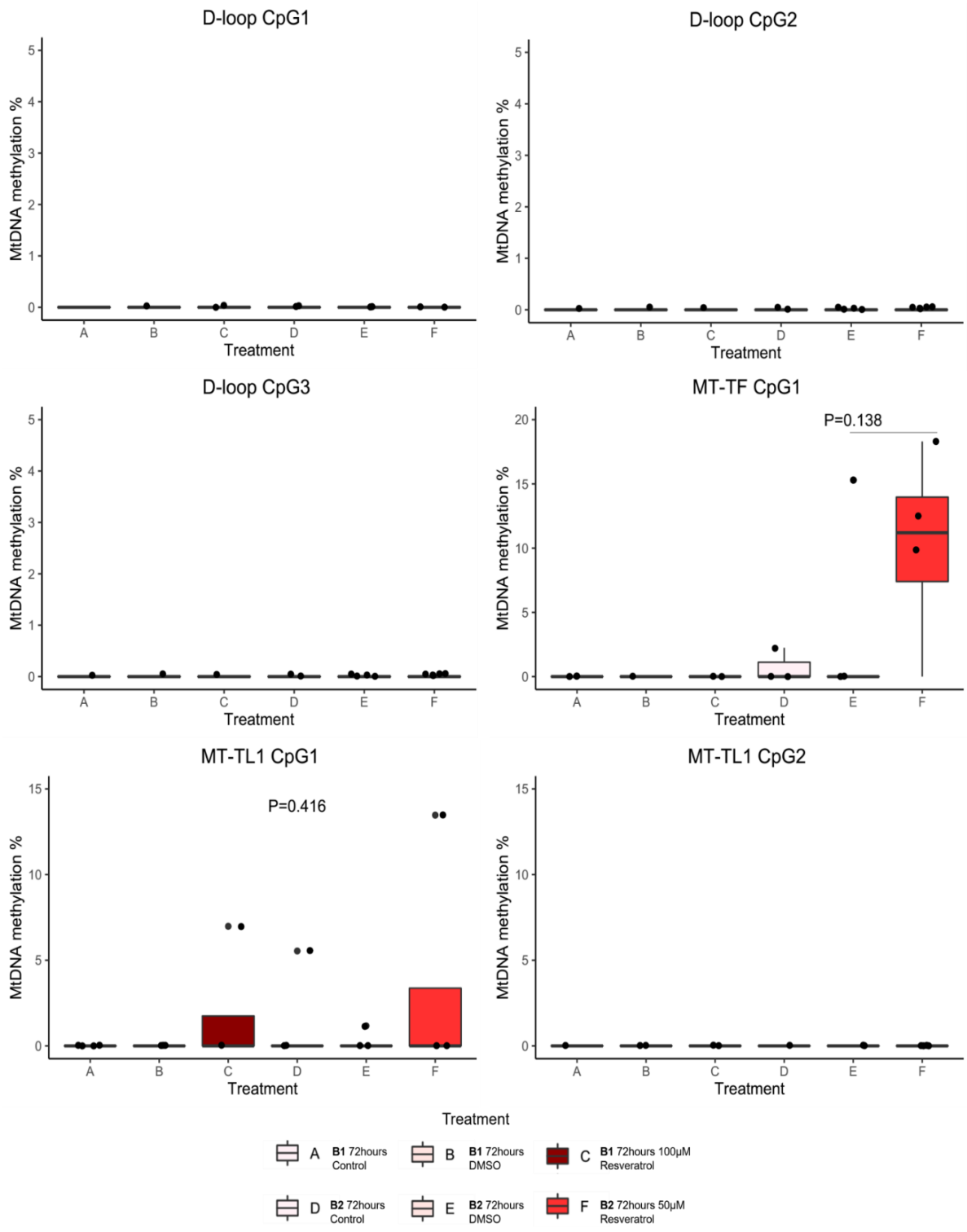


Figure 3.7 MtDNA methylation percentage at the genes *MT-TL1*, D-loop, and *MT-TF*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. The non-significant P values are reported in black. The position

D-loop CpG1, CpG2, CpG3 and MT-TL1 CpG2 have 0% methylation in all the sample analysed, therefore, there is no difference in the means. In the position MT-TF CpG1, the P value for the difference between treatment "E" (Batch 2, 72hours incubation, 10µl of DMSO) and "F" (Batch 2, 72hours incubation, 50 µM of Resveratrol dissolved into 10µl DMSO) is reported (P=0.138). In the position MT-TL1 CpG1, the P value of the ANOVA between all the treatment groups is reported. B1: Batch 1; B2: Batch 2; 72hours: 72 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 50µM Resveratrol: cell treated with 50 µM of Resveratrol dissolved into 10 µl DMSO; 100µM Resveratrol: cell treated with 100 µM of Resveratrol dissolved into 10 µl DMSO.

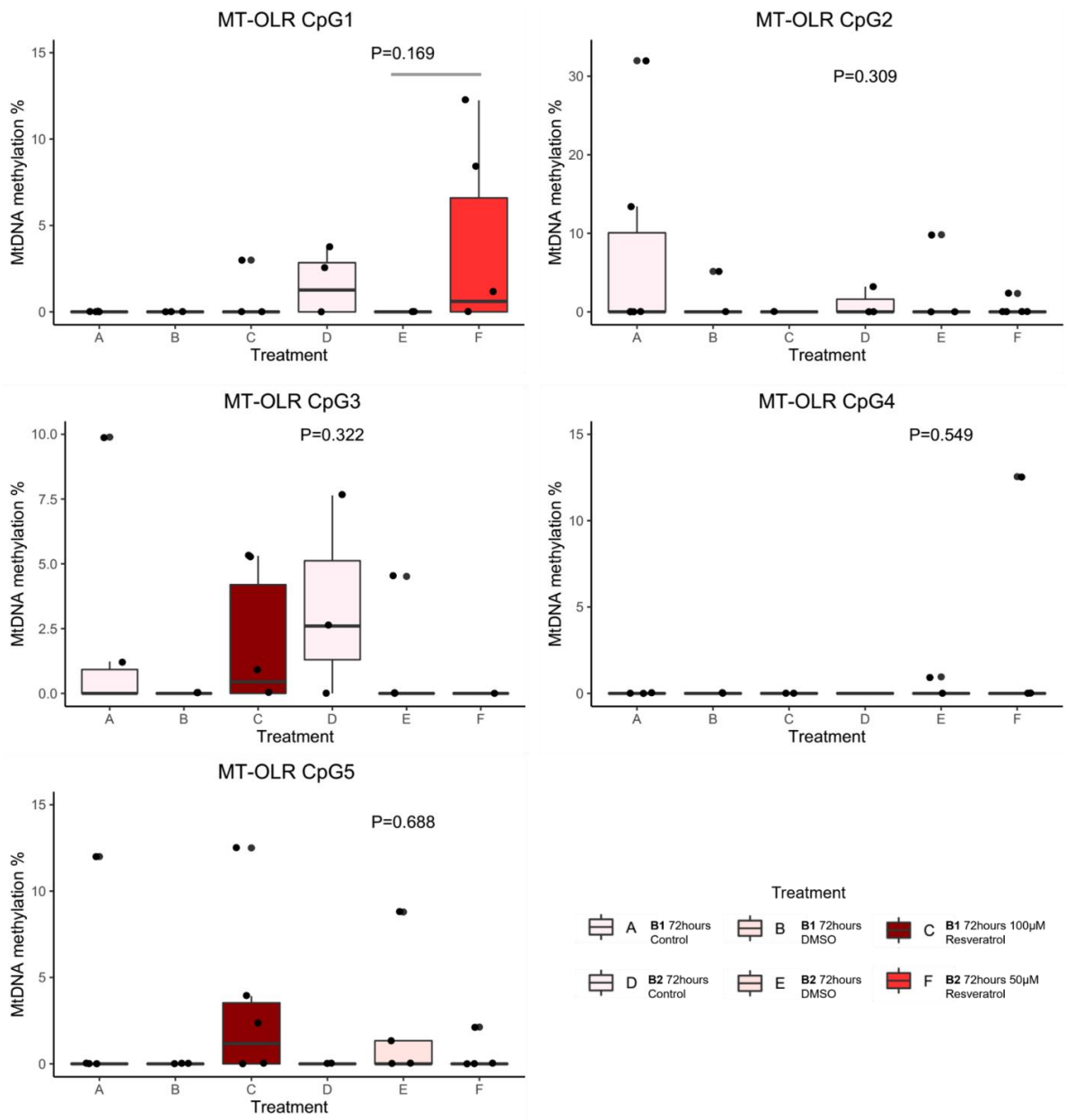


Figure 3.8 MtDNA methylation percentage at the genes *MT-OLR*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. These images represent the mtDNA methylation percentage at the positions MT-OLR CpG1, CpG2, CpG3, CpG4, and CpG5. The P values reported in black are non-significant. In the position MT-OLR CpG1, the P value of the difference between treatment "E" (Batch 2, 72hours incubation, 10µl of DMSO) and treatment "F" (Batch 2, 72hours incubation, 50µM of Resveratrol dissolved into 10µl DMSO) is reported. In the positions MT-OLR CpG2, CpG3, CpG4, and CpG5 the P values of the ANOVA between the treatment groups are reported. B1: Batch 1; B2: Batch

2; 72hours: 72 hours incubation; Control: untreated cells; DMSO: 10 µl DMSO added to the cell medium; 50µM Resveratrol: cell treated with 50 µM of Resveratrol dissolved into 10 µl DMSO; 100µM Resveratrol: cell treated with 100 µM of Resveratrol dissolved into 10 µl DMSO.

3.4.2.2 Quercetin does not induce mtDNA methylation changes

MEG-01 cells have been treated with 10 and 50 µM of Quercetin for 24 hours and the polyphenols have been dissolved in 10 µl of DMSO, and each treatment have been compared to its DMSO control treated with 10 µl of DMSO alone. Quercetin had no effect on mtDNA methylation level. This holds true for both doses of Quercetin, 10 and 50 µM at 24 hours incubation. The effect of Quercetin has been evaluated using ANOVA and the Tukey test. In the figures the mtDNA methylation percentage are reported for the controls, untreated cells, for the DMSO control, cells treated with 10 µl of DMSO alone, the 10 µM Quercetin dissolved in 10 µl of DMSO, and 50 µM Resveratrol dissolved in 10 µl of DMSO. All these cells have been incubated for 24 hours. The cells treated with Quercetin came from the same batch. Figure 3.9 shows the results for the *MT-CO1*, *MT-CO2*, and *MT-CO3* genes; figure 3.10 shows the results for the genes *MT-TL1*, D-loop, and *MT-TF*; figure 3.11 shows the result for *MT-OLR*.

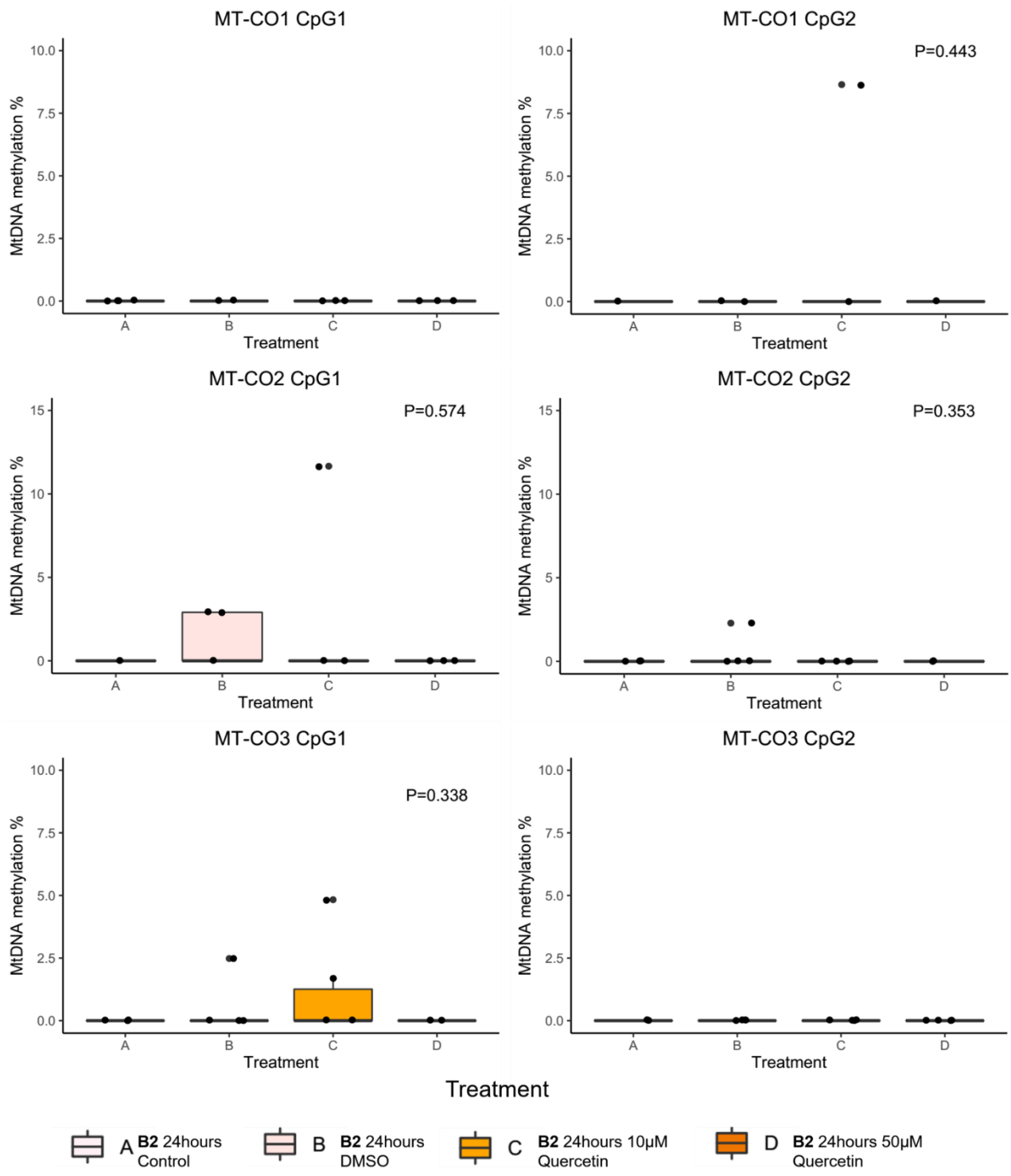


Figure 3.9 MtDNA methylation percentage at the genes *MT-CO1, MT-CO2,* and *MT-CO3*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. There are no significant P values for the genes MT-CO1, MT-CO2 and MT-CO3. The non-significant P values are reported in black. These P values have been calculated with ANOVA

between the treatment groups. MT-CO1 CpG1, and MT-CO3 CpG2 positions show 0% mtDNA methylation in all the samples, therefore there is no difference between the treatment groups. 24hours: 24 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 10µM Quercetin: cell treated with 10 µM of Quercetin dissolved into 10 µl DMSO; 50µMQuercetin: cell treated with 50 µM of Quercetin dissolved into 10 µl DMSO.

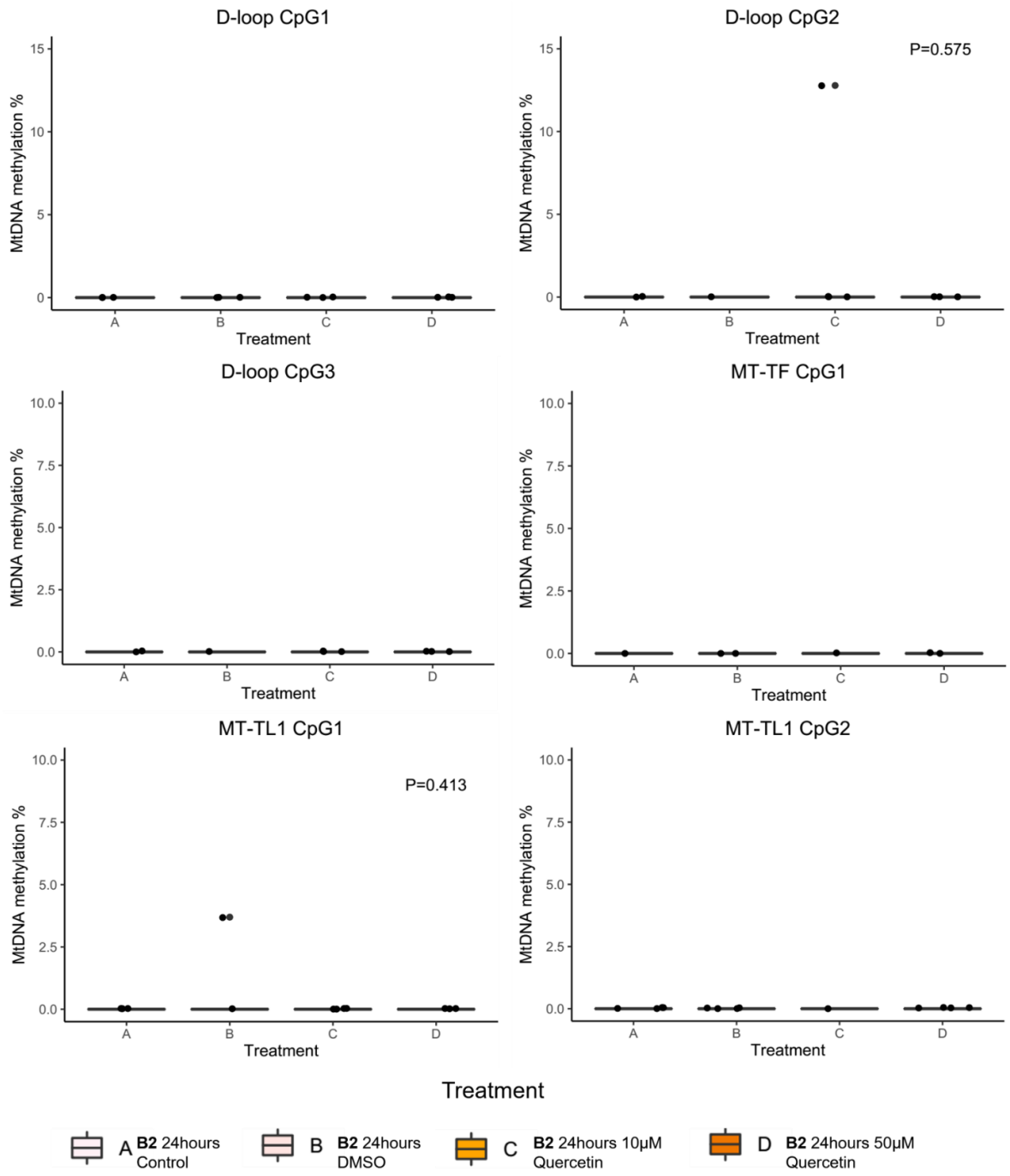

D-loop CpG1
D-loop CpG2
P=0.575
D-loop CpG3
MT-TF CpG1
MT-TL1 CpG1
P=0.413
MT-TL1 CpG2
MtDNA methylation %
Treatment
A
B
C
D
Treatment
A B2 24hours Control
B B2 24hours DMSO
C B2 24hours 10µM Quercetin
D B2 24hours 50µM Quercetin

Figure 3.10 MtDNA methylation percentage at the genes D-loop, *MT-TF* and *MT-TL1*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. There are no significant P values for the positions D-loop CpG2, and MT-Tl1 CpG1. The non-significant P values are reported in black. D-loop CpG1, D-loop CpG3, MT-TF CpG1, and MT-TL1 show 0% methylation in each treatment group.

24hours: 24 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 10µM Quercetin: cell treated with 10 µM of Quercetin dissolved into 10 µl DMSO; 50µMQuercetin: cell treated with 50 µM of Quercetin dissolved into 10 µl DMSO.

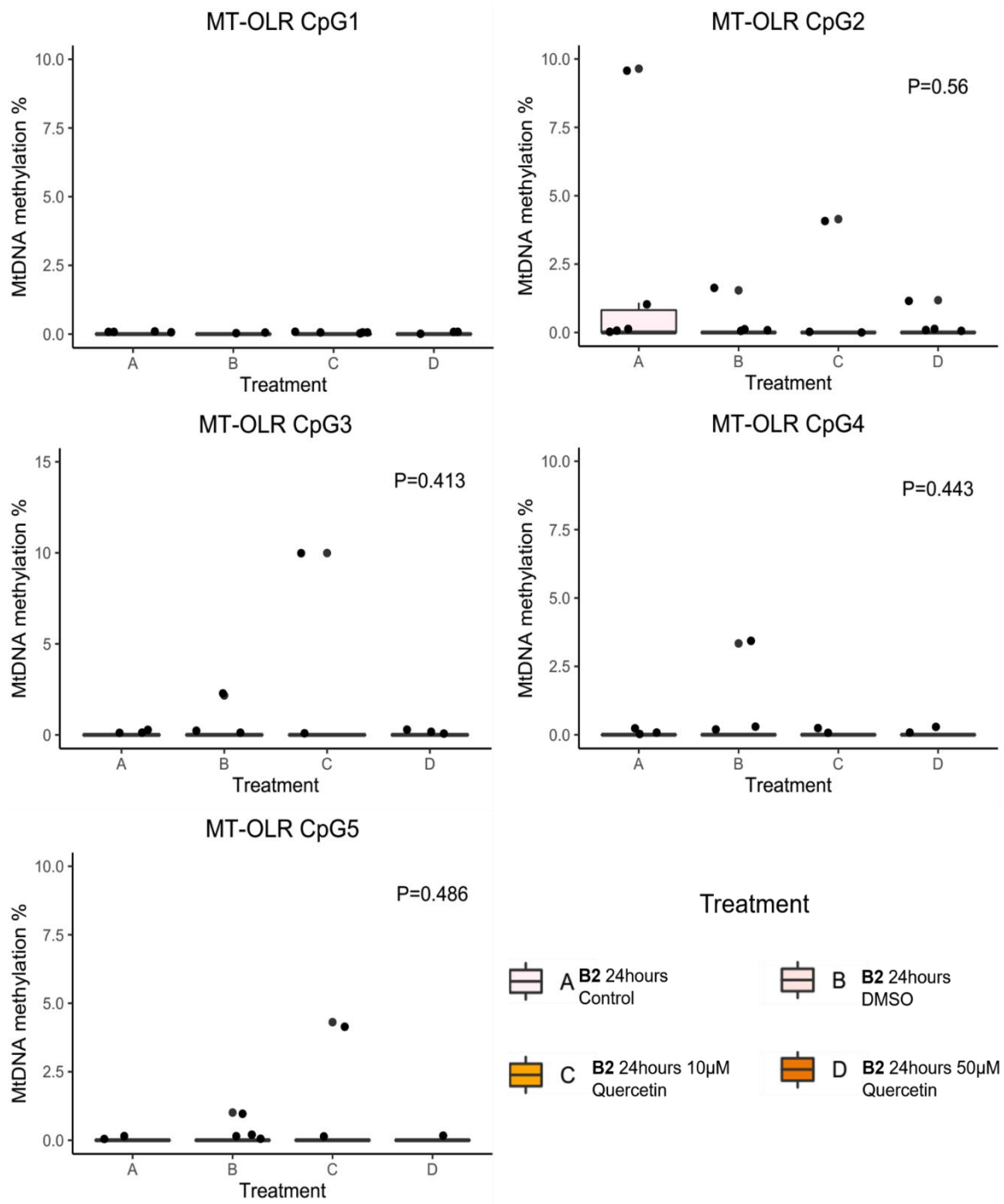


Figure 3.11 MtDNA methylation percentage at the genes *MT-OLR*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. There were no significant effects of Quercetin on mtDNA methylation for the positions MT-OLR CpG2, CpG3, CpG4, and CpG5. The positions MT-OLR CpG1 and CpG2 show 0% methylation in each treatment group. Therefore there is no difference between eh treatment groups and the P value cannot be calculated.

24hours: 24 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 10µM Quercetin: cell treated with 10 µM of Quercetin dissolved into 10 µl DMSO; 50µMQuercetin: cell treated with 50 µM of Quercetin dissolved into 10 µl DMSO.

3.4.2.3 Curcumin does not induce mtDNA methylation changes

MEG-01 cells have been treated with 50 and 100 µM of Curcumin for 24 hours and the polyphenols have been dissolved in 10 µl of DMSO, and each treatment has been compared to its DMSO control treated with 10 µl of DMSO alone. Curcumin had no effect on mtDNA methylation level. This holds true for both doses of Curcumin, 50 and 100 µM. The effect of Curcumin has been evaluated using ANOVA and the Tukey test. In the figures the mtDNA methylation percentage are reported for the controls, untreated cells, for the DMSO control, cells treated with 10 µl of DMSO alone, the 50 µM Curcumin dissolved in 10 µl of DMSO, and 100 µM Curcumin dissolved in 10 µl of DMSO. All these cells have been incubated for 24 hours. The cells treated with Curcumin came from the same batch. Figure 3.12 shows the results for the *MT-CO1, MT-CO2*, and *MT-CO3* genes; figure 3.13 shows the results for the genes *MT-TL1*, D-loop, and *MT-TF*; figure 3.14 shows the result for *MT-OLR*.

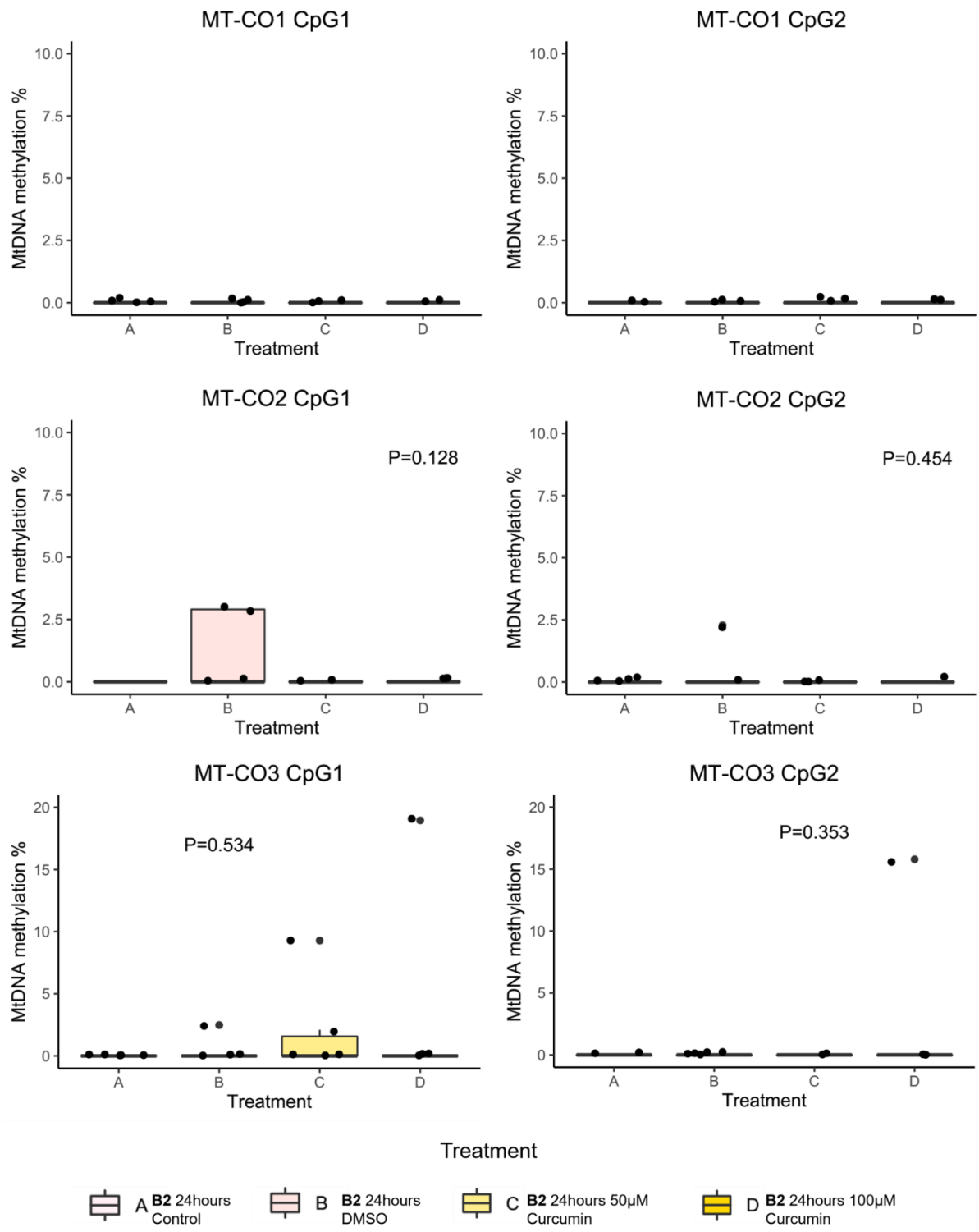


Figure 3. 12 MtDNA methylation percentage at the genes *MT-CO1, MT-CO2,* and *MT-CO3.*

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. There are no significant P values for the genes MT-CO2, and

MT-CO3. The non-significant P values are reported in black. These P values have been calculated with ANOVA between the treatment groups. MT-CO1 CpG1 and CpG2 positions show 0% mtDNA methylation in all the samples, therefore there is no difference between the treatment groups. 24hours: 24 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 50µM Curcumin: cell treated with 50 µM of Curcumin dissolved into 10 µl DMSO; 100µM Curcumin: cell treated with 100 µM of Curcumin dissolved into 10 µl DMSO.

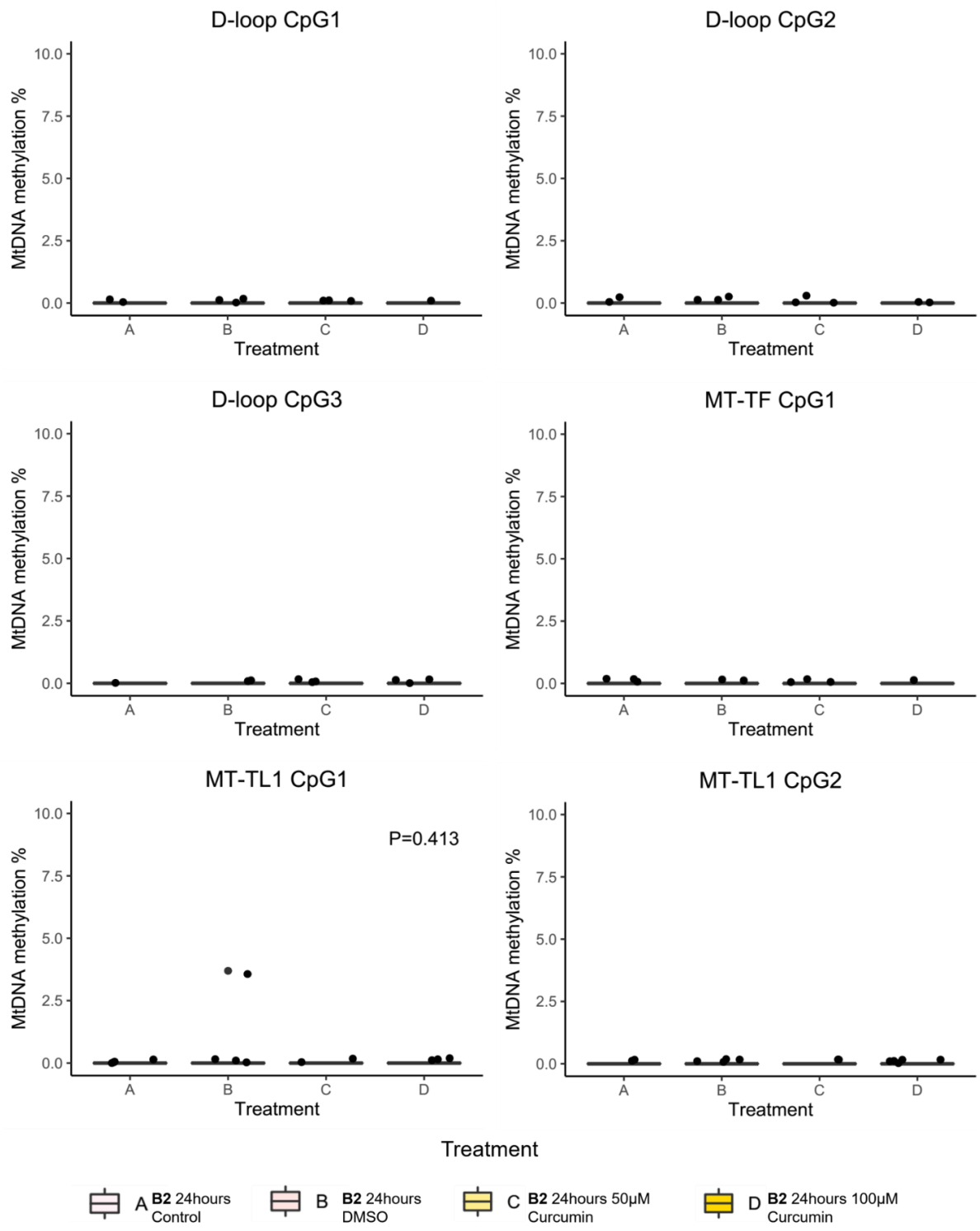


Figure 3. 13 MtDNA methylation percentage at the genes D-loop, *MT-TF* and *MT-TL1*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. There are no significant P values for the position MT-Tl1

CpG1. The non-significant P value is reported in black. D-loop CpG1, CpG2, and CpG3, MT-TF CpG1, and MT-TL1 CpG2 show 0% methylation in each treatment group. 24hours: 24 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 50µM Curcumin: cell treated with 50 µM of Curcumin dissolved into 10 µl DMSO; 100µM Curcumin: cell treated with 100 µM of Curcumin dissolved into 10 µl DMSO.

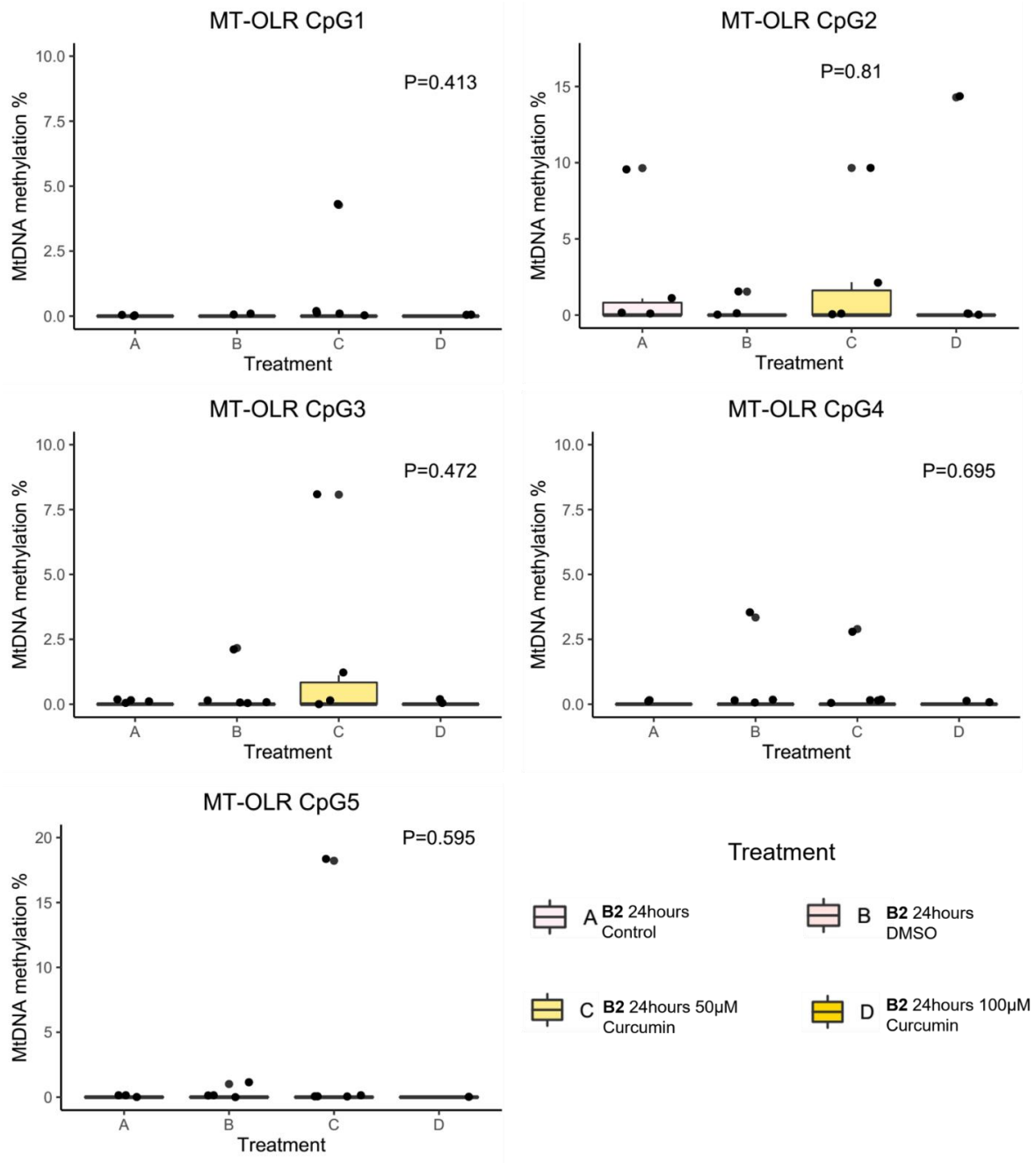


Figure 3. 14 MtDNA methylation percentage at the genes *MT-OLR*.

These graphs contain the boxplot overlapped with scatter plots of each treatment group. Each treatment group is made up by n=6 biological replicates. The analysis has been carried out using ANOVA. There are no significant P values for the positions MT-OLR CpG1-5. The non-significant P values are reported in black.

24hours: 24 hours incubation; Control: untreated cells; DMSO: 10µl DMSO added to the cell medium; 50µM Curcumin: cell treated with 50 µM of Curcumin dissolved into 10 µl

DMSO; 100µM Curcumin: cell treated with 100 µM of Curcumin dissolved into 10 µl DMSO.

3.4.3 Batch effect and incubation time

In the experiment design, there were two different batches, B1 and B2, and two different incubation times, 24 and 72 hours. Due to the number of treatment groups, the experiments were performed on two different days. Additionally, based on the viability assay 72 hours of Resveratrol treatment and 24 hours of Quercetin and Curcumin treatments were chosen.

This design allowed the analysis of the batch effect and the incubation time effect on mtDNA methylation level for each treatment group. To do this a linear model has been formulated to analyse the contribution of several variables such as treatment, dose, incubation, and batch, to the mtDNA methylation level of each CpG site. Table 3.1 reports a summary of the effects of dose, incubation, and batch to the mtDNA methylation variation for each CpG site (Table 3.1). The loci *MT-CO2* CpG1, *MT-TF* CpG1, and *MT-OLR* CpG1 show significant P value of the GLM, meaning that there is a trend in the mtDNA methylation variation. However, the effect of the interactions treatment*dose = "Effect of dose (same treatment≠ dose)", treatment*incubation = "Effect of incubation (same treatment ≠ incubation)", and treatment*batch = "Effect of batch (same treatment ≠ batch)" are not driving the mtDNA methylation changes.

Gene	Locus	**Effect of dose (same treatment ≠ dose)**		**Effect of incubation (same treatment ≠ incubation)**		**Effect of batch (same treatment ≠ batch)**		**P value of the GLM**
		estimate	P value	estimate	P value	estimate	P value	
MT-CO1	CpG1	na	na	na	na	na	na	na
	CpG2	-0.010	0.771	-0.002	0.871	0.250	0.818	0.656
MT-CO2	**CpG1**	**6.002**	**0.808**	**-9.505**	**0.789**	**6.657**	**1.000**	**< 0.001**
	CpG2	1.509	0.619	6.438	0.822	-7.599	1.000	0.960
MT-CO3	CpG1	0.040	0.236	-0.039	0.385	-1.648	0.692	0.541
	CpG2	2.002	0.319	7.136	0.792	6.245	1.000	0.500
D-loop	CpG1	na	na	na	na	na	na	na
	CpG2	2.196	0.917	-1.376	0.625	-8.871	1.000	0.904
	CpG3	na	na	na	na	na	na	na
MT-TL1	CpG1	0.002	0.903	-0.014	0.552	-0.728	0.643	0.166
	CpG2	na	na	na	na	na	na	na
MT-TF	**CpG1**	**-2.017**	**1.000**	**0.048**	**0.287**	**2.312**	**0.392**	**< 0.001**
MT-OLR	**CpG1**	**0.005**	**0.756**	**-0.036**	**0.156**	**-1.578**	**0.308**	**0.009**
	CpG2	0.031	0.489	0.022	0.774	7.602	0.092	0.250
	CpG3	0.025	0.539	-0.043	0.523	-0.657	0.870	0.876
	CpG4	0.006	0.712	0.002	0.928	0.191	0.902	0.544

CpG5	0.030	0.461	0.025	0.655	4.026	0.256	0.767

Table 3.1 Linear model analysis of batch effect.

*This is a summary of the results from the general linear model (GLM). The GLM has been performed to estimate the contribution of each variable (e.g. treatment, dose, incubation, and batch) to the changes in mtDNA methylation level. The last column reports the P value of the GLM, in other words, whether the mtDNA methylation level is different in different treatments group. Additionally, the estimate of the contribution of the variables "dose", "incubation", and "batch" for each treatment group is reported. In this table are reported the estimate and P value of the interactions treatment*dose = "Effect of dose (same treatment≠ dose)", treatment*incubation = "Effect of incubation (same treatment ≠ incubation)", and treatment*batch = "Effect of batch (same treatment ≠ batch)".*
The positions MT-CO1 CpG1, D-loop CpG1, D-loop CpG3, and MT-TL1 CpG2 cannot be computed because the mtDNA methylation level is 0% in all the samples.

3.5 Discussion

In the present study, the effect of polyphenolic treatment on MEG-01 cells has been evaluated. Based on our results, novel evidences are provided that the mtDNA methylation level in MEG-01 cells is generally low, and that depends mostly on the CpG site rather than the treatment. In fact, the D-loop assay, which include a total of three CpG sites, show 0% methylation regardless of the treatment, batch, or incubation time. While, the *MT-OLR* assay, which include a total of five CpG sites, show variable mtDNA methylation % ranging from 0-31%. Therefore, it may be speculated that the mtDNA methylation is not random but rather site-specific and possibly tightly regulated. This results seems to be in line with the group of Liao et al who investigated different diets on *yellow croaker* liver mitochondria. In their study, different diets altered the methylation status of *TR* and *ND4L*, and *RNR1* but not D-loop (Liao et al., 2015).

Interestingly, the 50 μM Resveratrol treatment for 72 hours significantly increased *MT-CO2* nt8113 CpG1 mtDNA methylation level. This is particularly significant as in Chapter 4 we found a similar effect of the Mediterranean diet on the human *MT-CO2* nt8113 CpG1. If the effect of Resveratrol on *MT-CO2* nt8113 CpG1 would be replicated this may support the human study results in Chapter 4, or so that MeDiet, or in this case polyphenols, have an effect on platelets mtDNA methylation level.

MT-CO2 is a mitochondrial gene that encodes for the subunit COII of complex IV (Cytochrome-C-Oxidase). This is a highly regulated enzyme in the electron transport chain (The UniProt Consortium, 2017). Resveratrol has been found both to have modulatory effects on the complex IV of the mitochondria (Gueguen et al., 2015; Ferretta et al., 2014) and direct effect on the DNA methylation (Xia et al., 2017; Naoi et al., 2019).

In the literature, *MT-CO2* nt8113 CpG1 hypermethylation has previously been reported in participants with CVD (Baccarelli & Byun, 2015). On the other hand, *MT-CO2* demethylation, induced by Valproic-Acid administration, has been reported to be associated with increased ROS production (Wolters et al., 2017). In MEG-01 cells' mitochondria, it is possible that Resveratrol regulates the ROS metabolism by actively increasing complex I activity and, indirectly after 72 hours, this may results in the alteration of mtDNA methylation level.

If the effect of 50 μM resveratrol on *MT-CO2* CpG1 is be validated, this would suggest two things. First, that the polyphenols have a CpG-specific effect on mtDNA methylation as for the nuclear DNA (Lubecka et al., 2016). Secondly, that the mtDNA methylation level in response Resveratrol, or more generally diet, can happen at the megakaryocytes level. Additionally, increased mtDNA methylation at *MT-CO2* nt8113 CpG1 upon 50 μM Resveratrol treatment, would resonate with the effect of higher MeDiet Score on mtDNA methylation described in Chapter 4.

Interestingly the lower dose of Resveratrol seemed to had an effect rather than the higher dose of 100 μM, this resonates with the literature which suggested a hormetic action of Resveratrol, which can exhibit different effects at different concentration (Xia et al., 2017).

In the SPHERE samples, higher Mediterranean diet adherence was also associated with a hypomethylation in the D-loop CpG1 (nt16383). In this experiment, it could not be replicated as D-loop methylation level were 0% in the controls and the treatment group with just one outlier in D-loop CpG2. Therefore, the hypomethylating effect of diet could not be established, as the controls had 0 %. It would be interesting to tests the effect of polyphenols on cells, that have been first stressed and whose methylation level is increased, to see whether polyphenols can reduce mtDNA methylation level in the D-loop.

Curcumin did not show any effect on mtDNA methylation level. This may be due to the short incubation time of 24 hours. While has been found that Curcumin treatment increases ROS in MEG-01 cell within 24 hours (Larasati et al., 2018), other studies suggest that the effect on

the nDNA methylation happens with long-term treatment. For example, Liu et al incubated MV4-11 cells with 3 μM of Curcumin for 72 hours (Liu et al., 2009), and Link et al treated the colon cancer cells with low doses of Curcumin for 6 or 240 days and concluded that Curcumin's methylation-modulatory activity was, perhaps, indirect (Link et al., 2013). This may explain why mtDNA methylation level did not change with 24 hours Curcumin incubation. However, given that MEG-01 samples have been incubated with Resveratrol for 72 hours, it is possible that its effect on mtDNA methylation level is indirect.

The experiment was conducted in two different batches and the batch effect was evident before the removal of the outliers in specific CpG positions. However, *MT-CO2* CpG1 did not show a batch and the different effects of Resveratrol at different concentrations may dependent on the hormetic effect of the Resveratrol molecule rather than the batch effect. Another important limitation is that the biological replicates show variation at specific CpG sites in the mtDNA methylation level, while at other CpG sites the mtDNA methylation level overlaps with the other biological replicates. This was evident before the removal of the outliers. For these reasons the results are not strong and cannot be considered reliable unless validated in a further experiment. Further, the possibility of nuclear DNA contamination was excluded by performing PCR using primers specific for the nuclear DNA gene PPARCG1A. The only sample who showed nDNA contamination is "Batch number 2, 50 μM Resveratrol, 72 h incubation, sample number 6 (B2_72h_R50_6)". This sample was excluded from analysis.

In conclusion, these experiment suggested that mtDNA methylation level in MEG-01 cells is CpG specific and, possibly, that at least part of the mitochondria DNA methylation patterns are established at the megakaryocytes level.

○

Chapter 4: Associations between the Mediterranean Diet Score and mitochondrial DNA methylation

4.1 Introduction

4.1.1 Diet

Dietary factors are the leading contributors to premature death globally (Stanaway et al., 2018), and the third leading factor for death in the Italian population (Monasta et al., 2019). More recently, Afshin et al confirmed that sub-optimal diet contributes to more deaths than any other risk factor, including smoking, and that diet affected health in all people regardless of age, sex, and socioeconomic development of the country. Therefore, they concluded that dietary factors are a major contributor to CVD risk globally (Afshin et al., 2019).

4.1.2 diet and DNA methylation

Diet, along with other lifestyle factors, influence CVD risk and also affect patterns of DNA methylation (Liyanage et al., 2016; Mathers et al., 2010; Mathers & Byun, 2016). In general higher intakes (or status) of methyl donors increase the level of mitochondrial DNA (mtDNA) methylation (Infantino et al., 2011; Menga et al., 2017; Saini et al., 2017), while dietary DNMT inhibitors reduce the risk of developing CVD (Pandey & Rizvi, 2009a; Widmer et al., 2013). There is strong evidence from both longitudinal cohort studies and intervention studies that higher adherence to a Mediterranean dietary pattern is associated with longer life expectancy and reduced CVD risk (Figure 1.9) (Estruch et al., 2018; Martínez-González et al., 2012; Martinez-Gonzalez & Martín-Calvo, 2016; Angelino et al., 2019).

4.1.3 Diet and platelets activity

Dietary pattern and food items have been demonstrated to be protective against CVD and other diseases (Meier et al., 2019). Although the relationship between diet and CVD has been

confirmed by several intervention studies (see section 1.5.2) (Trichopoulou et al., 2014; Gensous et al., 2020; Estruch et al., 2018; de Lorgeril et al., 1999), the mechanisms that link diet to improved clinical outcomes are still not well characterized *in vivo*. In the case of CVD, a prominent mechanism that accounts for the adverse outcome is thrombosis, or rather the process of blood clot (Violi et al., 2020).

This is especially true in a population of adults with overweight and obesity, where the participants are less responsive to anticoagulant drugs and more at risk of thrombotic events (Badimon et al., 2013; Anfossi et al., 2009; Vilahur et al., 2017; Bordeaux et al., 2010; Nansseu & Noubiap, 2015; Rothwell et al., 2018; Violi et al., 2020).

Mediterranean dietary patterns, plant-based food items, and polyphenolic compounds demonstrated to be effective in reducing platelets aggregability (Figure 4.1) (Vazhappilly et al., 2019; Hubbard et al., 2004b; Antonopoulou et al., 2006b; Gavriil et al., 2019; Bonaccio et al., 2014). For example, platelets count has been found to be associated with Mediterranean diet adherence in a population of 14,586 healthy volunteers (Bonaccio et al., 2014). Antonopoulou et al demonstrated that MeDiet intervention reduced platelets aggregation both in healthy and diabetic volunteers (Antonopoulou et al., 2006a). Also polyphenols-rich plant extracts and anthocyanins-rich juice have been found to reduce platelets aggregation (Gavriil et al., 2019; Santhakumar et al., 2015).

Additionally, the group of Carnevale et al investigated the effect of dark chocolate supplementation in healthy participants and smoker participants, whose platelets recruitment, platelets ROS and NOX2 activation were higher. They found that polyphenols rich dark chocolate was effective in reducing the platelets ROS in smoker participants but had no effect on the healthy ones (Carnevale et al., 2012).

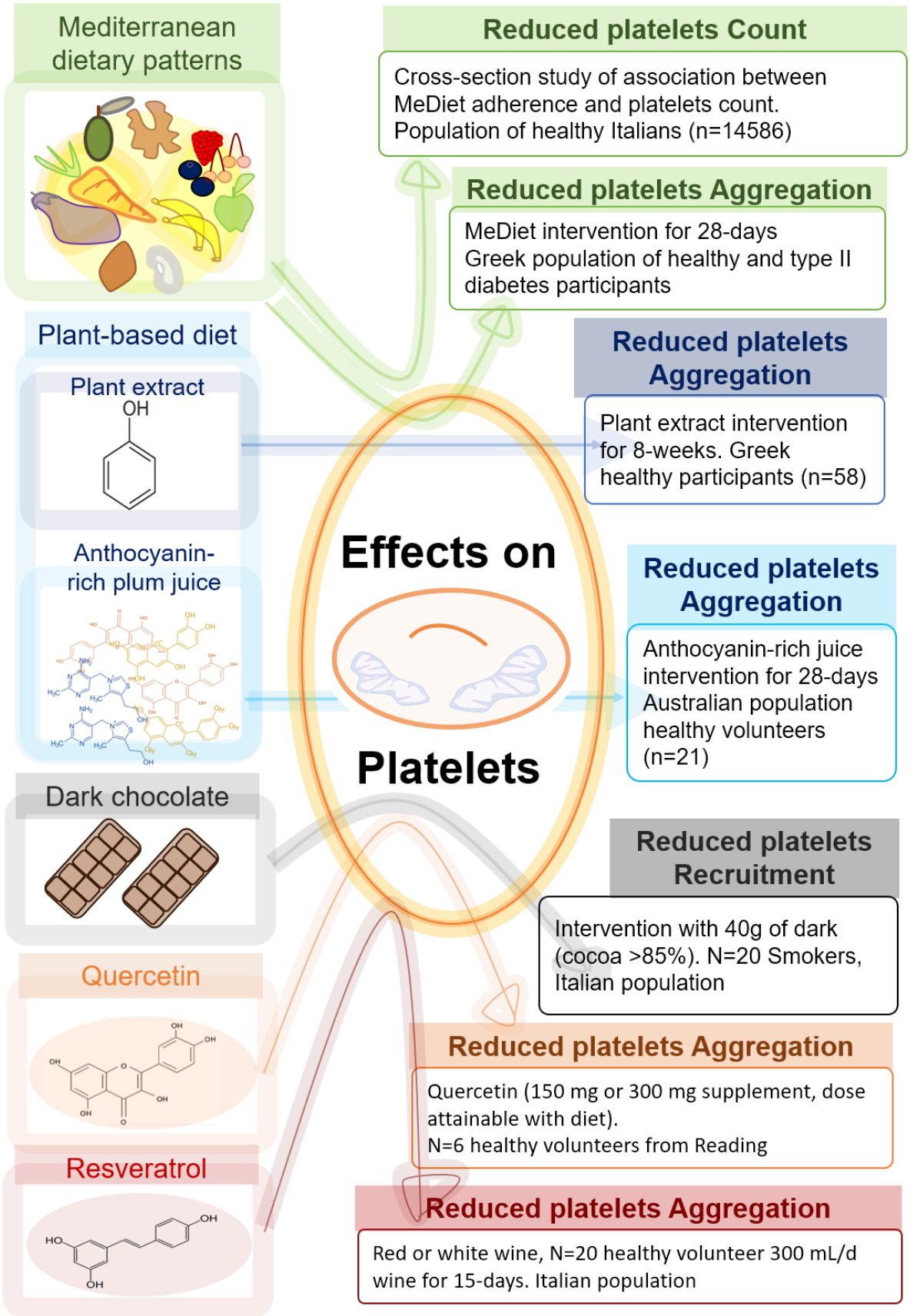


Figure 4. 1 Effects of dietary patterns, food items, and polyphenols on platelets

This image summarises some intervention studies that demonstrated the effect of the Mediterranean diet, plant extracts rich with polyphenols and individual polyphenols on platelets aggregation and count. For example, Mediterranean diet intervention studies demonstrated to reduce platelets count in a population of healthy Italians and reduce platelets aggregation in a group of healthy and diabetic Greeks volunteers (Bonaccio et al., 2014; Antonopoulou et al., 2006b).

Plant extract and anthocyanins-rich plum juice contain several types of vitamins and polyphenols. Particularly, the polyphenolic category of "flavonoids" includes Quercetin (Gavriil et al., 2019; Santhakumar et al., 2015). Similarly dark chocolate (Carnevale et al., 2012), Resveratrol-rich wine (Gresele et al., 2008) and Quercetin (Hubbard et al., 2004b) demonstrated to reduce platelets aggregation.

End of figure legend.

4.1.4 Diet and mtDNA methylation

To the best of my knowledge, there is no study that investigates the association between diet and mtDNA methylation in humans. However, this association has been investigated in yellow croaker fishes fed with different lipids sources (Liao et al., 2015) and piglets from mothers fed with different protein amounts (Jia et al., 2016, 2013). Among the two only Liao et al used the more accurate pyrosequencing to analyse mtDNA methylation level, whose changes in response to diet are anticipated to be very small. Liao et al found that olive oil and perilla oil-enriched diet, increase the mtDNA methylation level in the liver. The effect of these diets on the D-loop methylation level was investigated in the liver, muscle, and adipose tissue, but there was no difference.

4.1.5 SPHERE population and MeDiet

The overall aims of this study were to determine whether patterns of platelet mtDNA methylation at Baseline can predict the future CVD outcomes and to investigate possible associations with habitual diet (Figure 4.2).

In this chapter, MeDiet score has been extrapolated at Baseline and it has been used to evaluate the association between dietary patterns and cardiometabolic risk factors, mtDNA methylation level, and CVD outcome.

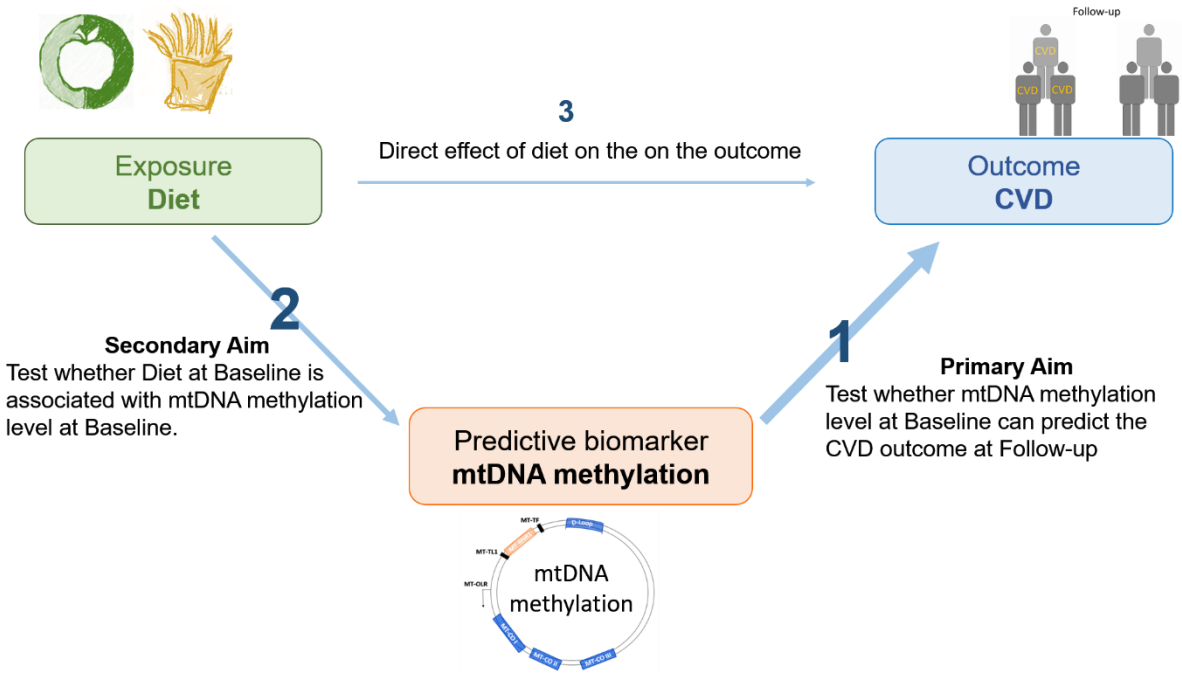


Figure 4. 2 Aims of the population study

4.2 Aims of Chapter 4

In this study, the adherence to the Mediterranean diet of n=134 participants was evaluated at Baseline using the MeDiet Score. The MeDiet Score was used to assess three main aims (Figure 4.2):

i) Define whether the diet at Baseline is cross-sectionally associated with cardiometabolic risk factors.

ii) Define whether the diet at Baseline can influence the mtDNA methylation at Baseline. (Secondary aim 2 of the population study)

iii) Define whether the diet at Baseline can influence the future CVD outcome at Follow-up.

4.3 Methods

4.3.1 Human participants

The human participants from the SPHERE cohort have been selected for the primary aim of finding correlations between the mtDNA methylation at Baseline and CVD outcome at Follow-up (See section 2.2 and Figure 2.2 and Figure 2.3). Given the availability of dietary information for 134 of them, the secondary aim, or rather the cross-sectional associations between mtDNA methylation and diet- were evaluated. Out of 200 individuals, 134 have filled the food frequency questionnaire (FFQ) reported (see section 2.8.2.1, Table 2.7). The demographic and clinical characteristics of these participants are summarised in table 4.1. Ethical approval was provided by the Institutional Review Board, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico at the University of Milan. Most of the SPHERE study participants were Caucasian (95.8% of cases), this is also the case for our set of 200 participants (Bollati, Iodice, et al., 2014).

4.3.2 MeDiet Score

See section 2.8.

4.3.3 Assessment of CVD risk at Baseline and Follow-up

At Baseline the common cardiometabolic risk factors based on anthropometric measures (e.g. BMI and waist circumference), lifestyle factors (e.g. smokers and years of education), and biochemical tests (e.g. fasting blood glucose and cholesterol analysis) were collected (Table 4.1).

4.3.3.1 Framingham risk score

To estimate individual CVD risk at Baseline, the Framingham risk score which uses the information on sex, age, systolic blood pressure (SBP), treatment for hypertension, smoking, type 2 diabetes, high-density lipoprotein (HDL), and total cholesterol (D'Agostino et al., 2008) was calculated. The Framingham risk score was available for n=132 participants out a total of n=134.

4.3.3.2 HeartScore

The HeartScore is used to predict the risk of fatal CVD within 10 years (Conroy et al., 2003; EAPC, 2012) using age, sex, SBP, cholesterol, HDL cholesterol, BMI, and smoking status. This score was developed by the European Society of Cardiology for the European population (EAPC, 2012) since we are investigating an Italian cohort, the "European Low Risk" model was selected. The HeartScore was available for n=32 participants therefore was excluded from the analysis.

4.3.4 CVD event definition

Details of CVD events were obtained from the hospital discharge registry of the Italian National Health Service. A CVD event was defined as any principal or any one of five secondary diagnoses of diseases of the circulatory system (3-digit ICD-9-CM codes from 390 to 459) (WHO, 2007). A detailed list of the CVD events of the 200 participants by Follow-up is summarised in Chapter 5, table 5.2. Briefly, 47 participants (56%) were diagnosed with hypertension and 37 participants (44%) were diagnosed with other CVD including atrial fibrillation and atherosclerosis (Table 5.2).

4.3.5 MtDNA methylation analysis

See section 2.4.1 for mtDNA extraction from platelets, and section 2.5 for mtDNA methylation quantification.

4.3.6 Data analysis

Data analysis was performed in collaboration with Valentina Bollati's Lab biostatistician at the University of Milan.

Univariate analyses were performed to assess the effect of MeDiet Score (dependent variable X), on the i) cardiometabolic risk factors at Baseline, ii) the mtDNA methylation level at Baseline, and iii) CVD status at Follow-up. Multivariate analysis was performed to better define the effect of MeDiet Score on the mtDNA methylation by adjusting for run, batch, nuclear DNA contamination, and HDL cholesterol. The mtDNA methylation % analysis was performed in duplicates, run1 and run2, and these values were analysed as repeated measure rather mean. The mtDNA methylation % was log-transformed to meet the requirements of normality. All reported P values were two-tailed, and those less than 0.05 were considered

statistically significant. Statistical analyses were performed with SAS software, version 9.4. The graphs were generated using R software, version 3.6.1 (2019-07-05).

The mediation analysis between the MeDiet Score (X, independent value), cardiometabolic risk factors (Y, dependent variable), and mtDNA methylation % (Mediator) was performed (Valeri & VanderWeele, 2013). For this analysis the direct effect and the P value of MeDiet Score on the Y variable were calculated using the beta value of the relationship between X and Y, adjusting for the mediator. Therefore, the direct effect was the association between the MeDiet "X" and the cardiometabolic risk factor "Y", removing the effect of mtDNA methylation level. The indirect effect was calculated using both the P value from the normal theory test for indirect effect and using the bootstrap method to estimate confidence limits for the indirect effect (Fritz & MacKinnon, 2007). These models have not been adjusted for any covariate.

4.4 Results

4.4.1 Characteristics of the human participants

The following table reports the characteristics of n=134 participants that have been included in the MeDiet analysis.

Variable	All participants (*n*=134)	CVD-free at Follow-up (*n*=70)	CVD-diagnosed during Follow-up (*n*=64)	*P* Value
Sex (n, %)				
Male	51 (38%)	26 (37%)	25 (39%)	0.819
Female	83 (62%)	44 (63%)	39 (61%)	
Age (mean, SD)	61.8 (±9.4)	61.0, ±8.8	62.7, ±10.0	0.314
BMI (mean, SD)	34.8 (±5.2)	34.6, ±4.9	35.0, ±5.7	0.658
BMI categorical (n, %)				
25.1-30.0 (Overweight)	30 (22%)	18 (25.7%)	12 (18.8%)	0.379
30.1-34.9 (Obesity I)	41 (31%)	18 (25.7%)	23 (35.9%)	

>35.1 (Obesity II and III)	63 (47%)	34 (48.6%)	29 (45.3%)	
Smoking status (n, %)				
Never	57 (41.4%)	29 (42.5%)	28 (43.8%)	
Former	61 (45.5%)	31 (44.3%)	30 (46.9%)	0.682
Current	16 (11.9%)	10 (14.3%)	6 (9.4%)	
Education, years of education (n, %)				
Primary school and other (<5 years)	23 (17.2%)	9 (12.9%)	14 (21.9%)	
Secondary school and high school (<13 years)	83 (61.9%)	46 (65.7%)	37 (57.8%)	0.196
University degree (>14 years)	23 (17.2%)	14 (20.0%)	9 (14%)	
SBP, mmHg (mean, SD)	127.2 (±14.5)	128.5, ±14.8	125.8, ±14.1	0.279
DBP, mmHg (mean, SD)	78.6, ±8.3	78.9, ±8.6	78.2, ±8.0	0.616
Fasting blood glucose, mmol/L (mean, SD)	5.9, ±1.3	5.7, ±1.3	6.0, ±1.4	0.257
Total cholesterol, mg/dl (mean, SD)	210.5, ±43.2	207.9, ±41.3	213.3, ±45.3	0.470
HDL cholesterol, mg/dL (mean, SD)	59.6, ±15.0	61.9, ±14.9	57.1, ±14.8	0.066
LDL cholesterol, mg/dL (mean, SD)	130.2, ± 38.3	129.3, ±36.1	131.2, ±40.7	0.782
Triglyceride (TC), mg/dL (mean, SD)	131.3, ±67.1	122.2, ±63.4	141.2, ±69.9	0.105
TC/HDL ratio (mean, SD)	3.7, ±1.1	3.5, ±089	4.0, ±1.3	**0.017**
Framingham risk score, median (Q1, Q3)	17.3 (8.4, 29.3)	16.8 (8.5, 26.0)	18.2 (8.4, 31.9)	0.565
Heart Score, median (Q1, Q3)	2.0 (1.0, 4.0)	3.0 (1.5, 4.0)	1.0 (1.0, 3.5)	0.113

Table 4.1. Characteristics of the participants.

The MeDiet Score was available for *n*=134 participants. The only variable that is associated with the CVD-status at the Follow-up is the TC/HDL ratio or cholesterol ratio, which is slightly higher in the participants who will be diagnosed CVD during the Follow-up.

4.4.2 MeDiet and CVD outcome

A univariate model was used to test whether the MeDiet Score at Baseline (independent variable "X") influences the CVD status at Follow-up (dependent variable "Y"). There is no difference in the distribution of MeDiet Score between those who will develop CVD from

those who will remain CVD-free at Follow-up (P= 0.8016) (Figure 4.3). Since the MeDiet Score does not predict the CVD status at Follow-up a mediation analysis could not be performed.

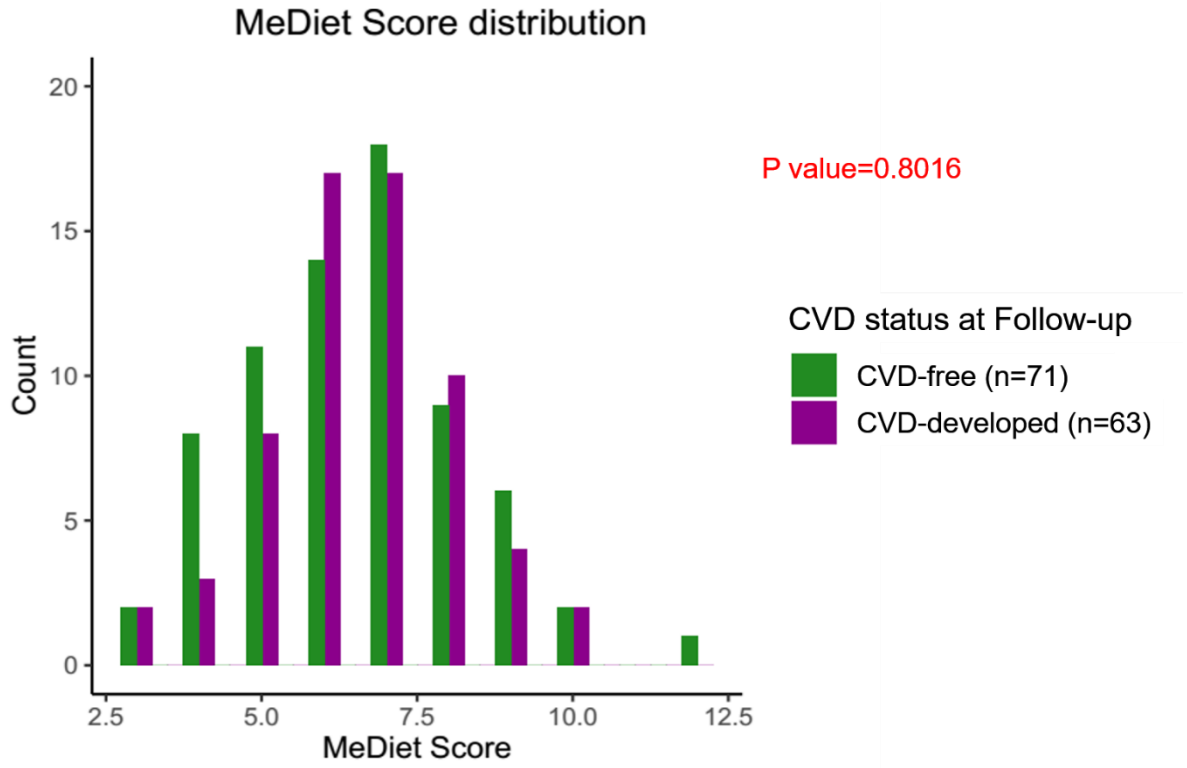


Figure 4. 3 MeDiet Score distribution in the population

There was a wide range of MeDiet scores within this cohort from 3-12 across from possible range of scores from zero to 14. There was no difference in the distribution of MeDiet Score between the participants who developed CVD during up to 5 years of Follow-up and those who remained CVD-free (P=0.8016).

4.4.3 Mediterranean diet distribution in the population

A total of 134 participants have completed the FFQ reported in table 2.7, 70 of them remained CVD-free at Follow-up (52%) and 64 developed CVD at Follow-up (48%) (Table 4.1). MeDiet Adherence was calculated using the MeDiet Score reported in table 2.8: the mean score was 6.7, the median was 7, and the range was (min=3, max=12).

The Mediterranean diet adherence has been calculated using the MeDiet Score. The MeDiet Score was considered the independent variable "X", and the cardiometabolic risks factors,

such as fasting blood glucose, BMI, and blood cholesterol were considered the dependent variable "Y". To estimate the effect of MeDiet Score on the dependent variables a univariate model has been performed (Table 4.2). The measure of association is a beta value or "estimate" which indicated the change in the outcome, or dependent variables, for each one unit change in the MeDiet Score (Table 4.2).

Figure 4.4 shows the association between the MeDiet Score and HDL cholesterol (P=0.004), thyroid-stimulating hormone (TSH) (P=0.030), 2-hours post-glucose insulin level (P=0.026), neck circumference (P=0.029), and Framingham risk score (P=0.041).

Dependent Variable	Estimate *	StdErr	Lower	Upper	P value
Age	0.237	0.496	-0.744	1.218	0.634
Weight	-1.263	0.913	-3.069	0.543	0.169
Height	-0.003	0.005	-0.013	0.007	0.591
BMI	-0.386	0.275	-0.930	0.157	0.162
circvita	-1.278	0.703	-2.669	0.113	0.071
Systolic blood pressure	-1.006	0.759	-2.507	0.495	0.187
Diastolic blood pressure	-0.077	0.440	-0.947	0.793	0.861
fc	-0.058	0.585	-1.216	1.100	0.921
diabete_n	-0.013	0.019	-0.051	0.024	0.474
diabete_hb	-0.036	0.021	-0.078	0.006	0.093
Uric Acid	-0.016	0.075	-0.164	0.133	0.833
Fibrinogen	3.985	3.221	-2.387	10.357	0.218
C-reactive protein	-0.008	0.040	-0.087	0.071	0.836
Total cholesterol	1.963	2.276	-2.539	6.464	0.390
HDL cholesterol	**2.247**	**0.767**	**0.731**	**3.764**	**0.004**
LDL cholesterol	0.540	2.020	-3.457	4.537	0.790
LDL/HDL	-0.060	0.044	-0.148	0.028	0.177
Total cholesterol/HDL	-0.099	0.057	-0.211	0.013	0.083
Triglyceride	-3.466	3.530	-10.448	3.516	0.328
Serum creatinine	0.015	0.033	-0.051	0.080	0.659
AST (U/l)	0.758	0.634	-0.496	2.012	0.234
ALT (U/l)	1.042	1.039	-1.013	3.096	0.318
Gamma-Glutamyltransferase	1.086	1.155	-1.200	3.371	0.349
Glucose	1.344	1.251	-1.131	3.819	0.285
Homocysteine	-0.327	0.313	-0.945	0.292	0.298
TSH	**0.156**	**0.071**	**0.016**	**0.297**	**0.030**
Glycated haemoglobin (mmolmol)	0.259	0.480	-0.690	1.209	0.590
Glycated haemoglobin %	0.096	0.050	-0.003	0.195	0.058
Postprandial glycaemia (mg/dl)	1.721	1.947	-2.132	5.573	0.379
Insulin level	-1.083	1.127	-3.312	1.146	0.338
2-hour post-glucose insulin level	**-7.862**	**3.486**	**-14.760**	**-0.963**	**0.026**
Urinary pH	0.038	0.039	-0.038	0.115	0.321
White blood cell count	0.044	0.086	-0.126	0.213	0.611
Neutrophils count	0.006	0.068	-0.128	0.140	0.930
Neutrophils %	-0.411	0.404	-1.209	0.388	0.311
Eosinophils count	0.008	0.007	-0.006	0.021	0.261
Eosinophils %	0.130	0.100	-0.069	0.329	0.199
Lymphocytes count	0.032	0.029	-0.025	0.088	0.271
Lymphocytes %	0.323	0.366	-0.401	1.048	0.379

Monocytes count	0.000	0.010	-0.019	0.019	0.999
Monocytes %	-0.021	0.105	-0.228	0.187	0.843
Basophil count	-0.001	0.001	-0.003	0.001	0.159
Basophil %	-0.020	0.014	-0.048	0.008	0.155
Granulocytes count	0.012	0.068	-0.123	0.147	0.860
Granulocytes %	-0.302	0.386	-1.066	0.463	0.437
Red blood cell count	0.009	0.023	-0.036	0.055	0.689
hgb	-0.073	0.069	-0.209	0.063	0.289
ht	-0.070	0.169	-0.405	0.265	0.680
Mean corpuscular volume	-0.267	0.363	-0.984	0.450	0.463
Platelets	-1.996	3.377	-8.677	4.685	0.555
Waist circumference	-1.335	0.698	-2.716	0.046	0.058
Neck circumference	**-0.494**	**0.223**	**-0.936**	**-0.052**	**0.029**
Index HOMA-IR	-0.171	0.130	-0.428	0.086	0.191
Waist to hip ration (WHR)	-0.002	0.019	-0.039	0.035	0.920
CD61 microvesicles per ml	-20.842	32.794	-86.562	44.878	0.528
Telomere length	0.018	0.014	-0.011	0.046	0.221
MtDNA copy number	0.018	0.017	-0.016	0.052	0.291
Glucose mmol	0.075	0.069	-0.063	0.212	0.285
Waist to Height Ratio	-0.007	0.004	-0.015	0.001	0.072
Non-HDL cholesterol	-0.285	2.228	-4.693	4.123	0.899
HOMA-IR	-0.326	0.437	-1.190	0.539	0.458
HOMA-β	-7.932	5.623	-19.057	3.192	0.161
QUICKI	0.001	0.001	0.000	0.003	0.069
Framingham Risk Score	**-1.882**	**0.912**	**-3.687**	**-0.077**	**0.041**
HeartScore	0.064	0.224	-0.394	0.522	0.778

Table 4.2 Association of MeDiet on the cardiometabolic risk factors at Baseline.

The number of participants used in this analysis is n=134. The estimate* is the gradient (slope) of the association between the MeDiet and the variable. This measure indicates the change in the variable of interest for each one-unit change in MeDiet Score.

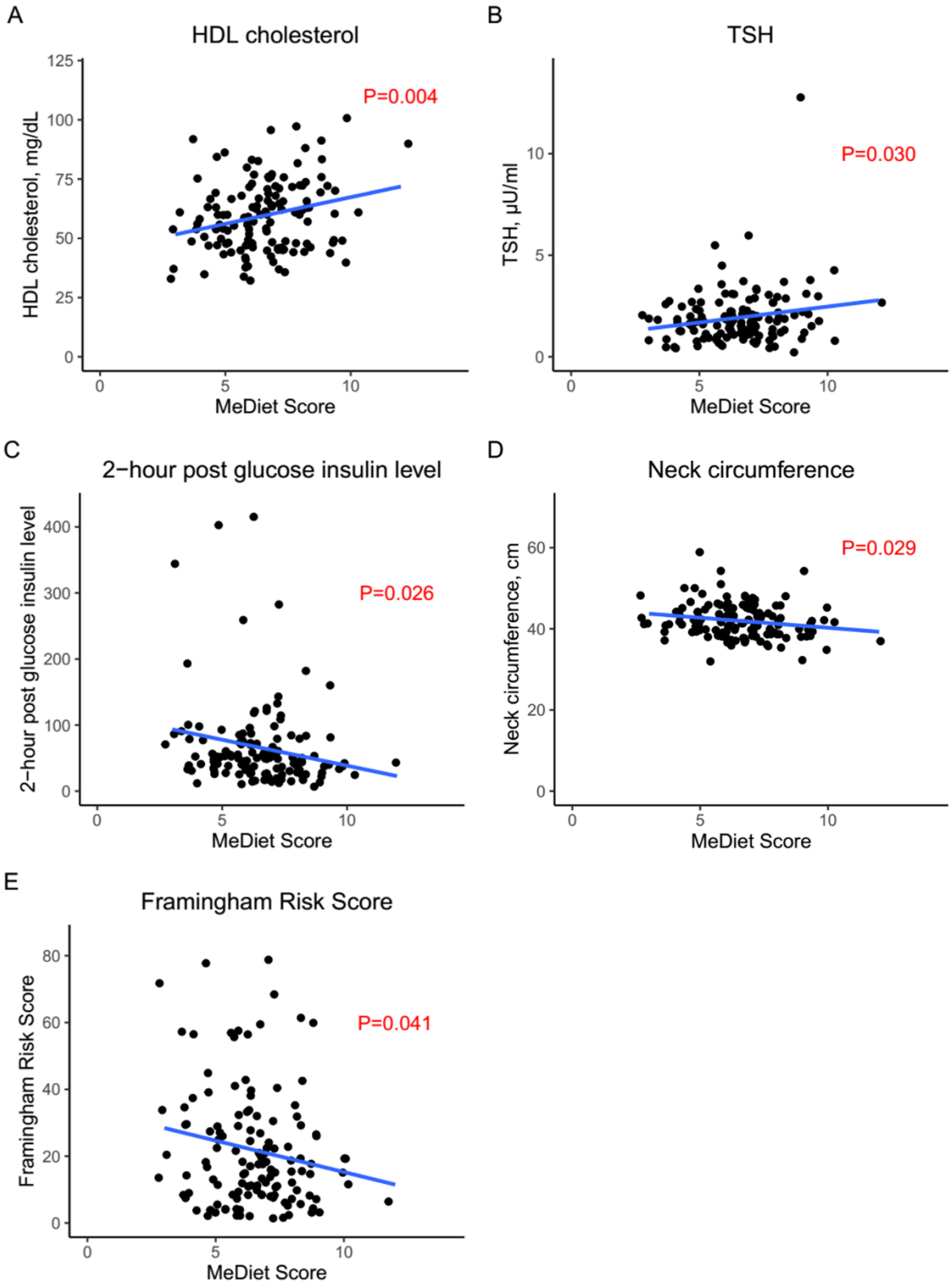

A
HDL cholesterol
P=0.004
HDL cholesterol, mg/dL
MeDiet Score
B
TSH
P=0.030
TSH, µU/ml
MeDiet Score
C
2−hour post glucose insulin level
P=0.026
2−hour post glucose insulin level
MeDiet Score
D
Neck circumference
P=0.029
Neck circumference, cm
MeDiet Score
E
Framingham Risk Score
P=0.041
Framingham Risk Score
MeDiet Score

Figure 4. 4 MeDiet Score and cardiometabolic risk factors.

Effect of MeDiet Score on A) HDL cholesterol (n=133), B) thyroid-stimulating hormone (TSH) (n=133), C) 2-hours post-glucose insulin level (n=129), D) neck circumference (n=132), and E) Framingham risk score (n=132). In red are reported the P values of the univariate model. The blue lines have been generated using the one-variable linear regression method with R (Y~X).

4.4.4 Associations between MeDiet score and platelet mtDNA methylation level

The associations between MeDiet (independent variable) and mtDNA methylation at baseline (dependent variable) were calculated using first a univariate model. The mtDNA methylation % was log-transformed to become normally distributed before analysis. The mtDNA methylation level was analysed using the repeated measure of the run1 and run2 rather than the average. Initially, in the univariate analysis, three CpG loci were associated with MeDiet Score at Baseline: *MT-CO2* nt8113 (P=0.020) and *MT-CO3* nt9444 (P=0.049) methylation were higher in participants with higher MeDiet Score, while D-loop nt16383 (P=0.024) was lower in participants with higher MeDiet Score. Then a multivariate analysis was performed, adjusting for run, batch, nuclear DNA contamination, and HDL cholesterol (Table 4.3 and Figure 4.5).

Multivariate analysis

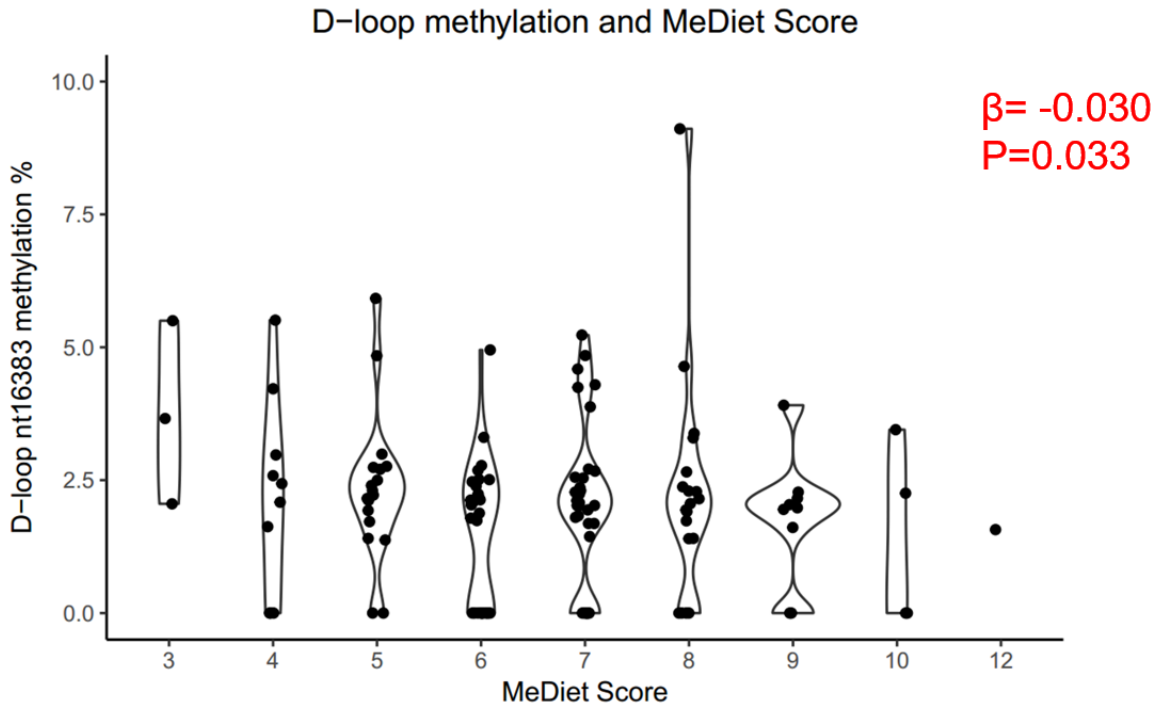
Outcome	Gene	Position (nt) *	Estimate	StdErr	95% CI		P value
					Lower	Upper	
mtDNA methylation	*MT-CO1*	6797	-0.015	0.019	-0.054	0.024	0.444
		6807	-0.007	0.015	-0.037	0.023	0.639
	MT-CO2	**8113**	**0.041**	**0.017**	**0.007**	**0.075**	**0.018**
		8117	-0.025	0.026	-0.079	0.029	0.353
	MT-CO3	9444	0.044	0.034	-0.026	0.113	0.212
		9449	0.062	0.100	-0.176	0.299	0.560
	D-loop	**16383**	**-0.030**	**0.014**	**-0.057**	**-0.002**	**0.033**
	MT-OLR	5737	0.011	0.019	-0.028	0.049	0.580
		5740	-0.004	0.015	-0.034	0.026	0.800
		5743	0.002	0.017	-0.032	0.036	0.912
	MT-TF	624	-0.013	0.030	-0.075	0.049	0.675
	MT-TL1	3247	0.008	0.007	-0.006	0.022	0.274
		3254	0.004	0.012	-0.020	0.029	0.723

Table 4.3 Multivariate analysis of the associations between MeDiet Score and mtDNA methylation.

In this multivariate analysis, the relationship between MeDiet and mtDNA methylation % (log transformed) was evaluated.

The Multivariate model has been adjusted for run, batch, nuclear DNA contamination, and HDL cholesterol.

Position*: the position of the CpG site is based on the NC_012920.1 version of the mtDNA.


D−loop methylation and MeDiet Score
β= -0.030
P=0.033
D−loop nt16383 methylation %
10.0
7.5
5.0
2.5
0.0
3
4
5
6
7
8
9
10
12
MeDiet Score

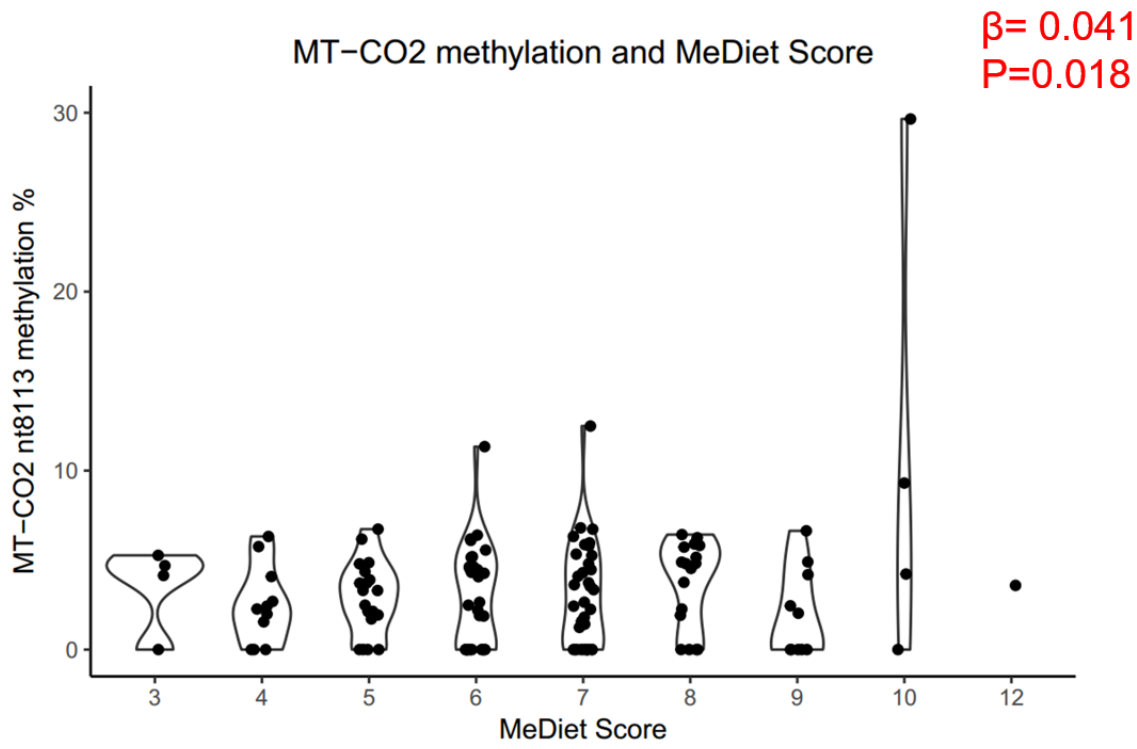

MT−CO2 methylation and MeDiet Score
β= 0.041
P=0.018
MT−CO2 nt8113 methylation %
30
20
10
0
3
4
5
6
7
8
9
10
12
MeDiet Score

Figure 4. 5 MeDiet Score association with mtDNA methylation at Baseline

On the X-axis there is the MeDiet Score (independent variable), while on the Y-axis the mtDNA methylation % (average of run1 and run2) is reported for two genes MT-CO2 nt8113 and D-loop nt16383 (dependent variables). The P values and the beta values reported have been calculated using the multivariate model adjusted for run, batch, nuclear DNA contamination, and HDL cholesterol.

End of figure legend.

4.4.5 MeDiet, metabolic risk factors, and mtDNA methylation

A mediation analysis to investigate the potential of mtDNA methylation to mediate the effect of the diet on the cardiometabolic risk factors was performed (Baseline). To perform a mediation analysis there are two basic assumptions. First that the X variable or the MeDiet Score is associated with the Y variable or the cardiometabolic risk factors. Therefore, the cholesterol HDL level, the TSH level, the neck circumference, 2-hours post-glucose insulin level, and the Framingham Score were used as "Y" variable. Secondly, to perform a mediation analysis, the X variable has to be associated with the mediator or the mtDNA methylation %; therefore two "mediators" were investigated *MT-CO2* nt8113 (Figure 4.6) and D-loop nt16383 (Figure 4.7) respectively. None of the mtDNA CpG sites showed a significant mediation effect on the MeDiet score and cardiometabolic risk factors.

MT-CO2 nt8113

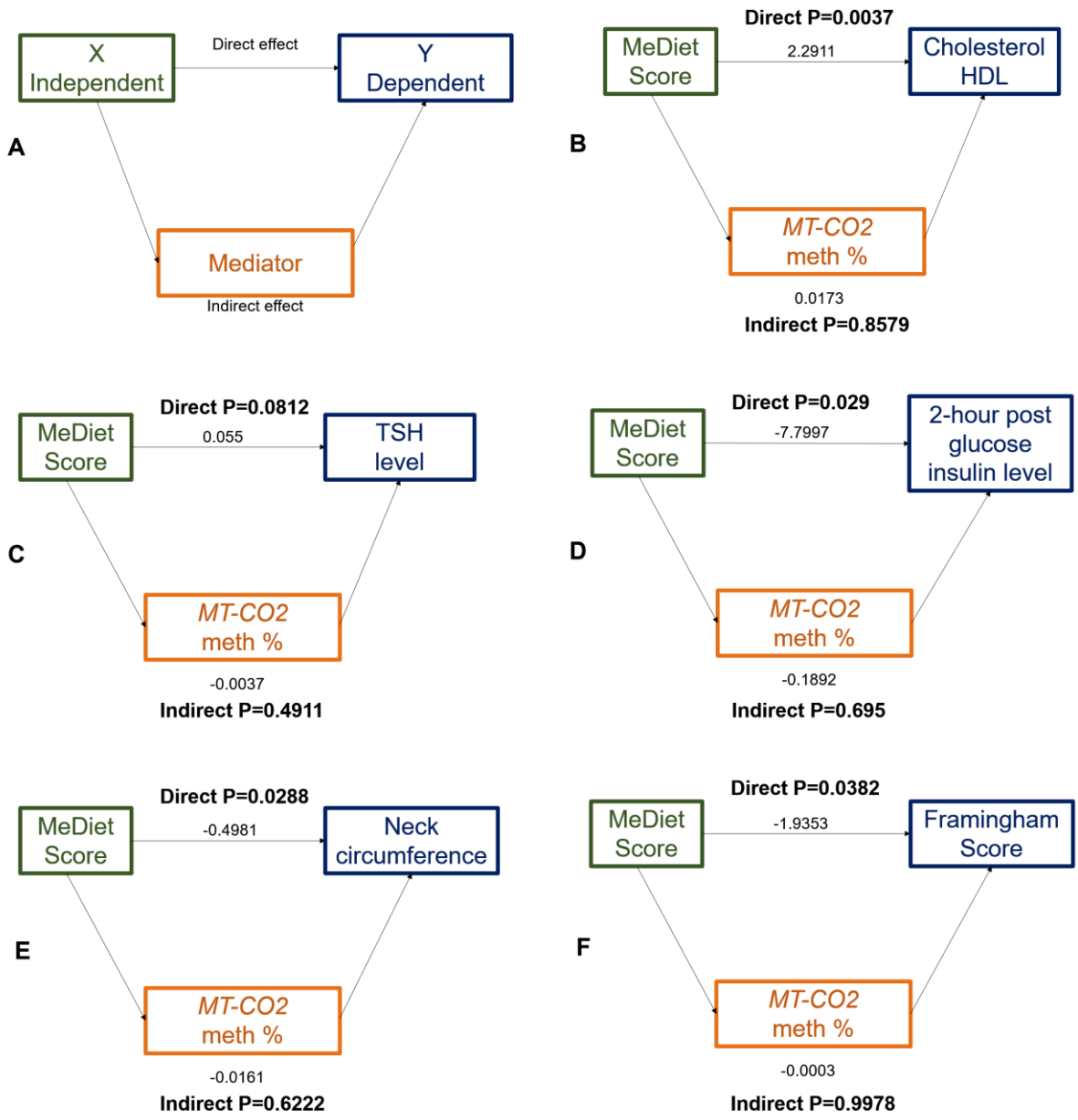


Figure 4. 6 Mediation analysis of the indirect effects of MT-CO2 methylation on the outcome

A) *Is the example of how the mediation analysis works. On the left green square, the is the independent variable "X", which in our case is the MeDiet Score. On the right blue square, there is the dependent variable "Y", which in this case is one of the cardiometabolic risk factors that have been found to be associated with the MeDiet Score. On the bottom of the figure there is an orange square there is the mediator or* MT-CO2 *nt8113 methylation %. The purpose of the analysis is to establish the effect of the MeDiet Score on the relationship "X→Y". The relationship between X and Y is reported on the top arrow "Direct effect", while the goodness of the mediator is expressed on the centre bottom of the figure "Indirect effect".* ***B, C, D, E, and F)*** *show the results for each of the cardiometabolic risk factors, namely Cholesterol HDL, TSH level, 2-hours post insulin glucose level, neck circumference, and Framingham Score.*

For this analysis, the mean of run1 and run2 of MT-CO2 *nt8113 methylation % have been used. The indirect P value reported have been generated using the normal theory test for the indirect effect.*

End of figure legend.

D-loop nt16383

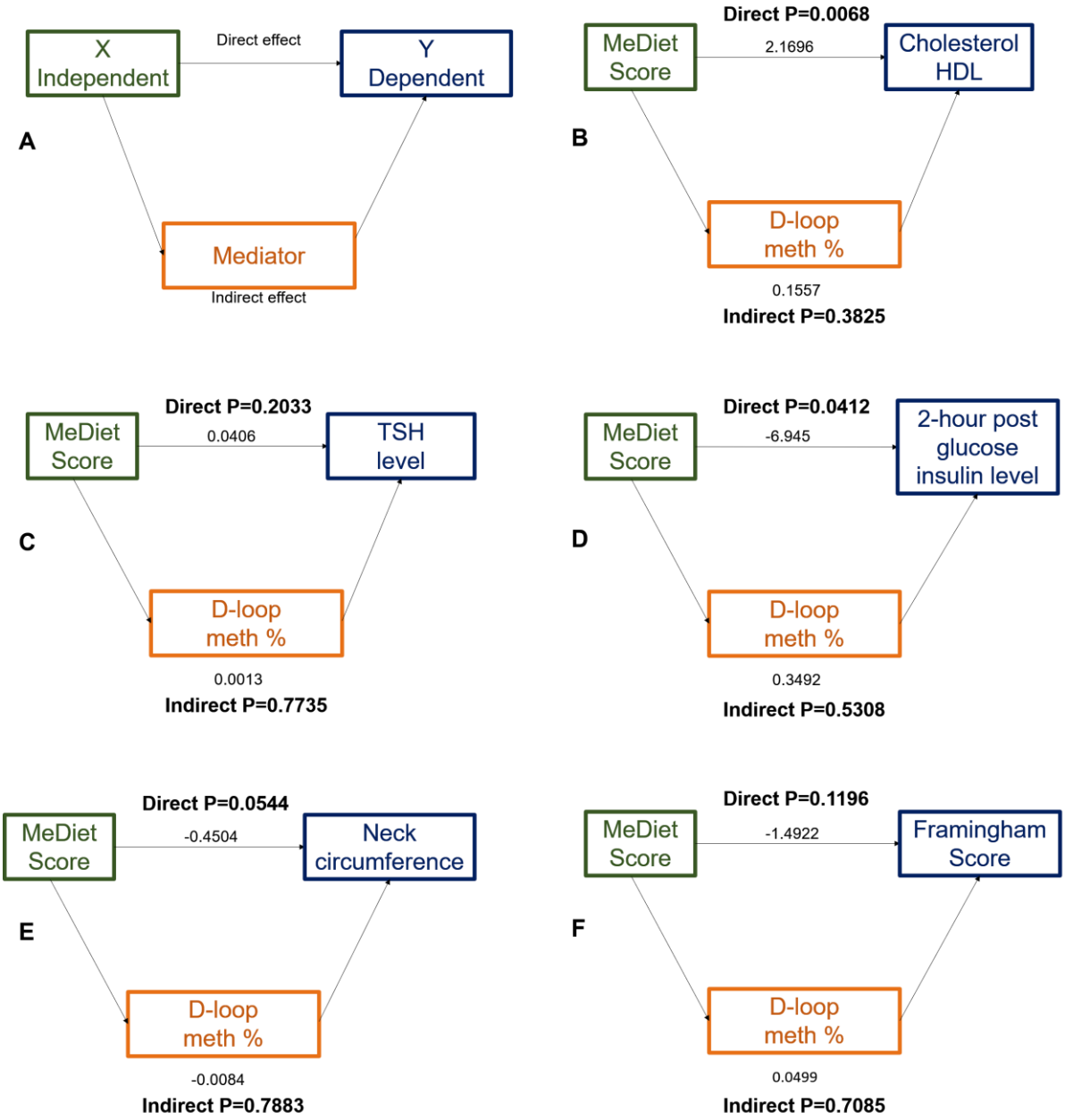


Figure 4.7 Mediation analysis of the indirect effects of D-loop methylation on the outcome

A) *Is the example of how the mediation analysis works. On the left green square, there is the independent variable "X", which in our case is the MeDiet Score. On the right blue square, there is the dependent variable "Y", which in this case is one of the cardiometabolic risk factors that have been found to be associated with the MeDiet Score. On the bottom of the figure there is an orange square there is the mediator or D-loop nt16383 methylation %. The purpose of the analysis is to establish the effect of the MeDiet Score on the relationship "X→Y". The relationship between X and Y is reported on the top arrow "Direct effect", while the goodness of the mediator is expressed on the centre bottom of the figure "Indirect effect".* ***B, C, D, E, and F)*** *show the results for each of the cardiometabolic risk factors, namely Cholesterol HDL, TSH level, 2-hours post insulin glucose level, neck circumference, and Framingham Score. For this analysis, the mean of run1 and run2 of D-loop nt16383 methylation % have been used. The indirect P value reported have been generated using the normal theory test for the indirect effect.*
End of figure legend.

4.5 Discussion

Main findings: To the best of my knowledge, this is the first study investigating the platelets mtDNA methylation in relations to diet quality in humans. Higher adherence to the MeDiet is associated with a wide range of health benefits, especially in later life, including reduced cardiovascular disease (Estruch et al., 2018; Arpón et al., 2017). In this cohort of 134 participants higher adherence to the Mediterranean diet is associated with hypomethylation of D-loop nt16383 (P=0.033) and increased methylation level of *MT-CO2* nt8113 (P=0.018) in platelets from older, overweight and obese individuals. However, there was no difference in MeDiet Score for individual who developed CVD over the following 5 years compared with those who remained CVD-free.

Interpretation of changes in mtDNA methylation patterns: The D-loop is critical for mitochondrial function since it initiates mtDNA replication and transcription. Increased D-

loop methylation has been associated with reduced mitochondrial functionality, and insulin resistance (Tong et al., 2017). Additionally, increased D-loop methylation has been previously associated with exposure to ambient air particles and inversely associated with mtDNA content (Janssen et al., 2015). Our results suggest that higher adherence to MeDiet lowers D-loop methylation which may protect against obesity-related comorbidities (e.g. insulin resistance). Interestingly, in our population one index of insulin resistance, the 2-hours post-glucose insulin level was significantly lower, at Baseline, in participants with higher MeDiet Score. However, the effect of the MeDiet Score on the 2-hours post-glucose insulin level was not mediated by D-loop methylation.

Higher MeDiet scores are associated with *MT-CO2* nt8113 CpG1 hypermethylation. *MT-CO2* gene encodes for a subunit of the Cytochrome-C-oxidase, a highly regulated enzyme involved in the oxidative metabolism. *MT-CO2* nt8113 hypermethylation has previously been reported in participants with CVD (Baccarelli & Byun, 2015). On the other hand, *MT-CO2* demethylation, induced by Valproic-Acid administration, has been reported to be associated with increased ROS production (Wolters et al., 2017). Increased adherence to MeDiet is known to reduce inflammation (Sureda et al., 2018), and possibly at the molecular level, to reduce free radicals. Given the involvement of Cytochrome C oxidase in the oxidative metabolism, our results suggest a possible role of MeDiet in mitochondrial ROS regulation via methylation of *MT-CO2*. However, in our population the effect of diet on the CVD outcome was not significant, therefore a mediation analysis with "diet → mtDNA methylation → CVD outcome" was not possible.

Further, the effect of MeDiet Score at Baseline was assessed on the major common cardiometabolic risk factors. Higher MeDiet Score is associated with increased HDL cholesterol (P=0.004) and TSH level (P=0.030), lower 2-hours post-glucose insulin level (P=0.026) and neck circumference (P=0.029). The positive association between MeDiet Score and HDL cholesterol has been previously reported in the Spanish cohort (Schröder et al., 2011; Papadaki et al., 2018). Therefore, even though the participants' number was low (n=134), still the MeDiet Score seems to detect cardiometabolic risks factor in our population. The 2-hours post-glucose insulin level, which is lower in participants with higher MeDiet Score, has been deemed "the earliest biomarker for diagnosing T2D" (DiNicolantonio et al., 2017), this remained valid in a cohort of adult females with obesity (Saxena et al., 2011). Therefore, in our cohort, it can be speculated that participants with a better diet have more favourable values of the 2-hours post-glucose insulin level, and

therefore are at less risk of developing diabetes. The neck circumference was lower in participants with higher MeDiet Score. This resonates with the literature that suggests the use of neck circumference as a biomarker of obesity and risk of developing obesity-related comorbidities (Ataie-Jafari et al., 2018; Preis et al., 2013). A higher level of Thyroid Stimulating Hormone (TSH), were found in participants with higher MeDiet Score. This may be due to the fact that hypothyroidism, or lower TSH levels, are associated with increased cardiovascular risk (Biondi & Klein, 2004).

Overall, even though the MeDiet Score is not associated with the main CVD risk factors such as BMI and blood pressure, it seems to be associated with some of the anthropometric and metabolic CVD risk factors. Interestingly, increased MeDiet Score is associated with lower Framingham risk score (P=0.041), which gives the participants' risk of developing severe CVD events in the following 10-years. In the literature, higher adherence to the Mediterranean dietary pattern was found to be associated with lower Framingham risk score (Veglia et al., 2019). However, in our population, the MeDiet Score and the Framingham risk score at Baseline were not associated with the incidence of mild CVD events in the following 5-years (Chapter 5). This is in accordance with our results from chapter 5 where the three genes associated with the future CVD outcome (*MT-CO1, MT-CO3* and *MT-TL1*) are not associated with the MeDiet or Framingham risk score.

A mediation analysis was performed to test whether the mtDNA methylation at the position *MT-CO2* nt8113 and D-loop nt16383 mediate the effect of MeDiet on the cardiometabolic risk factors, namely Cholesterol HDL, TSH level, 2-hours post-glucose insulin level, neck circumference, and Framingham risk score but this analysis was not significant. Implications for public health: The association between MeDiet and CVD status at Follow-up has been tested and resulted not significant. The lack of association between MeDiet Score and CVD outcome can be explained by several factors, such as the small sample number (n=134), the short age-range of our participants (50-70 years old), the short 5-years follow-up, and the mild CVD event that the participants developed, mostly hypertension. It can be speculated that different CpG sites of the mtDNA respond to different factors and that diet was not the sole causative agent of the CVD outcome in the following 5-years.

This study has limitations that merit consideration. The main limitation is the relatively small number of participants (n=134) for whom FFQ data were available. In addition, mtDNA methylation was measured on a single blood sample. The day-to-day variability in mtDNA methylation is not known so having multiple measurements on several days/ weeks may

provide more robust results for each participant. Additionally, having multiple blood samples across different year would help analysing the effect of diet on the CVD outcome.

In conclusion, for the first time, we observed associations between MeDiet adherence and mtDNA methylation. Validation of these findings in independent cohorts is required.

Chapter 5: Mitochondrial DNA methylation in platelets: predictor of CVD outcome in adults with overweight and obesity

5.1 Introduction

Obesity is recognised as a global epidemic (Jaacks et al., 2019). In adults and children alike (NCMP, 2018). Currently, in UK 66% of men and 58% of women live with overweight or obesity (CRUK, 2016). This trend has been increasing over the past four decades becoming a pressing issue, as BMI is one of the leading five risk factors in terms of attributable deaths and disability-adjusted life-years (NCD-RisC, 2016; Stanaway et al., 2018). Obesity is a heterogeneous disorder which 40-70% are caused by genetic factors (Comuzzie & Allison, 1998; Justice et al., 2017; Locke et al., 2015), mental disorders (Felitti et al., 2019), unhealthy lifestyle, socioeconomic status (Bann et al., 2018), ultra-processed food (Hall et al., 2019), and infections (Atkinson et al., 2005; Ponterio & Gnessi, 2015). Obesity is defined using BMI (Calle et al., 1999) which take into consideration only the weight and height of the participants without giving any information on the amount or distribution of body fat and the variety of metabolic manifestations (Neeland et al., 2018; Green et al., 2016).
Overweight and obesity are risk factors for cardiovascular diseases (CVD) development, this is attributed to insulin resistance (Reaven, 2011), inflammation (Karelis et al., 2005; Van Gaal et al., 2006; Wang & Nakayama, 2010), and the hyperaggregability of platelets (Bordeaux et al., 2010). However, not everyone with overweight and obesity develops CVD and the reasons why some individuals with obesity develop CVD while others remain CVD-free are poorly understood. Biomarkers for CVD risk prediction are based on the metabolic manifestations that in some cases coexist with excessive adiposity rather than the BMI. Biomarkers for CVD prediction include inflammation markers, such as C-reactive protein (CRP)(Ridker et al., 2005), uric acid (UA) (Ioachimescu et al., 2008; Wang et al., 2001), and fibrinogen (Appiah et al., 2015). Markers of platelet activation including lipoprotein-

associated phospholipase A2 (Lp-PLA2) are used in CVD risk assessment (Perk et al., 2012). In addition, obesity is associated with altered patterns of DNA methylation in the liver (Horvath et al., 2014) and in the blood (Dick et al., 2014), which may predict future CVD risk (Kim et al., 2010; Lind et al., 2018; Roetker Nicholas S. et al., 2018; Zhang et al., 2017; Agha Golareh et al., 2019).

Since patterns of DNA methylation are influenced by multiple environmental factors including diet that are risk factors for CVD, his is not surprising. DNA methylation is not only modified in participants with CVD (Rosa-Garrido et al., 2018; Zhang et al., 2018; Mathers et al., 2010), but also with exposure to CVD risk factors (Anderson et al., 2012; Gao et al., 2015; Joehanes et al., 2016; Rönn et al., 2015; Wahl et al., 2017). The associations between DNA methylation and CVD events are often stronger in individuals with pre-existing CVD risk markers, such as obesity (Kim et al., 2010; Mendelson et al., 2017).

Although nuclear DNA methylation has been the epigenetic mark investigated in most studies, growing evidence suggests that mitochondrial DNA (mtDNA) methylation is also associated with CVD risk and events (Baccarelli & Byun, 2015; Byun et al., 2016).

Mitochondrial dysfunction is implicated in obesity (Bournat & Brown, 2010; Zamora-Mendoza et al., 2018), CVD (Brown et al., 2017), and ageing (López-Otín et al., 2013), an occurs in a multiple cell types. For example, the mitochondrial role is central in platelet. As platelets are anucleated cells, their role in thrombosis and haemostasis it is regulated by mitochondria (Zharikov & Shiva, 2013). Intriguingly, platelets show hyperaggregability in adults with obesity and are unresponsive to anticoagulant treatment (Bordeaux et al., 2010). In CVD patients, the mtDNA in platelets is aberrantly methylated (Baccarelli & Byun, 2015) but whether this precedes disease development is not known.

5.2 Overview of chapter 5

Using the SPHERE population described in the Chapter 2, the mtDNA methylation level from platelets at Baseline when all the participants where otherwise healthy was measured.

5.2.1 Hypothesis

I hypothesized that aberrant platelet mtDNA methylation occurs in higher-risk individuals, such as adults with obesity, prior to developing CVD (Hypothesis 1).

Here, we tested that hypothesis by investigating whether platelet mtDNA methylation predicted future CVD events in 200 adults with overweight or obesity without CVD at the Baseline, 84 of whom developed CVD during five years Follow-up.

5.2.2 Aims of the study

1. To assess whether mtDNA methylation patterns at Baseline in adults with overweight and obesity predict the risk of CVD events in the following 5 years.
2. To investigate whether mtDNA methylation patterns are associated with well-recognised CVD risk factors such as: age, hypertension, cholesterol ratio, inflammation markers, smoking habit, and BMI.

5.3 Methods

5.3.1 Human samples and study design

The human participants and study design are described in section 2.2 (Figure 2.1, 2.2, and 2.3).

5.3.2 Assessment of CVD risk at Baseline and CVD events at Follow-up

To estimate individual CVD risk at Baseline, the Framingham risk score which uses information on sex, age, systolic blood pressure (SBP), treatment for hypertension, smoking, type 2 diabetes, high-density lipoprotein (HDL), and total cholesterol was calculated (D'Agostino et al., 2008) (see section 4.3.3.1). In addition, the HeartScore was used to predict the incidence of fatal CVD within 10 years (Conroy et al., 2003; EAPC, 2012) using age, sex, SBP, cholesterol, HDL cholesterol, BMI, and smoking status.
Details of CVD events were obtained from the hospital discharge registry of the Italian National Health Service. A CVD event was defined as any principal or any one of five secondary diagnosis of diseases of the circulatory system (3-digit ICD-9-CM codes from 390 to 459) (WHO, 2007). A detailed list of the CVD events of the participants by Follow-up is summarised in Table 5.2. Briefly, 47 participants (56%) were diagnosed with hypertension and 37 participants (44%) were diagnosed with other CVD including atrial fibrillation and atherosclerosis (Table 5.2).

Variable	All participants (*n*=200)	CVD-free at Follow-up (*n*=116)	CVD-diagnosed during Follow-up (*n*=84)	*P* Value
Sex (n, %)				
Male	78 (39%)	44 (38%)	34 (40%)	0.716
Female	122 (61%)	72 (62%)	50 (60%)	
Age (mean, SD)	62.5 (±10)	61.7, ±9.5	63.5, ±10.6	0.210
BMI (mean, SD)	35.5 (±5.1)	35.4, ±4.9	35.5, ±5.4	0.936
BMI categorical (n, %)				
25.1-30.0 (Overweight)	34 (17%)	22 (19%)	12 (14%)	0.762
30.1-34.9 (Obesity I)	62 (31%)	33 (28%)	29 (35%)	
>35.1 (Obesity II and III)	104 (52%)	61 (53%)	43 (51%)	
Smoking status (n, %)				
Never	89 (45%)	53 (46%)	36 (43%)	0.859
Former	91 (46%)	50 (43%)	41 (49%)	
Current	19 (10%)	13 (11%)	6 (7%)	
Education, years of education (n, %)				
Primary school and other (< 5 years)	34 (17%)	18 (16%)	16 (19%)	0.297
Secondary school and high school (<13 years)	129 (65%)	76 (66%)	53 (63%)	
University degree (>14 years)	32 (16 %)	21 (18%)	11 (13%)	
SBP, mmHg (mean, SD)	128.2 (±13.7)	129.1, ±13.3	127, ±14.1	0.268
DBP, mmHg (mean, SD)	78.9, ±8.5	79.2, ±8.5	78.4, ±8.5	0.517
Fasting blood glucose, mmol/L (mean, SD)	5.9, ±1.4	5.8, ±1.4	6.0, ±1.4	0.384
Total cholesterol, mg/dl (mean, SD)	206.6, ±42.9	204.5, ±42.4	209.5, ±43.8	0.421
HDL cholesterol, mg/dL (mean, SD)	58.6, ±15.0	60.0, ±15.5	56.8, ±14.3	0.141
LDL cholesterol, mg/dL (mean, SD)	128.3, ± 37.1	127.6, ±36.0	129.1, ±38.8	0.777
Triglyceride (TC), mg/dL (mean, SD)	126.4, ±61.6	121.0, ±57.6	133.7, ±66.2	0.153
TC/HDL ratio (mean, SD)	3.7, ±1.1	3.6, ±0.9	3.9, ±1.2	**0.039**
Framingham risk score, median (Q1, Q3)	18.2 (9.3, 28.9)	17.9 (9.6, 26.2)	18.3 (8.8, 30.5)	0.636
Heart Score, median (Q1, Q3)	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	0.232

Table 5.1. Characteristics of the participants

International Classification of Diseases, 9th revision, Clinical Modifications, 2007 (ICD-9-CM-2007)	**Frequency**	**Percentage (%)**	**Categorization mild vs severe**
401.1 Hypertension benign	34	40.48	Mild

401.9 Hypertension, Unspecified	13	15.48	Mild
411.1 Intermediate coronary syndrome	1	1.19	Severe
413.9 Forms of angina pectoris other than Angina decubitus and Prinzmetal Angina	1	1.19	Severe
414.01 Atherosclerosis of native coronary artery	2	2.38	Severe
414.8 Other specified forms of chronic ischemic heart disease	1	1.19	Severe
414.9 Chronic ischemic heart disease, unspecified	1	1.19	Severe
420.91 Acute idiopathic pericarditis	1	1.19	Severe
423.0 Hemopericardium	1	1.19	Severe
423.9 Unspecified disease of pericardium	1	1.19	Severe
424.0 Mitral valve disorders	1	1.19	Severe
425.1 Hypertrophic obstructive cardiomyopathy	1	1.19	Severe
426.0 Atrioventricular block, third-degree	1	1.19	Severe
426.3 Left bundle branch block	1	1.19	Severe
427.0 Tachycardia, paroxysmal supraventricular	1	1.19	Severe
427.31 Atrial fibrillation	2	2.38	Severe
427.5 Cardiac arrest	1	1.19	Severe
427.9 Cardiac dysrhythmia unspecified	1	1.19	Severe
428.1 Left heart failure	1	1.19	Severe
428.9 Heart failure, unspecified	1	1.19	Severe
433.10 Occlusion and stenosis of carotid artery without mention of cerebral infarction	1	1.19	Severe
436 Acute, but ill-defined, cerebrovascular disease	1	1.19	Severe
437.0 Cerebral atherosclerosis	1	1.19	Severe
437.1 Other generalised ischemic cerebrovascular disease	1	1.19	Severe
437.3 Cerebral aneurysm, unruptured	1	1.19	Severe

437.9 Unspecified cerebrovascular disease	1	1.19	Severe
438.21 Late effects of cerebrovascular disease, hemiplegia affecting dominant side	1	1.19	Severe
438.85 Other late effects of cerebrovascular disease, vertigo	1	1.19	Severe
441.03 Abdominal aneurysm, ruptured	1	1.19	Severe
441.4 Abdominal aneurysm without mention of rupture	1	1.19	Severe
442.9 Aneurysm of unspecified site	1	1.19	Severe
444.22 Arterial embolism and thrombosis of lower extremity	1	1.19	Severe
451.19 Phlebitis and thrombophlebitis of deep veins of lower extremities, other	2	2.38	Mild
455.0 Internal haemorrhoids without mention of complication	1	1.19	Mild
455.5 External haemorrhoids with other complication	1	1.19	Mild
Stenosis	1	1.19	Severe

Table 5.2 CVD events of the participants recorded during Follow-up.

5.3.3 Platelet mtDNA preparation and DNA methylation measurement

Plasma samples from 200 participants were used to isolate platelet mtDNA as described previously (Baccarelli & Byun, 2015). Briefly, platelet pellets obtained by centrifugation of 200 µl plasma at 1400x*g* were treated with DNaseI (30U, ROCHE) to eliminate cell-free nuclear DNA containing nuclear mitochondrial DNA segments (NUMTs). The EZ DNA Methylation Direct kit (Zymo Research; D5021) was used for extraction and bisulfite conversion of mtDNA simultaneously. To maximise bisulfite conversion efficiency (Liu et al., 2016), mtDNA was linearised with *Bam*HI (NEB) prior to proteinase K treatment. The bisulfite-converted mtDNA (20 µl) was stored at -80^{o}C until analysis.

Bisulfite-PCR reactions were performed using 1 µl of bisulfite-converted mtDNA, 9 µl water, 12 µl Hot-Start GoTaq® DNA Polymerase (Promega), 1 µl forward primer (10 pmole) and 1

µl reverse biotin-labelled primer (10 pmole) (See Chapter 2.2.2 for details on PCR and agarose gel). For the complete explanation see section 2.4.1 (Figure 2.1).

5.3.4. Nuclear DNA contamination assessment

Further, nuclear DNA contamination was assessed using the PPARCG1A primer (PPARGC1A-F: TTTTTTGTGTAKTTTGTTGTTTTAA and PPARGC1A-R : TTACAAAAAATTTTAATTATTATATAACCA) (see Chapter 2.5.2.2).

5.3.5 Pyrosequencing

See section 2.5. The full list of primers used for this experiment and the CpG analysed is reported in table 5.3.

Each sample was analysed in duplicate (Pearson's correlation coefficient = 0.74 for technical replicates; coefficient of variation 12.5%), and the mean of replicates was used for further analysis. The correlations between methylation at CpGs within each gene were low (Table 5.4) and each CpG was treated as a separate data point.

Assay	Primer sequence	Measured CpG (nt#)*	Product size (bp)	Annealing temperature (°C)
MT-CO1	Forward: TATTAATTGGTTTTTTAGGGTTTAT Reverse-biotin labelled: CAACAAATCATTTCATATTACTTCC Sequencing primer: TATTTATAGTAGGAAT Sequencing entry: AGAC/tGTAGATATAC/tGAGTATATTTTATTTT	6797 6807	177	52
MT-CO2	Forward: TTTATGAGTTGTTTTTATATTAGGTTTAAA Reverse-biotin labelled: ACTCCACAAATTTCAAAACATTAAC Sequencing primer: TAAAAATAGATGTAAT Sequencing entry: TTTC/tGGAC/tGTTTAAATTAAA	8113 8117	123	52
MT-CO3	Forward: TATATTATTTGTTTAAAAAGGTTTT Reverse-biotin labelled: AATAAAAAACTCAAAAAAATCCTAC Sequencing primer: TATATTATTTGTTTAAAAAGGTTTT Sequencing entry: CGATAYGGGATAATTTTATT	9444 9449	95	52
MT-TL1	Forward: TAGGGTTTGTTAAGATGGTAGAGTT Reverse-biotin labelled: ACAATAAAAAATAAAAAATTAACCATAAAT Sequencing primer: TAGGGTTTGTTAAGATGGTAGAGTT Sequencing entry: TAGGGTTTGTTAAGATGGTAGAGTT	3247 3254	117	52
D-loop	Forward: TGTGTAGATATTTAATTGTTATTA Reverse-biotin labelled: CAAATCTATCACCCTATTAACCAC Sequencing primer: TAATTAATTAATATATTT Sequencing entry: TAGTAAATATGTTC/tGTTT	16383	254	52
MT-TF	Forward: TAAAGTAATATATTGAAAATGTTTAGA Reverse-biotin labelled: TACTTAATACTTATCCCTTTTAAT Sequencing primer: TATTGAAAATGTTTA Sequencing entry: GAC/tGGGTTTATATTA	624	168	52
MT-OLR	Forward: AATTGGTTTTAATTTATTTTTTT Reverse-biotin labelled: AACCTCTTTTTACCAACTCC Sequencing primer: AATTGGTTTTAATTTATTTTTTT Sequencing entry: C/tGTC/tGTC/tGGGAAAAAAGGT	5737 5740 5743	126	52

Table 5.3 Primers for pyrosequencing assays.

5.3.6 Statistical analysis

Statistical analysis of the pyrosequencing data has been performed with the support of the bioinformatics team at Professor Valentina Bollati Lab in University of Milan, which has full access to the SPHERE database.

For normally distributed demographic and clinical characteristics and for DNA methylation levels, data were expressed as mean and standard deviation, otherwise by median and range. Frequencies and percentages were calculated for categorical variables. CVD-free and CVD-developed at Follow-up were compared using χ^2 test for categorical data or Student's t-test for continuous variables. Multivariate logistic regression, adjusted for age, BMI, nDNA contamination, fasting blood glucose, cholesterol ratio (TC/HDL), systolic blood pressure, and diastolic blood pressure, was performed to investigate the association between DNA methylation at each locus (CpG site) and the prevalence of CVD during Follow-up. Estimated effects were reported as odds ratios (ORs) and 95% confidence intervals (CIs) associated with an increase of 5-methylcytosine (5mC) % at each locus.

Receiver-Operating Characteristic (ROC) curves were generated to evaluate the diagnostic ability of the cholesterol ratio and mtDNA loci to distinguish between subjects with CVD-free and CVD-developed at Follow-up. The optimum threshold was selected by the Youden Index as the one that maximised Sensitivity (SE) + Specificity (SP)-1. The area under the Curve (AUC) and corresponding 95% CI, SE, SP and threshold were reported for cholesterol ratio and for the *MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254. For each CpG site, a dichotomous variable was created viz. "methylation level above the threshold" for the specific locus and "methylation level below the threshold". In addition, it was tested the utility of a score built as the sum of the index value (0, 1) for each locus (*MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254) in predicting CVD. The score has three categories: none of three loci display mtDNA methylation above threshold (Score 0); any one of the three loci has mtDNA methylation above threshold (Score 1); and any two or all three loci display mtDNA methylation threshold (Score 2).

Kaplan-Meier survival curves and log-rank tests were calculated stratifying CVD cases by each locus below (=low) or above (=high) the threshold. To evaluate the independent prognostic value of each single locus, and of their combination, on future CVD cases, the hazard ratios (HR) was calculated with Cox multivariable regression models adjusted for diastolic blood pressure (DBP), systolic blood pressure (SBP), fasting blood glucose

concentration, nuclear contamination, and cholesterol ratio. The same model was used to evaluate the prognostic role of cholesterol ratio, when evaluated as the predictor. The assumption of proportional hazard was checked with the log [log(survival)] plot and by the time-dependent covariate test. Cox multivariable regression models were also used to evaluate the potential prognostic role of the Framingham Risk Score and of the European HeartScore on CVD risk.

A sensitivity analysis was performed by excluding the participants who developed CVD within a year from Baseline in all Cox multivariable regression models. An additional sensitivity analysis was performed by stratifying the CVD cases into 'Mild' such as hypertension (*n*=51) and 'Severe' events, such as ischemic heart diseases (*n*=33). However, the Severe events (*n*=33) category did not provide enough power to be tested reliably (data not shown). All reported P values were two-tailed, and those less than 0.05 were considered statistically significant. Statistical analyses were performed with SAS software, version 9.4.

5.3.6.1 Survival Cox Regression

In order to test the biomarkers present at Baseline that are predictive of the outcome "CVD" at the Follow-up, Survival Cox Regression (Marioni et al., 2015; Perna et al., 2016; Y. Zheng et al., 2016) was used. The following analysis was performed using SAS9.4. The methylation percentage as calculated with pyrosequencing was not modified for this analysis.

Equation description

The probability of the end point (CVD development) is called the "hazard". The hazard function is as follow.

$$H(t) = H_0(t) \exp\left(\beta_1 X_{mtDNA\ methylation} + \beta_2 X_{Gender} + \beta_2 X_{Age} + \beta_n X_n\right)$$

H(t) = Hazard at time t

$H_0(t)$ = baseline hazard, the hazard when all the predictors equals 0

$X_{1,2,3,\ldots}$= predictors (mtDNA methylation level)

t= the exact time of CVD event for each individual

The exponential function of the covariates was used to insure that the hazard is positive and there is no intercept in the Cox hazard function. By dividing both sides by the baseline hazard $H_0(t)$, which is the same at every time point, and making the logarithm the hazard ratio is obtained:

$$ln\frac{H(t)}{H_0(t)} = \beta_1 X_{mtDNA\ methylation} + \beta_2 X_{Gender} + \beta_2 X_{Age} + \beta_n X_n$$

After having assessed the three categories as described is the results section "Model for predicting the future CVD risk" the hazard ratio was analysed for each of the three categories adjusting for diastolic and systolic blood pressure, fasting blood glucose, cholesterol ratio, and nuclear DNA contamination.

5.4 Results

5.4.1 Characteristics of participants

The mean age of participants (n=200) was 62 years (SD=10) and 61% (*n*=122) were female. The participants were overweight or obese (mean BMI=35.5, SD=5.1) and without CVD diagnosis at Baseline. These participants were followed for up to five years and the incidence of CVD was recorded (Figure 5.1). At Baseline, those participants who developed CVD during Follow-up were BMI- and sex-matched to those who remained CVD-free. In addition, smoking status, education levels, blood pressure (systolic and diastolic (SBP and DBP)), fasting blood glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglyceride levels at Baseline were not significantly different by future CVD status (P>0.05) (Table 5.1). Total cholesterol to HDL cholesterol ratio (TC/HDL) was lower at Baseline in those who remained CVD-free compared to participants who developed CVD (CVD-free: mean=3.7, SD=1.1; CVD-developed: mean=3.9, SD=1.2; P=0.039) (Table 5.1).

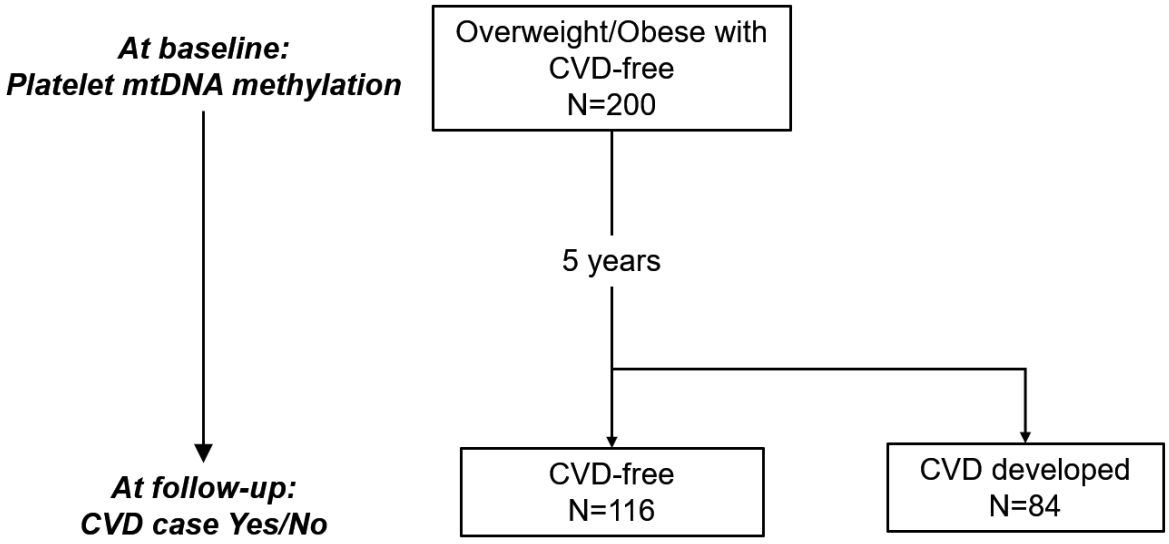


Figure 5.1 Study flowchart

5.4.2 Distribution of mtDNA methylation in the participants

Using pyrosequencing, 13 CpG sites distributed within seven mitochondrial genomic regions were analysed (Figure 5.2). First, the Pearson correlation coefficients was calculated between extent of methylation at each of the CpG sites located within an individual gene and for each of the seven different assays included in the study. The methylation percentage of any single CpG site was independent of methylation at other CpG sites and, therefore, each of the CPG was analysed individually (Table 5.4). In table 5.5, the methylation percentage at each CpG site it is reported for the CVD-free at Follow-up and CVD-developed at Follow-up groups (Table 5.5).

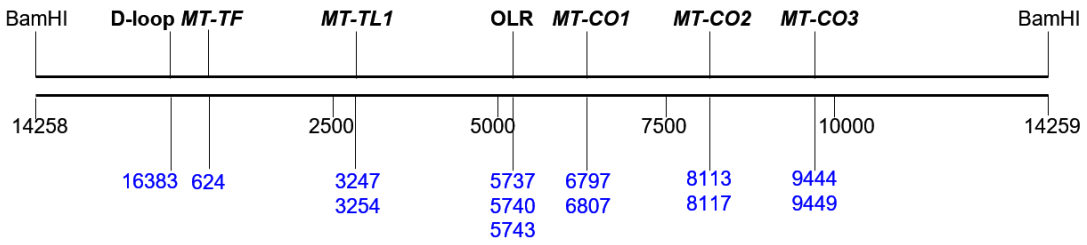


Figure 5.2 CpG locations within the mitochondrial genome

Mitochondrial DNA was linearised using BamHI. The gene names and that of the displacement loop (D-loop), and the origin-of-replication of the light-strand (OLR) are annotated on the upper side. The nucleotide position of the CpG sites that have been analysed are annotated below in blue text.

MtDNA gene	Nucleotide position	CVD-free at the Follow-up (n=116)					CVD-developed at the Follow-up (n=84)				
		Mean (%)	SD	Median	Q1	Q3	Mean (%)	SD	Median	Q1	Q3
MT-CO1	6797	3.1	3.1	2.6	0	5.3	2.9	3	2.5	0	4
	6807	10.8	4.8	10.3	7.6	13.6	12.5	4.8	12.7	9.7	15.1
MT-CO2	8113	3.8	3.5	3.7	1.2	5.7	3.2	3.2	2.5	0	5.2
	8117	0.4	0.8	0	0	0	0.6	1.2	0	0	1.1
MT-CO3	9444	0.7	2	0	0	0.7	1.3	1.9	0	0	2.2
	9449	0.1	0.7	0	0	0	0.2	0.7	0	0	0
MT-TL1	3247	6.6	1.5	6.5	5.8	7.3	6.2	1.5	6.2	5.6	6.7

	3254	2.4	1.5	2.7	1.5	3.6	3	1.6	3.1	1.8	4.1
D-loop	6383	2.1	2.1	2.1	0	2.7	2.4	1.6	2.2	1.7	3
MT-TF	624	0.7	1.7	0	0	0	1.2	2.5	0	0	1.6
MT-OLR	5737	1.1	1.4	0.8	0	1.5	1.3	1.2	1	0.3	2
	5740	3.8	1.7	4.1	2.8	4.9	4.1	2.1	4.2	2.4	5.4
	5743	3.5	1.9	3.7	2.3	4.4	3.7	2.1	3.8	2.2	5.3

Table 5.5. Summary of Methylation percentage for each CpG position quantified using pyrosequencing.

5.4.3 Platelet mtDNA methylation at Baseline by future CVD status

In this study, 13 CpG sites distributed within seven mitochondrial genomic regions were analysed (Figure 5.2). Methylation at Baseline was lower in those participants who remained CVD-free compared with those who developed CVD during Follow-up at nt6807 of *MT-CO1* (CVD-free: mean=10.8±4.8%; CVD-developed: mean=12.5±4.8%; P=0.014), nt9444 of *MT-CO3* (CVD-free: mean=0.7±2%; CVD-developed: mean=1.3±1.9%; P=0.042), and nt3254 of *MT-TL1* (CVD-free: mean=2.4±1.5%; CVD-developed: mean=3.0±1.6%; P=0.008) (Figure 5.3a-c). No significant differences in methylation were present for the other CpG sites measured.

Then was tested the methylation at the three loci in relation to the development of CVD during Follow-up. The odds ratios (OR) for developing CVD during Follow-up were 1.08 (95% CI 1.02-1.16) for nt6807 of *MT-CO1*, 1.22 (95% CI 1.02-1.46) for nt9444 of *MT-CO3*, and 1.30 (95% CI 1.05-1.61) for nt3254 of *MT-TL1*, adjusted for age, BMI, fasting blood glucose, cholesterol ratio, SBP and DBP (Figure 5.4). Logistic regression demonstrated that there were no significant associations between mtDNA methylation of *MT-CO1*, *MT-CO3* and *MT-TL1* and conventional CVD risk biomarkers at the Baseline, including insulin resistance (HOMA-IR), age, cholesterol level, serum uric acid, and BMI (Appendix A).

Position (nt#)	*MT-CO1* 6797	*MT-CO1* 6807	*MT-CO2* 8113	*MT-CO2* 8117	*MT-CO3* 9444	*MT-CO3* 9449	*MT-TL1* 3247	*MT-TL1* 3254	D-loop 16383	*MT-TF* 624	*MT-OLR* 5737	*MT-OLR* 5740	*MT-OLR* 5743
MT-CO1 6797	1	0.77647 <.0001	0.1761 0.0146	0.10858 0.1349	-0.10996 0.1269	-0.09612 0.1836	-0.14134 0.0482	0.22877 0.0013	0.46573 <.0001	0.01773 0.8052	0.08875 0.2149	0.29655 <.0001	0.33849 <.0001
MT-CO1 6807	0.77647 <.0001	1	0.1618 0.025	0.13884 0.0554	0.04579 0.5261	0.01438 0.8426	-0.15348 0.0317	0.28669 <.0001	0.50499 <.0001	0.06199 0.3881	0.15897 0.0257	0.34708 <.0001	0.36526 <.0001
MT-CO2 8113	0.1761 0.0146	0.1618 0.025	1	0.35736 <.0001	0.24829 0.0005	0.20411 0.0046	0.35101 <.0001	0.05281 0.4646	0.16581 0.0257	0.46865 <.0001	0.34074 <.0001	0.20646 0.0038	0.14159 0.0489
MT-CO2 8117	0.10858 0.1349	0.13884 0.0554	0.35736 <.0001	1	0.01637 0.8222	0.11327 0.1197	0.0784 0.2785	0.10325 0.153	0.21243 0.0042	0.18628 0.0095	0.1935 0.0069	0.29934 <.0001	0.22649 0.0015
MT-CO3 9444	-0.10996 0.1269	0.04579 0.5261	0.24829 0.0005	0.01637 0.8222	1	0.55779 <.0001	0.42585 <.0001	0.04138 0.5647	-0.11953 0.1061	0.41703 <.0001	0.55029 <.0001	0.18602 0.0089	0.14008 0.0502
MT-CO3 9449	-0.09612 0.1836	0.01438 0.8426	0.20411 0.0046	0.11327 0.1197	0.55779 <.0001	1	0.16685 0.0197	-0.01355 0.8509	-0.03266 0.6608	0.21639 0.0024	0.34343 <.0001	0.11986 0.0943	0.13468 0.0605
MT-TL1 3247	-0.14134 0.0482	-0.15348 0.0317	0.35101 <.0001	0.0784 0.2785	0.42585 <.0001	0.16685 0.0197	1	0.08669 0.2234	-0.14277 0.0525	0.31831 <.0001	0.38255 <.0001	0.22996 0.0011	0.15047 0.0343
MT-TL1 3254	0.22877 0.0013	0.28669 <.0001	0.05281 0.4646	0.10325 0.153	0.04138 0.5647	-0.01355 0.8509	0.08669 0.2234	1	0.29298 <.0001	0.14493 0.0416	0.13576 0.0559	0.30944 <.0001	0.39609 <.0001
D-loop 6383	0.46573 <.0001	0.50499 <.0001	0.16581 0.0257	0.21243 0.0042	-0.11953 0.1061	-0.03266 0.6608	-0.14277 0.0525	0.29298 <.0001	1	0.05739 0.4378	0.28847 <.0001	0.32449 <.0001	0.40303 <.0001

MT-TF 624	0.01773 0.8052	0.06199 0.3881	0.46865 <.0001	0.18628 0.0095	0.41703 <.0001	0.21639 0.0024	0.31831 <.0001	0.14493 0.0416	0.05739 0.4378	1	0.30585 <.0001	0.05395 0.4492	0.06999 0.3272
MT-OLR 5737	0.08875 0.2149	0.15897 0.0257	0.34074 <.0001	0.1935 0.0069	0.55029 <.0001	0.34343 <.0001	0.38255 <.0001	0.13576 0.0559	0.28847 <.0001	0.30585 <.0001	1	0.5669 <.0001	0.54853 <.0001
MT-OLR 5740	0.29655 <.0001	0.34708 <.0001	0.20646 0.0038	0.29934 <.0001	0.18602 0.0089	0.11986 0.0943	0.22996 0.0011	0.30944 <.0001	0.32449 <.0001	0.05395 0.4492	0.5669 <.0001	1	0.81336 <.0001
MT-OLR 5743	0.33849 <.0001	0.36526 <.0001	0.14159 0.0489	0.22649 0.0015	0.14008 0.0502	0.13468 0.0605	0.15047 0.0343	0.39609 <.0001	0.40303 <.0001	0.06999 0.3272	0.54853 <.0001	0.81336 <.0001	1

Table 5.4. Correlation matrix of mtDNA methylation at CpG position included in the study.

Correlation size and interpretation: Very high positive (negative) correlation 0.90 to 1.00 (−0.90 to −1.00); High positive (negative) correlation 0.70 to 0.90 (−0.70 to −0.90); Moderate positive (negative) correlation 0.50 to 0.70 (−0.50 to −0.70); Negligible correlation 0.30 to 0.50 (−0.30 to −0.50); Negligible correlation 0.00 to 0.30 (0.00 to −0.30). “nt” stands for “nucleotide” position and it is based on the mtDNA sequence “NC_012920.1”.

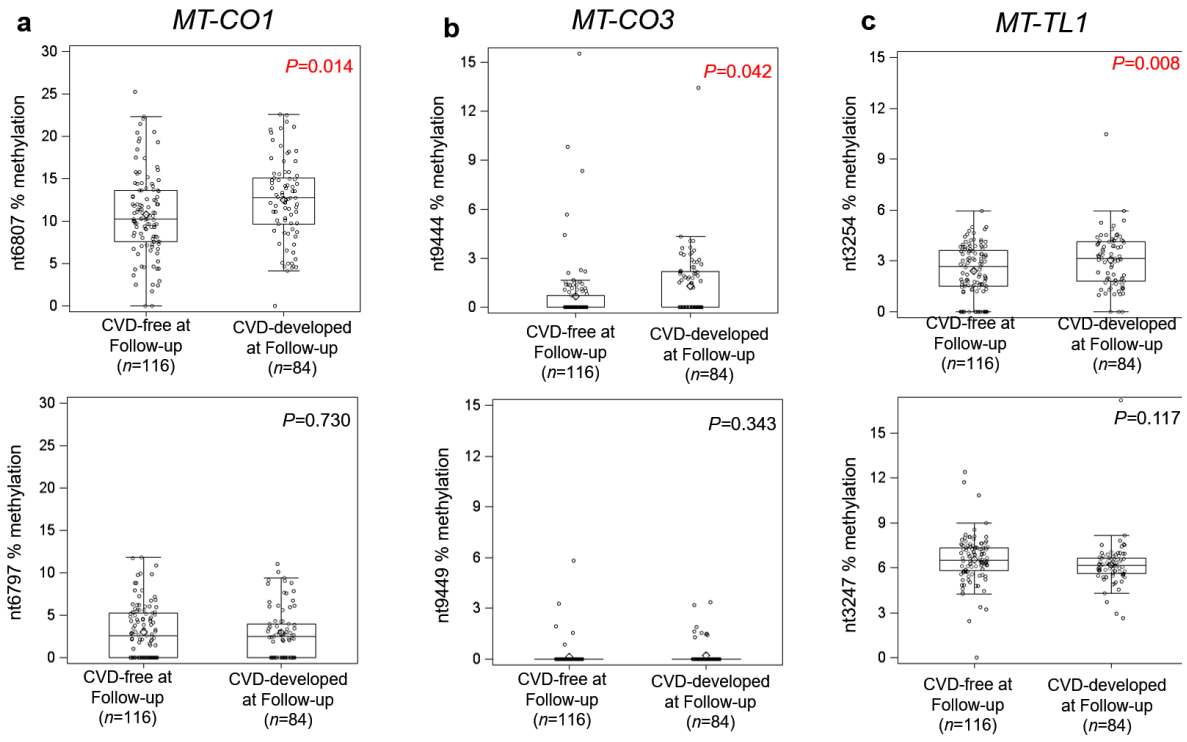


Figure 5.3 Distribution of mtDNA methylation at Baseline among individuals who remained CVD-free and who develop CVD during Follow-up

Methylation at two CpG positions for MT-CO1 (A), MT-CO3 (B) and MT-TL1 (C), examined by pyrosequencing. The top panels report the CpG sites whose methylation significantly differs between the CDV-free and CVD-developed at Follow-up. The P values reported for MT-CO1 nt6807, MT-CO3 nt9444, and MT-TL1 nt3254 were calculated by t-test.

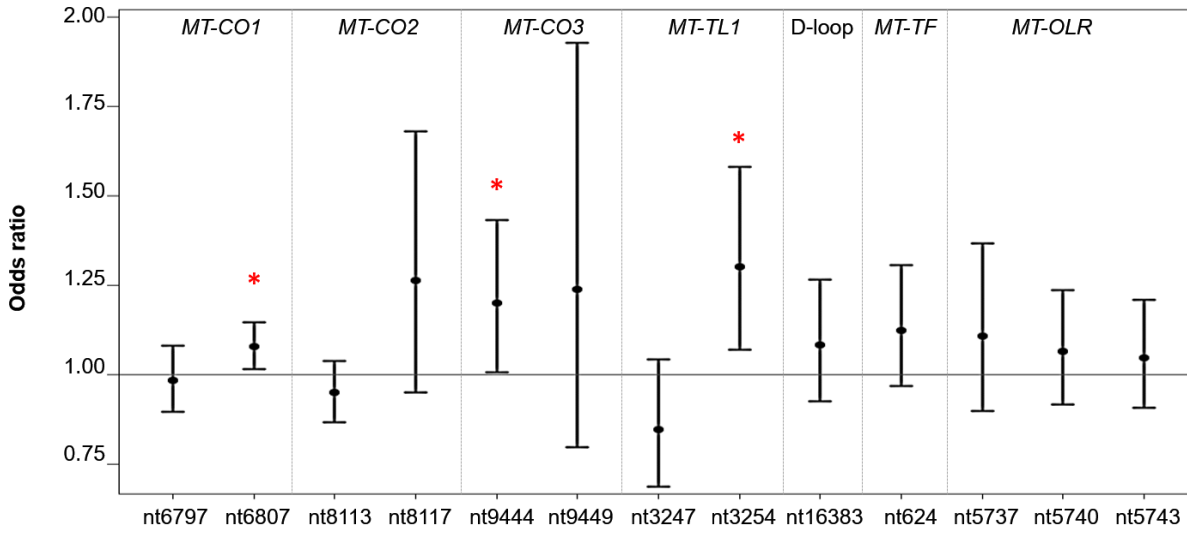


Figure 5.4 Odds ratios for the estimated contribution of each CpG site to future risk of CVD

The estimated effect of mtDNA methylation at each CpG site on the CVD outcome at Follow-up, expressed as odds ratio (ORs) with 95% CI. Statistically significant positions are indicated by red asterisks. The analysis was performed by a multivariate logistic model adjusted for age, BMI, fasting blood glucose, cholesterol ratio, SBP and DBP.

5.4.4 Utility of platelet mtDNA methylation to predict CVD risk

Receiver-operating characteristic (ROC) curves were generated to determine the optimal threshold of mtDNA methylation (%) for each CpG site at Baseline to discriminate between CVD-free and CVD-developed individuals at Follow-up (Figure 5.5). Thresholds of 12% for *MT-CO1* nt6807 (P=0.049), 1.5% for *MT-CO3* nt9444 (P=0.001), and 3% for *MT-TL1* nt3254 (P=0.22) yielded maximum discrimination between CVD-free and CVD-developed participants (Table 2a). TC/HDL cholesterol, which differed between groups at Baseline, was not a predictor of CVD risk during Follow-up (P=0.38) (Figure 5.5d, Table 5.6).
The threshold values that maximised sensitivity and specificity to predict CVD risk were used to create dichotomous variables "methylation level above the threshold" and "methylation level below the threshold" for each of the significant CpG sites within *MT-CO1*, *MT-CO3* and *MT-TL1*. Using these values, overall scores were calculated for each individual participant as follows: methylation not above the thresholds at any of the three loci (Score 0); methylation above the threshold at any one locus (Score 1); and methylation above the

threshold at any two or all three loci (Score 2) (Table 5.7). Compared with Score 0, the hazard ratio (HR) for developing CVD for Score 1 was 1.38 (95% CI, 0.68-2.78) and for Score 2 was 2.68 (95% CI, 1.41-5.08) (Figure 5.6a). During Follow-up, 65% of the individuals with Score 2 developed CVD, while only 21% of individuals with Score 0 developed CVD (Figure 5.6a and Table 5.7). Participants with Score 2 had a lower median time without-CVD (35.1 months) than participants with Score 1 (54.8 months). More than half of the participants with Score 0 were CVD-free at the end of the Follow-up period.

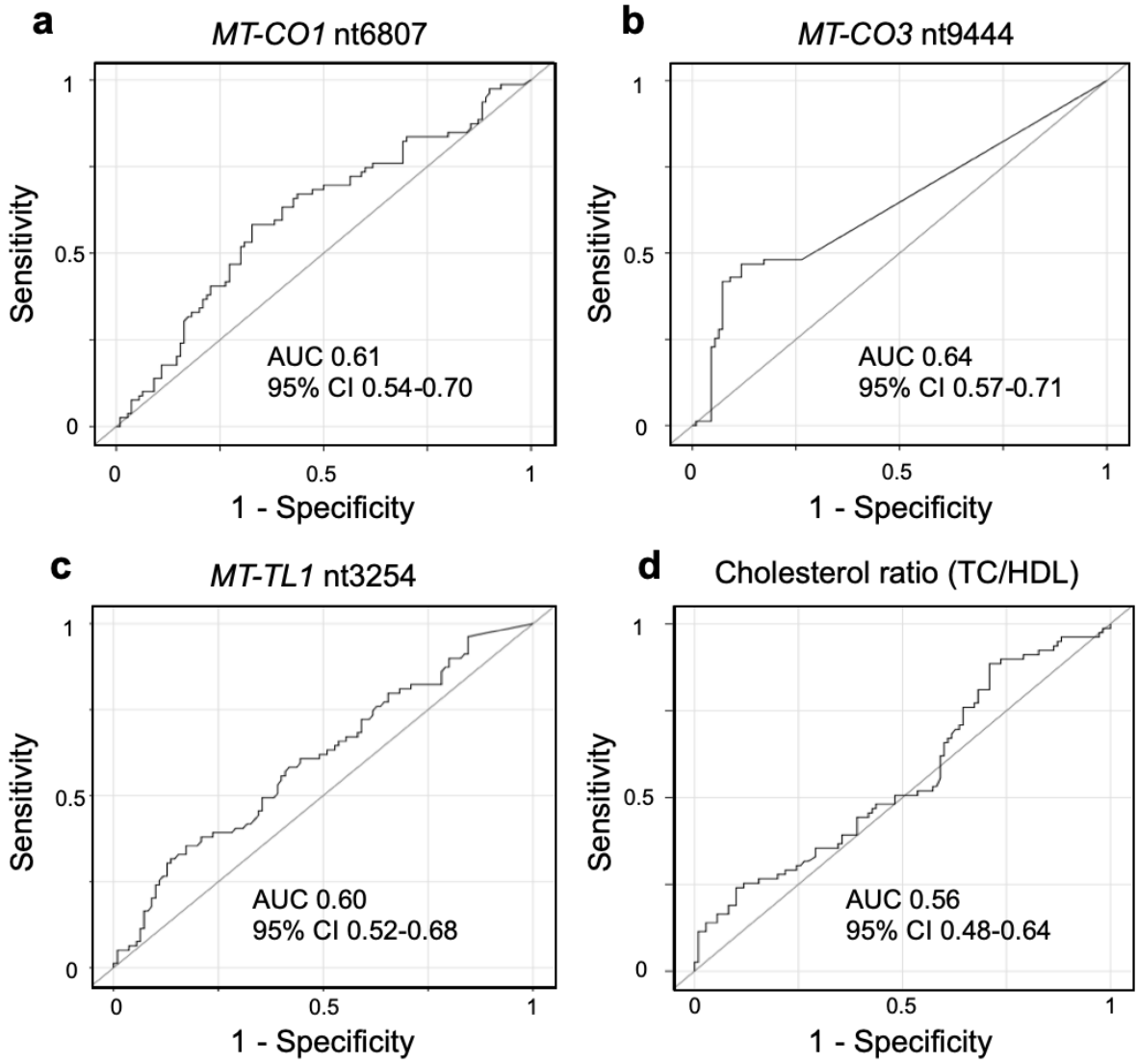


Figure 5.5 ROC curves for methylation at three loci and cholesterol ratio for prediction of CVD outcome.

	Category	Median survival time (months)*	At risk	CVD during Follow-up	CVD free	Log-rank *P* value
All patients		43.8	200	84	116	
MT-CO1 nt6809	<12.0	47.5	114	35	79	**0.049**
(% methylation)	≥12.0	38.3	83	47	36	
MT-CO3 nt9444	<1.5	47.0	146	44	102	**0.001**
(% methylation)	≥1.5	33.0	51	38	13	
MT-TL1 nt3254	<3.0	45.7	105	37	68	0.22
(% methylation)	≥3.0	42.1	94	46	48	
Cholesterol ratio	<3.5	42.1	102	43	59	0.38
(ratio)	≥3.5	45.3	94	41	53	

Table 5.6 MtDNA methylation thresholds for each CpG site and outcomes of survival analysis

Survival analysis for the participants stratified according to the methylation score at the single gene positions, MT-CO1, MT-CO3, and MT-TL1, and to the cholesterol ratio.

* Median: time in months without-CVD.

Discrimination ability of methylation at three CpG sites (MT-CO1 nt6807, MT-CO3 nt9444, and MT-TL1 nt3254) (a, b, and c) and the cholesterol ratio (TC/HDL) (d) to predict CVD incidence within 5 years of baseline. The area under the ROC Curve (AUC) and 95% CI values are annotated.

Score*	Median survival time (months)	At risk	CVD during Follow-up	CVD-free	% CVD-developed at Follow-up	Log-rank P-value
0	~ 60	61	13	48	21%	
1	54.8	63	21	42	33%	0.003
2	35.1	69	45	24	65%	

Table 5.7 Score to predict future CVD events based on methylation levels at MT-CO1 nt6809, MT-CO3 nt9444, and MT-TL1 nt3254

*Participants with Score 2 (two or three CpG sites with methylation above the thresholds) had a lower median time without-CVD (35.1 months) than the participants with Score 1 (54.8 months) and Score 0 (the median survival time is not reached).

This analysis was performed on a total of 193 participants, for whom the methylation % of all the three genes was available.

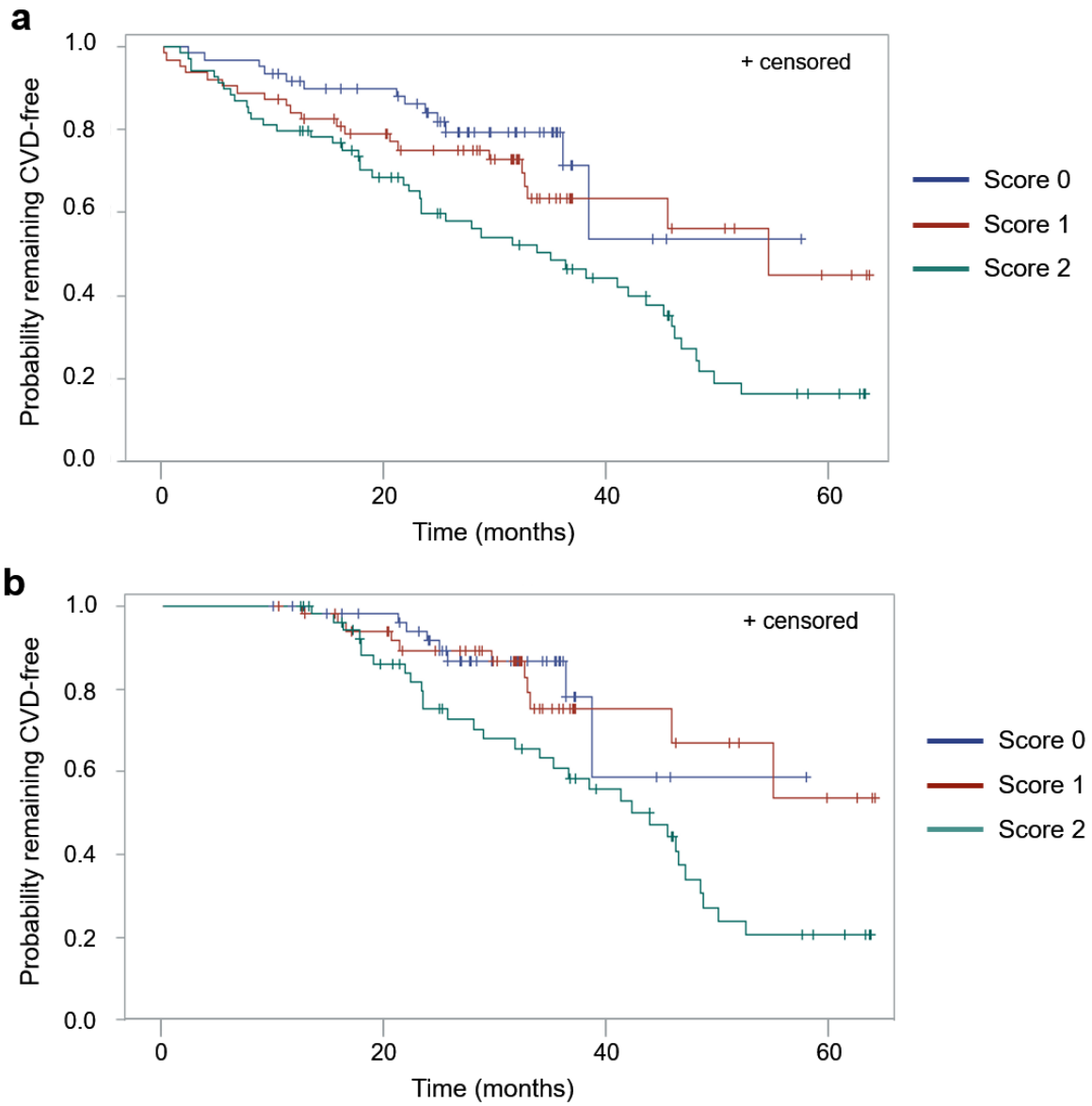


Figure 5.6 Kaplan-Meier curves for probability of remaining CVD-free by methylation score

a) *Time CVD-free from Baseline (months) among participants categorised by methylation at MT-CO1 nt6807, MT-CO3 nt9444 and MT-TL1 nt3254. b) Sensitivity analysis in which all participants who developed CVD within one year of Baseline were excluded.*

5.4.5 Comparison with existing risk prediction models

Conventional CVD risk prediction scores were calculated at Baseline using the Framingham Risk Score (D'Agostino et al., 2008) and the European HeartScore (Conroy et al., 2003). These scores were not different between those who remained CVD-free and the CVD-developed group (P=0.636 and P=0.232, respectively), demonstrating the potential utility of mtDNA methylation as a predictor of CVD development.

5.4.6 Sensitivity analysis

A sensitivity analysis was performed by excluding participants who developed CVD within a year from Baseline, but this did not change the relationships previously observed (Figure 5.6b). The HR for those who Scored 2 remained significantly higher than those who Scored 1 (HR=2.17, 95% CI 1.06-4.47) and was even higher in comparison with those who Scored 0 (HR=2.53, 95% CI 1.12-5.72) (Figure 5.6b). An additional sensitivity analysis was performed by stratifying the CVD cases into 'Mild', such as hypertension (*n*=51), and 'Severe' events, such as ischemic heart diseases (*n*=33) (Table 5.2). The model was tested in the Mild subgroup and showed that the mtDNA methylation score was a significant (P<0.001) predictor of future risk of developing CVD. The HR for those who Scored 2 was significantly higher than for those Scored 1 (HR=2.27, 95% CI 1.13-4.44, P=0.021) and those who Scored 0 (HR=4.34, 95% CI 1.76-10.73, P<0.002). No such relationships were apparent in the Severe subgroup of CVD events (*n*=33), due to lack of power (P=0.086) (data not shown).

5.4.6 Validation

Validation was performed diving the 200 participants in two groups, the test set (*n*=150) and the validation set (*n*=50). The test and validation set have the same proportion of CVD-free and CVD-developed at Follow-up participants. The same Cox survival analysis was

performed on both the test and the validation set. This analysis was adjusted by systolic and diastolic blood pressure, fasting blood glucose, cholesterol ratio, and nuclear contamination (figure 5.7a, 5.7b). Both in the test and in the validation sets the relationship previously observed remained significant (P=0.045, and P=0.034 respectively). In the test set (*n*=150) the HR for those who Scored 2 remained significantly higher than for those who Scored 1 (HR=1.59, 95%CI 0.86-2.93, P=0.137) and those who Scored 0 (HR=2.45, 96%CI 1.14-5.28, P=0.022). In the validation set the HR for those who Scored 2 remained significantly higher than those who Score 1 (HR=8.73, 95%CI 1.66-45.88, P=0.011) and those who Scored 0 (HR=4.18, 95%CI 1.22-14.31, P=0.023).

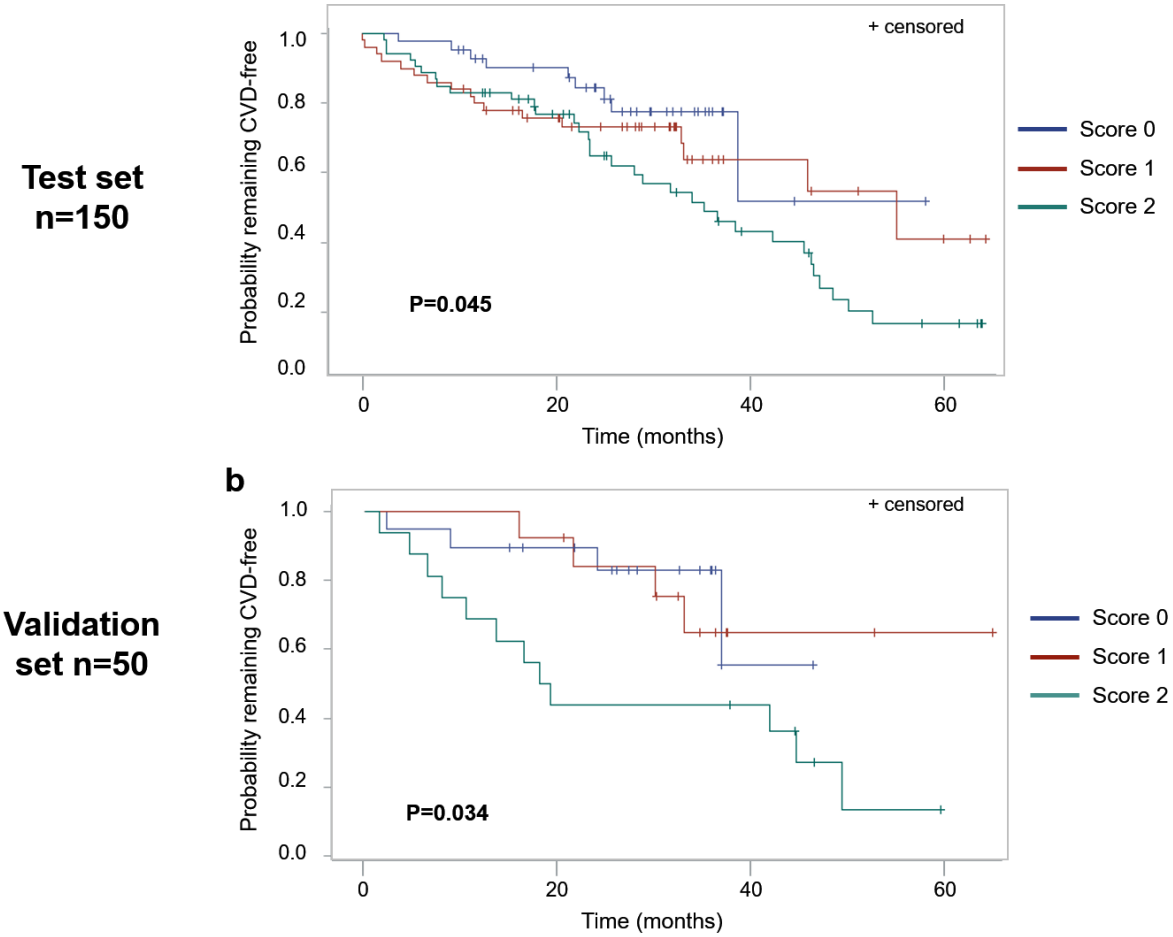


Figure 5.7 Validation of the model

A validation analysis was performed by dividing the initial population (n=200) into the test (n=150) and validation (n=50) sets. In the top panel, there are the Kaplan-Meier curves for the probability of remaining CVD-free by methylation score in the test set; while in the lower panel "b" the results for the validation set are reported.

5.5 Discussion

To the best of my knowledge, this is the first study investigating platelet mtDNA methylation in relation to the future development of CVD. In this nested case-control study of 200 adults with overweight and obesity, higher mtDNA methylation at three loci (*MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254) in platelets was associated with higher risk of developing CVD within five years. Further, participants with Score 2 (high methylation at two or three loci) developed CVD significantly sooner than the participants with Score 1 and Score 0. Thus, mtDNA methylation at the three loci may be a novel predictive biomarker for the future risk of developing CVD.

These results are consistent with previous findings showing that mtDNA methylation in platelets from participants which CVD is altered, including hypermethylation of *MT-CO1*, *MT-CO3* and *MT-TL1* (Baccarelli & Byun, 2015). Further, mtDNA methylation has been reported to be modified by particulate matter exposure in blood (Byun et al., 2016; Xu et al., 2017), which is known to be correlated with CVD incidence.

In this study, I have demonstrated that mtDNA methylation in platelets is modified prior to the disease development and therefore it can be used as a predictor of CVD risk among individuals with overweight and obesity.

However, the field remains at a nascent stage, with little understanding of the mechanisms underpinning how mtDNA methylation levels may be implicated in the aetiology of CVD and/or platelet activation. Recently, it has been demonstrated that mtDNA methylation is associated with the expression of mitochondrial-derived peptides (MDP) with cytoprotective function (Breton et al., 2019) suggesting that mtDNA methylation level may be indicative of the overall stress to which the cell is exposed. Additionally, *in vitro* studies have shown that the presence of 5-methylcytosine can alter mitochondrial transcription factor (TFAM) binding and transcription initiation (Dostal & Churchill, 2019).

MtDNA methylation levels in blood are associated with blood pressure and heart rate variability in individuals with CVD-related environmental and occupational exposures (Byun et al., 2013; Janssen et al., 2015; Byun et al., 2016; Xu et al., 2017). However, in platelets, there was no association between mtDNA methylation level and the most common CVD risk factors including age, BMI, blood pressure, blood glucose concentration, cholesterol, and uric acid in individuals with overweight and obesity. Therefore, this study supports the idea that altered mtDNA methylation in platelets precedes the development of CVD, and may serve as a non-invasive, easy-to-access biomarker to distinguish individuals with higher CVD risk.

Adults with overweight or obesity may, therefore, benefit from identification to facilitate early primary prevention and monitoring to reduce their personal risk of CVD.

In these participants were observed low levels of mtDNA methylation and subtle, but detectable, differences between individuals who developed CVD during Follow-up and those who remained CVD-free. Such subtle changes in methylation are not confined to the mitochondrial epigenome, as changes in methylation of <5% are frequently reported in aging, in response to environmental exposures (Breton et al., 2017a), and during disease initiation (Leenen et al., 2016b). It is not known whether these small changes in DNA methylation reflect changes in gene expression. Regardless, they may serve as a biomarker of a cascade of other biological reactions (Devall et al., 2016b; Feng et al., 2012; Ghosh et al., 2015; Gentilini et al., 2015; Feinberg & Irizarry, 2010), such as MDP regulation (Breton et al., 2019).

This study has limitations that merit consideration. The outcome was diagnosis of any of a heterogeneous group of CVD that ranged from mild (e.g. hypertension) to more severe events within 5 years. The mtDNA methylation model remained strong in predicting the 'mild' CVD events, but the lack of statistical power prevented examination of its ability to predict more 'severe' cases. Further, replication of these findings is imperative. Such a validation would require access to data and samples from a cohort that had collected plasma or platelets and had follow-up data on CVD incidence as part of a prospective study of individuals with overweight and obesity. A partial validation was attempted by dividing the population of 200 individuals into a test set (n=150) and a validation set (n=50) with the same proportions of CVD-free and CVD-developed at Follow-up participants in both, which showed that the mtDNA methylation markers predicted CVD risk in both the test (P=0.045) and validation sets (P=0.034). Finally, as most of the participants were Caucasian, additional studies are needed to validate these findings in individuals with different ethnicities.

In conclusion, this study demonstrated that mtDNA methylation of *MT-CO1*, *MT-CO3*, and *MT-TL1* in platelets from adults with overweight and obesity may predict CVD risk during the following five years. These findings require confirmation in a larger, independent study.

Chapter 6. General Discussion

6.1 Overview

During this PhD, the ability of mitochondrial DNA methylation to respond to dietary compounds and to serve as an indicator of cardiovascular risk has been investigated. I will first discuss the controversy surrounding the existence and validity of mtDNA methylation, with an emphasis on the methodological adjustments that I took to obtain accurate and reproducible results.

After laying the foundation for the correct measurement and interpretation of mtDNA methylation, I summarise the key findings, strengths and limitations of the three experimental chapters of the thesis: Chapter 3, 4, and 5.

The primary hypothesis of this study is that mtDNA methylation from platelets is altered prior to the development of CVD and that can be therefore used as a predictive biomarker (Hypothesis 1).

To test this a population of adults with overweight and obesity, which are at higher risk of developing CVD, was selected from the SPHERE cohort. MtDNA methylation from platelets at Baseline (n=200) of healthy participants with overweight and obesity was tested against the future CVD outcome at Follow-up (Chapter 5).

Additionally, the hypothesis that diet is one of the main driving factor of CVD risk was tested by measuring the association between mtDNA methylation at Baseline and the MeDiet Score at Baseline (Chapter 4).

Further, as the main items of the Mediterranean diet are represented by polyphenols-rich food such fruit and vegetables three polyphenolic compounds: Resveratrol, Curcumin, and Quercein were selected. Therefore it was tested whether the treatment of platelet precursor cell, MEG-01, with Resveratrol, Quercetin, and Curcumin, *in vitro*, alters patterns of mtDNA methylation (Chapter 3).

Finally, I will describe the future perspective on using mtDNA methylation as an indicator of exposure and predictor of CVD outcome.

6.2 MtDNA methylation controversy

The presence, distribution, and biological significance of mtDNA methylation is still controversial (Mechta et al., 2017; Liu et al., 2016; Hong et al., 2013). The controversy surrounding mtDNA methylation revolves around both methodological aspects and biological

aspects. Methodologically the study of mtDNA methylation pose some challenges due to i) the presence of mtDNA sequences inside the nDNA, called “NUMTs” that may bias the results, ii) circular mtDNA structure that may cause incomplete bisulfite conversion and therefore false positives iii) low levels of mtDNA methylation that require an extremely accurate detection method.

During this PhD, I focus on the development of thorough protocols for the analysis of mtDNA methylation to avoid i) contamination from NUMTs ii) false positives due to circular mtDNA structure, and iii) low levels of mtDNA methylation that are masked by white noise (see figure 1.7and figure 2.9) (Vos et al., 2020; Liu et al., 2016; Owa et al., 2018; Bintz et al., 2014; Lambertini & Byun, 2016; Byun & Barrow, 2015a).

The scepticism about mtDNA methylation is also due to the biological significance and effects of mtDNA methylation. Interestingly, the presence of 5mC and 5hmC- the methylation output- and of DNMT and TET enzymes, involved in the active methylation and oxidation of mtDNA, has been confirmed by several studies (Shock et al., 2011; Saini et al., 2017; Bellizzi et al., 2013; Dzitoyeva et al., 2012b; Dou et al., 2019). This suggests the presence of a complex and tightly regulated epigenetic landscape within the mitochondria, which is dynamic and constantly remodelled (Bacalini et al., 2017). The study of the effects of mtDNA methylation is still a nascent field. Although several studies have reported the association between mtDNA methylation and gene expression (Breton et al., 2019; Mishra & Kowluru, 2015b; Patil et al., 2019; Dou et al., 2019), the mechanisms that regulate this association and the effects on the mitochondrial functions remain elusive. Recently, one study suggested that the binding of TFAM to the promoter region of mtDNA is modified by the presence of 5mC (Dostal & Churchill, 2019), and another study reported the association between mtDNA methylation level and MDP and mitochondrial function (Breton et al., 2019).

All this considered, the study of mtDNA methylation and its use as an indicator of exposure or pathological conditions can be carried out using *ad hoc* methodological adjustments, however, the evaluation of the effects of mtDNA methylation requires more caution. Therefore, during this PhD, I accurately measured mtDNA methylation level from platelets and explored their association the future risk of developing CVD and with diet at Baseline. Additionally, I tested the effects of polyphenolic treatment on MEG-01 mtDNA methylation.

6.3 Main findings

6.3.1 The effect of polyphenols on mtDNA methylation

In this chapter, the effect of polyphenols has been tested on MEG-01 cell mtDNA methylation. Resveratrol, Quercetin, and Curcumin were administered at different doses and incubation times. Despite the high variability in the biological replicates, which made it difficult to draw robust conclusions, my findings suggest that mtDNA methylation is not affected by treatment with Quercetin and Curcumin for up to 24 hours. The only significant difference in mtDNA methylation level has been recorded for the treatment of MEG-01 cells with 50 μM Resveratrol for 72 hours. This treatment increased the mtDNA methylation of 12%. This effect was site-specific only the CpG1 of *MT-CO2* was altered by the treatment (Figure 6.1).

This may seem counterintuitive due to the fact that Resveratrol is a DNMT inhibitor (Aldawsari et al., 2016) and that the opposite effect would be expected. However, it should be considered that Resveratrol regulates serval mechanisms in the nucleus, cytoplasm and mitochondria (Calabrese et al., 2010; Lubecka et al., 2016; Gresele et al., 2008). The pleiotropic effect of Resveratrol may results, after 72 hours of treatment, in the increased mtDNA methylation at the CpG2 of the *MT-CO2* gene.

More generally, based on our results, novel pieces of evidence are provided that the mtDNA methylation level in MEG-01 cells are generally low, and that the methylation % is mostly associated to the CpG site rather than the treatment. For example, the D-loop assay, which includes a total of three CpG sites, show 0% methylation regardless of the treatment, batch, or incubation time. While, the *MT-OLR* assay, which includes a total of five CpG sites, show variable mtDNA methylation % ranging from 0-31%. Therefore, it can be speculated that the mtDNA methylation is not random but rather site-specific and possibly tightly regulated.

Intriguingly, 72h Resveratrol treatment at 50 μM is associated with significantly higher mtDNA methylation at the CpG1 of the gene *MT-CO2* (nt8113). This is particularly interesting, as, in the SPHERE Study, higher adherence to the Mediterranean dietary pattern was also associated with hypermethylation of *MT-CO2* nt8113 in platelets from older adults with overweight and obesity.

Moreover, in the SPHERE Study, higher Mediterranean diet adherence was associated with hypomethylation in the D-loop CpG1 (nt16383). In the in *vitro* experiment, this could not be

replicated as D-loop methylation level were 0% in the controls and in the treatment group with just one outlier in D-loop CpG2. Therefore, the hypomethylating effect of diet could not be established, as the controls had 0 %. This may be due to the *in vitro* experiment design, which does not allow to replicate the same conditions of a cohort of older adults with overweight and obesity.

Considering all these results, the effect of the polyphenols Resveratrol, Curcumin, and Quercetin could not be accurately tested due to the high variability between the biological replicates. However, these results if confirmed seems to suggest that polyphenolic treatment does not have a direct effect on mtDNA methylation. Regardless of the effect of polyphenols, these data seem to suggest that mtDNA methylation happens at the megakaryocytes level and, possibly, the mitochondria that are distributed into the platelets may already have an mtDNA methylation signature. What influences this signature remains unclear.

6.3.2 Association between diet and mtDNA methylation

To the best of my knowledge, this is the first study that investigated the methylation of mtDNA from platelets in relation to diet quality in humans. In both observational and intervention studies, higher adherence to the MeDiet is associated with a wide range of health benefits, especially in later life, including reduced cardiovascular disease (Estruch et al., 2018; Arpón et al., 2017). In this sub-set of 134 participants in the SPHERE Study, higher adherence to the Mediterranean diet was associated with hypomethylation of D-loop nt16383 (and increased methylation level of *MT-CO2* nt8113 in platelets from older, individuals with overweight and obesity (Figure 6.1). However, there was no difference in MeDiet Score at baseline for individual who developed CVD over the following 5 years compared with those who remained CVD-free.

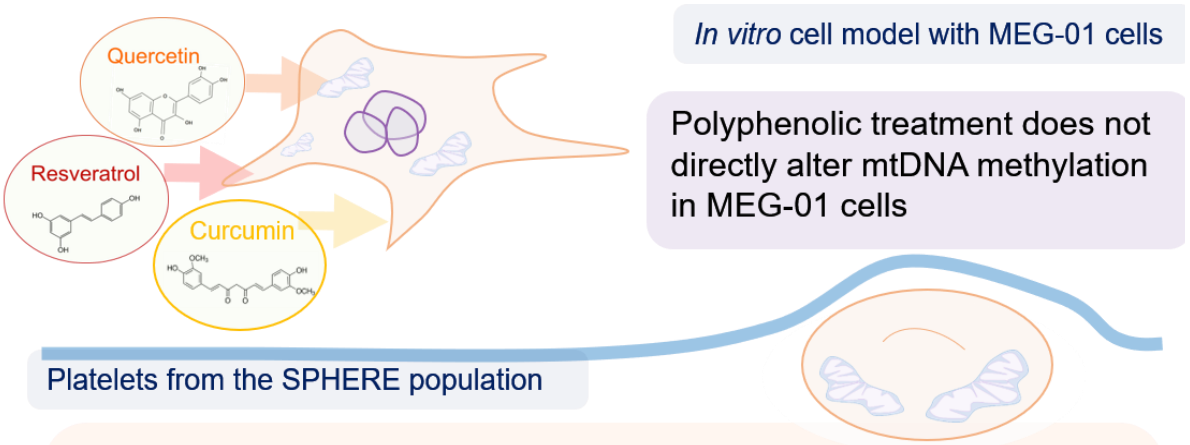
D-loop is the region on the mtDNA that controls replication and it has been previously associated with reduced mitochondrial functionality and insulin resistance (Zheng et al., 2015; Tong et al., 2017). The D-loop is normally enriched with methylated and hydroxymethylated cytosines (Shock et al., 2011). In our population, the lower mtDNA methylation of the D-loop is associated with higher Mediterranean Diet adherence but not with the CVD outcome. Accordingly, higher MeDiet Score is associated with the hypermethylation of *MT-CO2* nt8113, which has been previously associated both with lower ROS (Wolters et al., 2017) and with CVD (Baccarelli & Byun, 2015).

Notably, the MeDiet Score was not associated with the CVD outcome at Follow-up (P=0.8016), however, it correlates with some cardiometabolic risk factors at Baseline. The higher MeDiet scores are positively associated with HDL cholesterol (P=0.004) and TSH level (P=0.030), and negatively associated with 2-hours post-glucose insulin level (P=0.026), neck circumference (P=0.029), and Framingham risk score (P=0.041). This suggests that in our population of older adults with overweight and obesity, the MeDiet Score is associated with some anthropometric and metabolic factors but not with the incidence of mild CVD outcomes at Follow-up. The three genes whose methylation status was associated with the future CVD outcome (*MT-CO1*, *MT-CO3* and *MT-TL1*) (described in Chapter 5) were not associated with the MeDiet or Framingham risk score.

Altogether these results seem to suggest that MeDiet is associated with the mtDNA methylation level at Baseline, but it is not associated with the CVD outcome at Follow-up. This may be because the driver for the CVD event it may not be the diet but some other environmental or lifestyle factor or a combination of them.

6.3.3 MtDNA methylation predicts future CVD outcomes

To the best of my knowledge, this is the first study investigating methylation of mtDNA from platelets in relation to the future development of CVD. In this nested case-control study of 200 adults with overweight and obesity, higher mtDNA methylation at three loci (*MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254) in platelets was associated with a higher risk of developing CVD within five years (Figure 6.1). Further, participants with Score 2 (high methylation at two or three loci) developed CVD significantly sooner than the participants with Score 1 and Score 0. Thus, mtDNA methylation at the three loci may be a novel predictive biomarker for the future risk of developing CVD.



MtDNA methylation at Baseline at the position *MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254 is a predictor of the future CVD outcome.

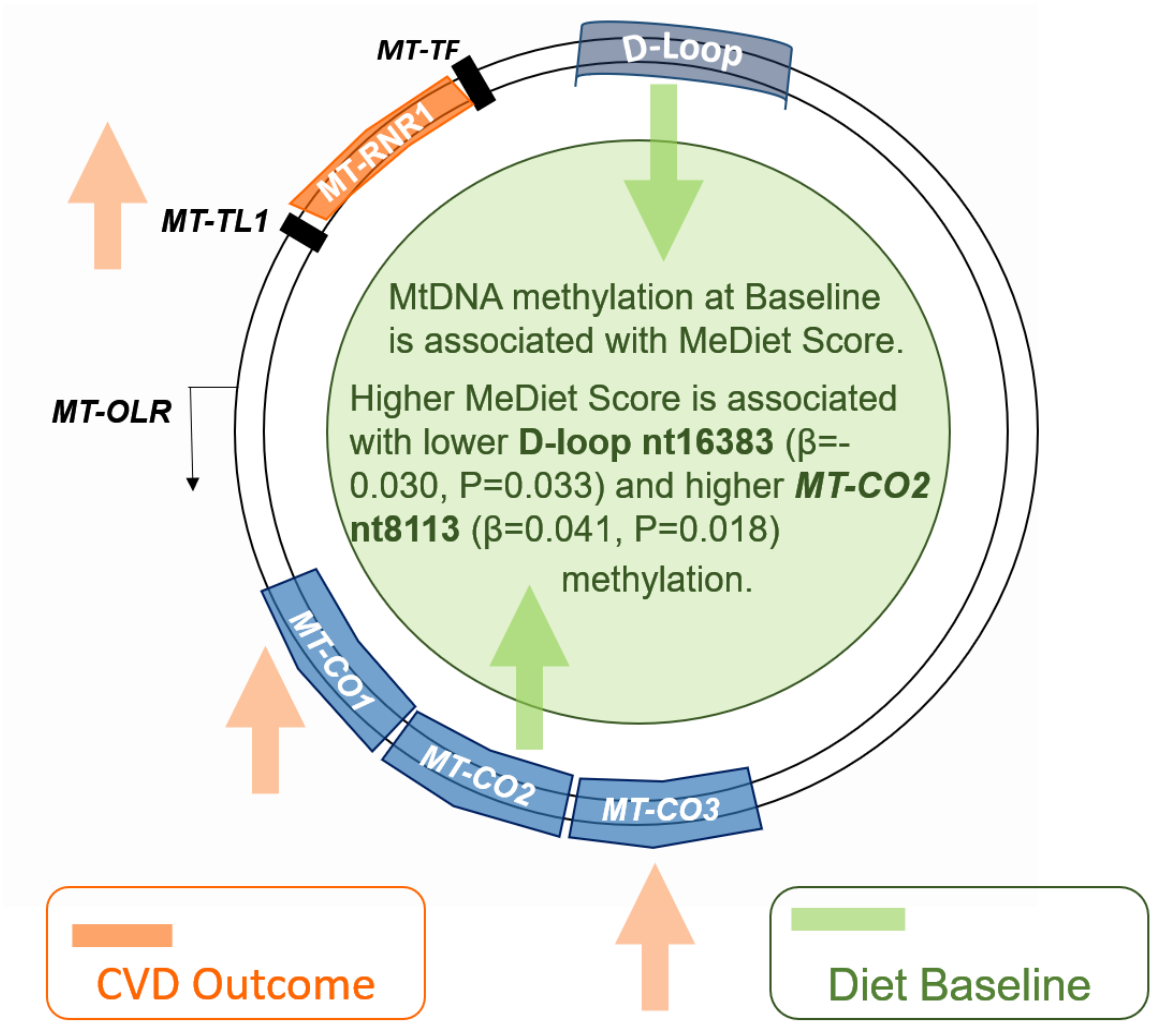


Figure 6.1 Overview of the results

This image outlines the main results of this PhD work. In the upper part, there are the results of the in vitro study using MEG-01 cell. This experiment aimed to evaluate the effects of polyphenolic treatment on mtDNA methylation level. The results suggest that Resveratrol, Quercetin, and Curcumin do not have a direct effect on mtDNA methylation.

In the middle part, there are the main results from the population study. This study was carried out on a population of 200 adults with overweight and obesity but otherwise healthy individuals from the SPHERE study. I aimed to demonstrate that mtDNA methylation level from platelets at Baseline predicts the future CVD outcome. The orange arrows indicate the three genes MT-CO1 nt6807, MT-CO3 nt9444, and MT-TL1 nt3254, whose methylation is higher in the participants who will develop CVD within 5-years of follow-up.

The green arrows indicate the two genes D-loop nt16383 and MT-CO2 nt8113, whose methylation is associated with the MeDiet Score at Baseline.

End of figure legend.

6.3 Strengths and limitations

The main strength of the population study is that the methodology used to quantify the mtDNA methylation level was structured to be accurate and precise, as it was possible to distinguish very low methylation level from the background noise. This is of paramount importance in the testing of mtDNA methylation as a predictive biomarker on a healthy population. Another essential aspect of the identification of predictive biomarker is the choice of the cell type. The use of platelets not only provide the technical advantage of not having nDNA, but also provide a highly sensitive cell type able to respond to obesity, CVD, diet, and other CVD-related risk factors. Platelets are dysregulated in obesity (Anfossi et al., 2009; Bordeaux et al., 2010; Santilli et al., 2012; Tamminen et al., 2003), and to have an altered mtDNA methylation profile in participants with CVD (Baccarelli & Byun, 2015). Further, it has been demonstrated that platelets' activity and count can be modified by the Mediterranean diet (Bonaccio et al., 2014; Antonopoulou et al., 2006b). Furthermore, the population of 200 adults with overweight and obesity but otherwise healthy at Baseline was selected so that, at Baseline, those who developed CVD during the Follow-up period and

those who remained CVD-free were indistinguishable using conventional CVD risk biomarkers.

The main limitation of the *in vitro* study is the variability of biological replicates at specific CpG sites. This limitation was addressed by removing outliers and when this was done, the correlation coefficient between biological replicates was 0.96 ($P<0.001$). Despite this adjustment, the replication of these data is needed to warrant a robust interpretation. The main limitation of the investigation of the association between Mediterranean diet adherence and mtDNA methylation is the restricted number of participants for whom the diet information was available. Only 134 out of the 200 participants selected for this study completed the FFQ at recruitment which reduced the power of the analysis.

6.4 Conclusion

Mitochondrial DNA methylation is a controversial issue. The controversy depends both on the ability to precisely quantify mtDNA methylation level at single CpG sites and on the functional consequences and biological meaningfulness of mtDNA methylation.

The presence of mtDNA methylation has been confirmed in many studies that adapted their methodologies to the specific characteristic of mtDNA (Shock et al., 2011; Baccarelli & Byun, 2015; Liu et al., 2016; Byun & Barrow, 2015a). There has been a further debate about the functional consequences of mtDNA methylation and its biological significance.

Regarding the functional consequences, there are some studies that suggested a correlation between mtDNA methylation and gene expression (Breton et al., 2019; Mishra & Kowluru, 2015b; Patil et al., 2019; Dou et al., 2019; Shock et al., 2011). However, in my case, I have investigated mtDNA methylation from human platelets which are anucleated cells with a short life-span. It is possible to speculate that, given the short life-span of 7-10 days and high turnover of platelets (Melchinger et al., 2019; Leeksma & Cohen, 1955; Harker et al., 2000), mtDNA methylation does not cause any functional consequences in platelets' number and function. This is supported by our data from chapter 5 in which there is no association between methylation percentage at the loci *MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254 and the platelet count.

Even though mitochondria are key regulators in platelets survival and activation, it is unlikely that low mtDNA methylation level, such as the methylation that was recorded in this study's

healthy volunteers (mean= 3.13%; SD=4.06), may affect mitochondrial gene expression. It is possible, however, that mtDNA methylation may regulate MDP synthesis (Breton et al., 2019).

To the best of my knowledge, there is no study that confirms the presence of mtDNMT in platelet's mitochondria. Therefore, I hypothesized that mtDNA methylation happens at the megakaryocytes level. This was tested using MEG-01 cells, an *in vitro* model of megakaryocyte. As suggested by the *in vitro* study, the mtDNA methylation happens at the megakaryocytes level and then the platelets are assembled with the machinery present inside the megakaryocytes.

Importantly, a single-base resolution method has been used to quantify mtDNA methylation patterns in platelets. In fact, it has been suggested that mtDNA methylation patterns are highly specific for each disease, organ, and even specialized regions of the same organ, such the brain cortex vs the brain cerebellum (Devall et al., 2017). For example, Devall et all demonstrated that post-mortem brain samples from the cortex and cerebellum and pre-mortem blood show different mtDNA methylation patterns, and possibly a tissue-specific signature. This study has been conducted using MeDIP-seq data from three healthy individuals. MeDIP-seq is a whole genome sequencing and does not allow single-base resolution which resulted in the use of 100 bp "window" or cut-offs (Devall et al., 2017). Nonetheless, this study found that although mtDNA methylation level is low, a highly specific mtDNA methylation signature is present in different tissue and organs' regions. These findings resonate with my results. During my PhD, pyrosequencing was used which allows quantifying the mtDNA methylation level at each CpG base.
Interestingly, the mtDNA methylation % of CpGs located within the same gene are not correlated. This was true both in the *in vitro* and population studies. The *in vitro* studies confirmed the highly specific changes upon treatment with polyphenolic compounds in megakaryocytes. For example, the CpG site *MT-CO2* nt8113 – and not *MT-CO2* nt8117 just 5 nucleotides away – has the ability to specifically respond to Resveratrol treatment. Within the SPHERE population, different CpG sites are markers for different exposures/outcomes. The CpGs whose methylation level predict the future CVD outcome (*MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254) are different from the CpG sites whose methylation is associated with dietary patterns at Baseline (D-loop nt16383, and *MT-CO2* nt8113).

The mtDNA methylation signature at the positions *MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254 resonates with the literature which reports a higher mtDNA methylation in these loci in platelets from participants with CVD (Baccarelli & Byun, 2015). This support the hypothesis that mtDNA methylation is highly specific depending on the tissue and disease status.

In summary, we concluded that the mtDNA methylation is an excellent biomarker of future risk of developing cardiovascular diseases and that this methylation pattern can be used as an indicator of exposure to environmental factors such as diet.

6.5 Future Research

Although preventable CVD is the leading cause of death worldwide. CVD are multifactorial disorders with a high environmental component. The biggest driver of CVD is dietary factors, lifestyle and environmental exposure (e.g environmental pollution and smoking) (Meier et al., 2019). The need for biomarkers to stratify the population according to the risk and according to the exposure is paramount. MtDNA methylation has demonstrated to be a good biomarker of exposure to environmental pollution in these studies (Byun & Barrow, 2015a; Byun et al., 2013, 2016; Vos et al., 2020). Even more significantly, mtDNA methylation has been found to be altered in placenta from women exposed to particulate matter (Janssen et al., 2015) and to predict the bodyweight of the new-born (Vos et al., 2020).

In this study, I demonstrated that mtDNA methylation predicts the future incidence of mild CVD outcome and it is associated with dietary patterns at Baseline. The sensitivity of mtDNA to change according to the environment and to predict the future CVD outcome and to respond to the environment make it a great candidate biomarker for prevention and personalised interventions.

Recently, DNA methylation patterns in the nuclear DNA have been used to predict all-cause mortality, Grim Age, Telomere length, and risk of diseases. Some of these chips, such as the GrimAge (Lu, Quach, et al., 2019), include not only the epigenetic clock but also information about metabolism.

This chip can easily provide a tool for the epidemiological screening of the population, for informing public health strategies and to direct research into specific molecular mechanism of diseases and ageing. Given the fact that mtDNA methylation can capture differences between two population which are not distinguishable using conventional CVD risk factors, it would be important to implement this type of analysis with routine tests. The most comprehensive analysis would be creating a chip which entails primers that anneal specifically with the

mtDNA and can record the methylation level at the specific CpG site. This would be important to inform policy making and risk stratification and more importantly to empower each individual.

Appendices

Appendix A. Multivariate analysis for the association between potential risk factors and mitochondrial DNA methylation at Baseline.

Variable	*MT-CO1* nt6807				*MT-CO3* nt9444				*MT-TL1* nt3254			
	Estimate	CI	SE	P value	Estimate	CI	SE	P value	Estimate	CI	SE	P value
Age	-0.001	-0.006, 0.003	0.002	0.524	-0.003	-0.013,0.007	0.005	0.582	-0.003	-0.006, 0.000	0.002	0.083
BMI	0.001	-0.007, 0.009	0.004	0.783	-0.007	-0.028, 0.013	0.010	0.492	0.005	-0.002, 0.011	0.003	0.148
Serum Uric Acid	0.010	-0.023, 0.042	0.016	0.554	-0.024	-0.107, 0.059	0.042	0.564	-0.012	-0.036, 0.012	0.012	0.330
ALT	0.000	-0.002, 0.003	0.001	0.760	-0.002	-0.007, 0.002	0.002	0.334	-0.001	-0.002, 0.001	0.001	0.539
AST	0.001	-0.003, 0.005	0.002	0.478	-0.005	-0.012, 0.002	0.003	0.133	-0.001	-0.004, 0.002	0.002	0.385
Basophil	-1.406	-3.819, 1.007	1.222	0.252	-5.863	-11.877, 0.151	3.005	0.056	1.238	-0.574, 3.050	0.917	0.179

Basophil percentage	-0.113	-0.283, 0.058	0.086	0.194	-0.475	-0.913, -0.036	0.219	**0.034**	0.052	-0.075, 0.179	0.064	0.419
Waist circumference	0.001	-0.002, 0.005	0.002	0.520	-0.005	-0.016, 0.005	0.005	0.287	0.000	-0.003, 0.003	0.001	0.941
HDL cholesterol	-0.002	-0.006, 0.001	0.002	0.170	0.007	-0.003, 0.018	0.005	0.181	0.000	-0.003, 0.003	0.001	0.995
LDL cholesterol	-0.001	-0.002, 0.001	0.001	0.250	0.000	-0.004, 0.004	0.002	0.982	-0.001	-0.002, 0.001	0.001	0.326
Total cholesterol	-0.001	-0.002, 0.000	0.001	0.192	0.001	-0.002, 0.004	0.002	0.628	0.000	-0.001, 0.001	0.000	0.790
Serum creatinine	-0.015	-0.076, 0.045	0.031	0.620	-0.030	-0.135, 0.076	0.053	0.573	-0.008	-0.057, 0.042	0.025	0.761
Eosinophils count	0.077	-0.287, 0.441	0.184	0.677	-0.483	-1.626, 0.660	0.571	0.401	0.118	-0.158, 0.393	0.140	0.401
Eosinophils %	0.004	-0.021, 0.028	0.012	0.762	-0.039	-0.117, 0.038	0.039	0.312	0.004	-0.014, 0.023	0.009	0.635
Heart Rate	0.002	-0.002, 0.005	0.002	0.386	0.000	-0.010, 0.010	0.005	0.987	0.001	-0.001, 0.004	0.001	0.333
Fibrinogen	-0.001	-0.001, 0.000	0.000	**0.007**	0.000	-0.002, 0.002	0.001	0.991	0.000	0.000, 0.001	0.000	0.351

Gamma-Glutamyltransferase	0.000	-0.002, 0.002	0.001	0.900	0.000	-0.004, 0.005	0.002	0.857	-0.001	-0.002, 0.001	0.001	0.267
White blood cell count	0.010	-0.013, 0.034	0.012	0.395	0.002	-0.063, 0.067	0.033	0.957	0.017	-0.001, 0.035	0.009	0.070
Red blood cells count	-0.029	-0.132, 0.073	0.052	0.574	-0.018	-0.261, 0.224	0.121	0.879	0.019	-0.057, 0.096	0.039	0.620
Granulocytes count	0.008	-0.021, 0.036	0.014	0.601	-0.010	-0.089, 0.068	0.039	0.796	0.020	-0.002, 0.041	0.011	0.077
Glycated haemoglobin	-0.002	-0.009, 0.006	0.004	0.639	-0.030	-0.047, -0.013	0.008	**0.001**	0.005	-0.001, 0.010	0.003	0.130
Complete Blood count	0.015	-0.020, 0.049	0.017	0.402	0.028	-0.051, 0.108	0.040	0.477	-0.027	-0.054, -0.001	0.013	**0.041**
Haematocrit	0.005	-0.009, 0.019	0.007	0.495	0.010	-0.023, 0.043	0.016	0.553	-0.011	-0.022, -0.001	0.005	**0.038**
Lymphocyte count	0.042	-0.034, 0.118	0.039	0.279	0.072	-0.113, 0.256	0.092	0.441	0.033	-0.027, 0.093	0.030	0.279
Lymphocyte %	0.000	-0.006, 0.006	0.003	0.980	0.007	-0.008, 0.021	0.007	0.351	-0.001	-0.005, 0.003	0.002	0.671
Mean Corpuscolar Volume	0.003	-0.003, 0.010	0.003	0.305	0.005	-0.012, 0.021	0.008	0.578	-0.006	-0.010, -0.001	0.002	**0.021**

Monocytes count	0.099	-0.131, 0.328	0.116	0.396	0.008	-0.608, 0.624	0.308	0.979	0.000	-0.179, 0.178	0.091	0.997
Monocytes %	0.008	-0.015, 0.030	0.011	0.494	-0.005	-0.064, 0.054	0.030	0.873	-0.011	-0.028, 0.006	0.009	0.208
Neutrophils count	0.007	-0.021, 0.036	0.015	0.611	-0.007	-0.087, 0.072	0.040	0.858	0.019	-0.003, 0.041	0.011	0.089
Neutrophils %	-0.001	-0.006, 0.005	0.003	0.834	-0.005	-0.019, 0.010	0.007	0.524	0.001	-0.003, 0.005	0.002	0.573
Homocysteine	-0.003	-0.010, 0.005	0.004	0.496	-0.006	-0.024, 0.013	0.009	0.538	0.001	-0.005, 0.006	0.003	0.793
Diastolic Blood Pressure	0.004	-0.003, 0.010	0.003	0.256	-0.020	-0.036, -0.004	0.008	**0.017**	-0.003	-0.008, 0.002	0.002	0.191
Systolic Blood Pressure	-0.002	-0.005, 0.002	0.002	0.373	0.012	0.003, 0.021	0.005	**0.009**	0.002	0.000, 0.005	0.001	0.095
C-Reactive protein	-0.043	-0.096, 0.010	0.027	0.113	-0.006	-0.152, 0.140	0.073	0.937	0.023	-0.017, 0.064	0.020	0.256
Platelets	0.000	-0.001, 0.001	0.000	0.794	0.000	-0.002, 0.002	0.001	0.995	0.000	0.000, 0.001	0.000	0.196
LDL/HDL	-0.079	-0.250, 0.091	0.086	0.362	-0.069	-0.654, 0.516	0.292	0.815	-0.138	-0.265, -0.011	0.064	**0.033**

TC/HDL	-0.020	-0.059, 0.020	0.020	0.325	-0.053	-0.154, 0.048	0.050	0.297	-0.005	-0.036, 0.026	0.016	0.743
Triglyceride	0.001	0.000, 0.001	0.000	0.159	0.000	-0.002, 0.003	0.001	0.711	0.001	0.000, 0.001	0.000	**0.009**
TSH	0.016	-0.007, 0.038	0.012	0.181	-0.001	-0.049, 0.047	0.024	0.959	0.008	-0.010, 0.026	0.009	0.367
Waist circumference	0.001	-0.003, 0.004	0.002	0.625	-0.006	-0.016, 0.004	0.005	0.262	-0.001	-0.003, 0.002	0.001	0.654
Neck circumference	-0.005	-0.013, 0.003	0.004	0.222	0.013	-0.003, 0.030	0.008	0.116	-0.002	-0.008, 0.004	0.003	0.441
Inedx-HOMA-IR *	0.000	-0.009, 0.008	0.004	0.960	-0.026	-0.073, 0.020	0.023	0.259	0.000	-0.006, 0.007	0.003	0.960
Waist to hip ratio	0.003	-0.014, 0.020	0.009	0.694	0.076	-0.173, 0.325	0.125	0.545	-0.016	-0.106, 0.073	0.045	0.722
waist-to-height ratio	0.345	-0.253, 0.942	0.303	0.256	-0.667	-2.252, 0.918	0.792	0.403	0.309	-0.163, 0.781	0.239	0.197
Not HDL cholesterol	-0.001	-0.002, 0.001	0.001	0.227	0.000	-0.004, 0.005	0.002	0.923	0.000	-0.001, 0.001	0.001	0.720
HOMA-IR †	-0.001	-0.005, 0.004	0.002	0.810	-0.008	-0.018, 0.001	0.005	0.080	0.000	-0.004, 0.004	0.002	0.925

HOMA-b ‡	0.000	0.000, 0.000	0.000	0.998	0.000	-0.001, 0.000	0.000	0.343	0.000	0.000, 0.000	0.000	0.412
QUICKI §	-1.382	-4.645, 1.881	1.655	0.405	13.652	2.570, 24.735	5.540	**0.017**	-1.012	-3.434, 1.411	1.227	0.411
Glucose	-0.011	-0.041, 0.018	0.015	0.444	0.116	0.050, 0.181	0.033	**0.001**	-0.027	-0.051, -0.002	0.012	**0.033**

Logistic regression adjusted for run, batch, nuclear contamination. DNA methylation variables were logtransformed. In bold are highlighted the significant P values.

*Index-HOMA-IR: Index-homeostatic model assessment (HOMA)-Insulin Resistance; † HOMA-IR: Homeostatic Model Assessment (HOMA)-Insulin Resistance; ‡HOMA-b: HOMA-beta; § QUICKI: quantitative insulin sensitivity check index

References

Adler, B.J., Kaushansky, K. & Rubin, C.T. (2014) Obesity-driven disruption of haematopoiesis and the bone marrow niche. *Nature Reviews. Endocrinology*. 10 (12), 737–748.

Afshin, A., Sur, P.J., Fay, K.A., Cornaby, L., Ferrara, G., Salama, J.S., Mullany, E.C., Abate, K.H., Abbafati, C., Abebe, Z., Afarideh, M., Aggarwal, A., Agrawal, S., Akinyemiju, T., Alahdab, F., Bacha, U., Bachman, V.F., Badali, H., Badawi, A., et al. (2019) Health effects of dietary risks in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*. 393 (10184), 1958–1972.

Aggarwal, B.B. (2010) Targeting Inflammation-Induced Obesity and Metabolic Diseases by Curcumin and Other Nutraceuticals. *Annual review of nutrition*. 30173–199.

Agha Golareh, Mendelson Michael M., Ward-Caviness Cavin K., Joehanes Roby, Huan TianXiao, Gondalia Rahul, Salfati Elias, Brody Jennifer A., Fiorito Giovanni, Bressler Jan, Chen Brian H., Ligthart Symen, Guarrera Simonetta, Colicino Elena, Just Allan C., Wahl Simone, Gieger Christian, Vandiver Amy R., Tanaka Toshiko, et al. (2019) Blood Leukocyte DNA Methylation Predicts Risk of Future Myocardial Infarction and Coronary Heart Disease. *Circulation*. 140 (8), 645–657.

Aguirre, L., Fernández-Quintela, A., Arias, N. & Portillo, M.P. (2014) Resveratrol: Anti-Obesity Mechanisms of Action. *Molecules*. 19 (11), 18632–18655.

Ahmed, A.S.I., Sheng, M.H., Wasnik, S., Baylink, D.J. & Lau, K.-H.W. (2017) Effect of aging on stem cells. *World Journal of Experimental Medicine*. 7 (1), 1–10.

Akbari, M., Lankarani, K.B., Tabrizi, R., Ghayour-Mobarhan, M., Peymani, P., Ferns, G., Ghaderi, A. & Asemi, Z. (2019) The Effects of Curcumin on Weight Loss Among Patients With Metabolic Syndrome and Related Disorders: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Frontiers in Pharmacology*. 10.

Aldawsari, F.S., Aguayo-Ortiz, R., Kapilashrami, K., Yoo, J., Luo, M., Medina-Franco, J.L. & Velázquez-Martínez, C.A. (2016) Resveratrol-salicylate derivatives as selective DNMT3 inhibitors and anticancer agents. *Journal of enzyme inhibition and medicinal chemistry*. 31 (5), 695–703.

Anderson, O.S., Sant, K.E. & Dolinoy, D.C. (2012) Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *The Journal of Nutritional Biochemistry*. 23 (8), 853–859.

Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M. & Howell, N. (1999a) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nature Genetics*. 23 (2), 147.

Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M. & Howell, N. (1999b) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nature Genetics*. 23 (2), 147.

Anfossi, G., Russo, I. & Trovati, M. (2009) Platelet dysfunction in central obesity. *Nutrition, metabolism, and cardiovascular diseases: NMCD*. 19 (6), 440–449.

Angelino, D., Godos, J., Ghelfi, F., Tieri, M., Titta, L., Lafranconi, A., Marventano, S., Alonzo, E., Gambera, A., Sciacca, S., Buscemi, S., Ray, S., Galvano, F., Del Rio, D. & Grosso, G. (2019) Fruit and vegetable consumption and health outcomes: an umbrella review of observational studies. *International Journal of Food Sciences and Nutrition*. 70 (6), 652–667.

Anthony, B. & Link, D.C. (2014) Regulation of Hematopoietic Stem Cells by Bone Marrow Stromal Cells. *Trends in immunology*. 35 (1), 32–37.

Antonopoulou, S., Fragopoulou, E., Karantonis, H.C., Mitsou, E., Sitara, M., Rementzis, J., Mourelatos, A., Ginis, A. & Phenekos, C. (2006a) Effect of Traditional Greek Mediterranean Meals on Platelet Aggregation in Normal Subjects and in Patients with Type 2 Diabetes Mellitus. *Journal of Medicinal Food*. 9 (3), 356–362.

Antonopoulou, S., Fragopoulou, E., Karantonis, H.C., Mitsou, E., Sitara, M., Rementzis, J., Mourelatos, A., Ginis, A. & Phenekos, C. (2006b) Effect of Traditional Greek Mediterranean Meals on Platelet Aggregation in Normal Subjects and in Patients with Type 2 Diabetes Mellitus. *Journal of Medicinal Food*. 9 (3), 356–362.

Appiah, D., Schreiner, P.J., MacLehose, R.F. & Folsom, A.R. (2015) Association of Plasma γ' Fibrinogen With Incident Cardiovascular Disease: The Atherosclerosis Risk in Communities (ARIC) Study. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 35 (12), 2700–2706.

Arpón, A., Milagro, F.I., Razquin, C., Corella, D., Estruch, R., Fitó, M., Marti, A., Martínez-González, M.A., Ros, E., Salas-Salvadó, J., Riezu-Boj, J.-I. & Martínez, J.A. (2017) Impact of Consuming Extra-Virgin Olive Oil or Nuts within a Mediterranean Diet on DNA Methylation in Peripheral White Blood Cells within the PREDIMED-Navarra Randomized Controlled Trial: A Role for Dietary Lipids. *Nutrients*. 10 (1), .

ASRM, P.C. of the A.S. for R.M. (2015) Obesity and reproduction: a committee opinion. *Fertility and Sterility*. 104 (5), 1116–1126.

Ataie-Jafari, A., Namazi, N., Djalalinia, S., Chaghamirzayi, P., Abdar, M.E., Zadehe, S.S., Asayesh, H., Zarei, M., Gorabi, A.M., Mansourian, M. & Qorbani, M. (2018) Neck circumference and its association with cardiometabolic risk factors: a systematic review and meta-analysis. *Diabetology & Metabolic Syndrome*. 10 (1), 72.

Atkins, J.L., Whincup, P.H., Morris, R.W., Lennon, L.T., Papacosta, O. & Wannamethee, S.G. (2014) Sarcopenic Obesity and Risk of Cardiovascular Disease and Mortality: A Population-Based Cohort Study of Older Men. *Journal of the American Geriatrics Society*. 62 (2), 253–260.

Atkinson, R.L., Dhurandhar, N.V., Allison, D.B., Bowen, R.L., Israel, B.A., Albu, J.B. & Augustus, A.S. (2005) Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. *International Journal of Obesity*. 29 (3), 281.

Aune, D., Keum, N., Giovannucci, E., Fadnes, L.T., Boffetta, P., Greenwood, D.C., Tonstad, S., Vatten, L.J., Riboli, E. & Norat, T. (2018) Dietary intake and blood concentrations of antioxidants and the risk of cardiovascular disease, total cancer, and all-cause mortality: a systematic review and dose-response meta-analysis of prospective studies. *The American Journal of Clinical Nutrition*. 108 (5), 1069–1091.

Baaten, C.C.F.M.J., Moenen, F.C.J.I., Henskens, Y.M.C., Swieringa, F., Wetzels, R.J.H., Oerle, R. van, Heijnen, H.F.G., Cate, H. ten, Holloway, G.P., Beckers, E.A.M., Heemskerk, J.W.M. & Meijden, P.E.J. van der (2018) Impaired mitochondrial activity explains platelet dysfunction in thrombocytopenic cancer patients undergoing chemotherapy. *Haematologica*. haematol.2017.185165.

Bacalini, M.G., D'Aquila, P., Marasco, E., Nardini, C., Montesanto, A., Franceschi, C., Passarino, G., Garagnani, P. & Bellizzi, D. (2017) The methylation of nuclear and mitochondrial DNA in ageing phenotypes and longevity. *Mechanisms of Ageing and Development*. 165156–161.

Baccarelli, A. & Bollati, V. (2009) Epigenetics and environmental chemicals. *Current opinion in pediatrics*. 21 (2), 243–251.

Baccarelli, A.A. & Byun, H.-M. (2015) Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. *Clinical Epigenetics*. 744.

Bacos, K., Gillberg, L., Volkov, P., Olsson, A.H., Hansen, T., Pedersen, O., Gjesing, A.P., Eiberg, H., Tuomi, T., Almgren, P., Groop, L., Eliasson, L., Vaag, A., Dayeh, T. & Ling, C. (2016) Blood-based biomarkers of age-associated epigenetic changes in human islets associate with insulin secretion and diabetes. *Nature Communications*. 711089.

Badimon, L., Hernández Vera, R., Padró, T. & Vilahur, G. (2013) Antithrombotic therapy in obesity. *Thrombosis and Haemostasis*. 110 (4), 681–688.

Bagheri, H., Ghasemi, F., Barreto, G.E., Rafiee, R., Sathyapalan, T. & Sahebkar, A. (2020) Effects of curcumin on mitochondria in neurodegenerative diseases. *BioFactors*. 46 (1), 5–20.

Balduini, C.L. & Noris, P. (2014) Platelet count and aging. *Haematologica*. 99 (6), 953–955.

Ballinger, S.W. (2005) Mitochondrial dysfunction in cardiovascular disease. *Free Radical Biology and Medicine*. 38 (10), 1278–1295.

Bann, D., Johnson, W., Li, L., Kuh, D. & Hardy, R. (2018) Socioeconomic inequalities in childhood and adolescent body-mass index, weight, and height from 1953 to 2015: an analysis of four longitudinal, observational, British birth cohort studies. *The Lancet. Public Health*. 3 (4), e194–e203.

Bannister, A.J. & Kouzarides, T. (2011) Regulation of chromatin by histone modifications. *Cell Research*. 21 (3), 381–395.

Barrow, T.M., Byun, H.-M., Li, X., Smart, C., Wang, Y.-X., Zhang, Y., Baccarelli, A.A. & Guo, L. (2018) The effect of morphine upon DNA methylation in ten regions of the rat brain. *Epigenetics*. 12 (12), 1038–1047.

Beaulieu, L.M., Lin, E., Mick, E., Koupenova, M., Weinberg, E.O., Kramer, C.D., Genco, C.A., Tanriverdi, K., Larson, M.G., Benjamin, E.J. & Freedman, J.E. (2014) Interleukin 1 Receptor 1 and Interleukin 1β Regulate Megakaryocyte Maturation, Platelet Activation, and Transcript Profile During Inflammation in Mice and Humans. *Arteriosclerosis, Thrombosis, and Vascular Biology*. ATVBAHA.113.302700.

Bell, J.T., Tsai, P.-C., Yang, T.-P., Pidsley, R., Nisbet, J., Glass, D., Mangino, M., Zhai, G., Zhang, F., Valdes, A., Shin, S.-Y., Dempster, E.L., Murray, R.M., Grundberg, E., Hedman, A.K., Nica, A., Small, K.S., MuTHER Consortium, Dermitzakis, E.T., et al. (2012) Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population. *PLoS genetics*. 8 (4), e1002629.

Bellizzi, D., D'Aquila, P., Scafone, T., Giordano, M., Riso, V., Riccio, A. & Passarino, G. (2013) The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern. *DNA research: an international journal for rapid publication of reports on genes and genomes*. 20 (6), 537–547.

Bintz, B.J., Dixon, G.B. & Wilson, M.R. (2014) Simultaneous Detection of Human Mitochondrial DNA and Nuclear-Inserted Mitochondrial-origin Sequences (NumtS) using Forensic mtDNA Amplification Strategies and Pyrosequencing Technology. *Journal of Forensic Sciences*. 59 (4), 1064–1073.

Biondi, B. & Klein, I. (2004) Hypothyroidism as a risk factor for cardiovascular disease. *Endocrine*. 24 (1), 1–13.

Blanch, M., Mosquera, J.L., Ansoleaga, B., Ferrer, I. & Barrachina, M. (2016) Altered Mitochondrial DNA Methylation Pattern in Alzheimer Disease–Related Pathology and in Parkinson Disease. *The American Journal of Pathology*. 186 (2), 385–397.

Bloom, D.E., Cafiero, E.T., Jané-Llopis, E., Abrahams-Gessel, S., Bloom, L.R., Fathima, S., Feigl, A.B., Gaziano, T., Mowafi, M., Pandya, A., Prettner, K., Rosenberg, L., Seligman, B., Stein, A.Z. & Weinstein, C. (2011) *GlobalEconomicBurdenNonCommunicableDiseases. Geneva: World Economic Forum.* [Online] [online]. Available from: http://www3.weforum.org/docs/WEF_Harvard_HE_GlobalEconomicBurdenNonCommunicableDiseases_2011.pdf (Accessed 29 April 2017).

Bollati, V., Favero, C., Albetti, B., Tarantini, L., Moroni, A., Byun, H.-M., Motta, V., Conti, D.M., Tirelli, A.S., Vigna, L., Bertazzi, P.A. & Pesatori, A.C. (2014) Nutrients Intake Is Associated with DNA Methylation of Candidate Inflammatory Genes in a Population of Obese Subjects. *Nutrients*. 6 (10), 4625–4639.

Bollati, V., Iodice, S., Favero, C., Angelici, L., Albetti, B., Cacace, R., Cantone, L., Carugno, M., Cavalleri, T., De Giorgio, B., Dioni, L., Fustinoni, S., Hoxha, M., Marinelli, B., Motta, V., Patrini, L., Pergoli, L., Riboldi, L., Rizzo, G., et al. (2014) Susceptibility to particle health effects, miRNA and exosomes: rationale and study protocol of the SPHERE study. *BMC public health*. 141137.

Bonaccio, M., Di Castelnuovo, A., De Curtis, A., Costanzo, S., Persichillo, M., Donati, M.B., Cerletti, C., Iacoviello, L. & de Gaetano, G. (2014) Adherence to the Mediterranean

diet is associated with lower platelet and leukocyte counts: results from the Moli-sani study. *Blood*. 123 (19), 3037–3044.

Bordeaux, B.C., Qayyum, R., Yanek, L.R., Vaidya, D., Becker, L.C., Faraday, N. & Becker, D.M. (2010) Effect of obesity on platelet reactivity and response to low-dose aspirin. *Preventive Cardiology*. 13 (2), 56–62.

Bordoni, L., Sawicka, A.K., Szarmach, A., Winklewski, P.J., Olek, R.A. & Gabbianelli, R. (2020) A Pilot Study on the Effects of l-Carnitine and Trimethylamine-N-Oxide on Platelet Mitochondrial DNA Methylation and CVD Biomarkers in Aged Women. *International Journal of Molecular Sciences*. 21 (3), .

Bordoni, L., Smerilli, V., Nasuti, C. & Gabbianelli, R. (2019) Mitochondrial DNA methylation and copy number predict body composition in a young female population. *Journal of Translational Medicine*. 17 (1), 399.

Bournat, J.C. & Brown, C.W. (2010) Mitochondrial dysfunction in obesity. *Current Opinion in Endocrinology, Diabetes, and Obesity*. 17 (5), 446–452.

Bray, A.W. & Ballinger, S.W. (2017) Mitochondrial DNA mutations and cardiovascular disease. *Current Opinion in Cardiology*.

Breton, C.V., Marsit, C.J., Faustman, E., Nadeau, K., Goodrich, J.M., Dolinoy, D.C., Herbstman, J., Holland, N., LaSalle, J.M., Schmidt, R., Yousefi, P., Perera, F., Joubert, B.R., Wiemels, J., Taylor, M., Yang, I.V., Chen, R., Hew, K.M., Freeland, D.M.H., et al. (2017b) Small-Magnitude Effect Sizes in Epigenetic End Points are Important in Children's Environmental Health Studies: The Children's Environmental Health and Disease Prevention Research Center's Epigenetics Working Group. *Environmental Health Perspectives*. 125 (4), 511–526.

Breton, C.V., Song, A.Y., Xiao, J., Kim, S.-J., Mehta, H.H., Wan, J., Yen, K., Sioutas, C., Lurmann, F., Xue, S., Morgan, T.E., Zhang, J. & Cohen, P. (2019) Effects of air pollution on mitochondrial function, mitochondrial DNA methylation, and mitochondrial peptide expression. *Mitochondrion*. 4622–29.

Brown, D.A., Perry, J.B., Allen, M.E., Sabbah, H.N., Stauffer, B.L., Shaikh, S.R., Cleland, J.G.F., Colucci, W.S., Butler, J., Voors, A.A., Anker, S.D., Pitt, B., Pieske, B., Filippatos, G., Greene, S.J. & Gheorghiade, M. (2017) Expert consensus document: Mitochondrial function as a therapeutic target in heart failure. *Nature Reviews Cardiology*. 14 (4), 238–250.

Burdge, G.C. & Lillycrop, K.A. (2010) Nutrition, Epigenetics, and Developmental Plasticity: Implications for Understanding Human Disease. *Annual Review of Nutrition*. 30 (1), 315–339.

Butland, B., Jebb, S., Kopelman, P., McPherson, K., Thomas, S., Mardell, J. & Parry, V. (2007) *Tackling obesities: future choices - project report (2nd edition) - 07-1184x-tackling-obesities-future-choices-report.pdf*. [Online] [online]. Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/287937/07-1184x-tackling-obesities-future-choices-report.pdf (Accessed 7 November 2016).

Byun, Colicino, E., Trevisi, L., Fan, T., Christiani, D.C. & Baccarelli, A.A. (2016) Effects of Air Pollution and Blood Mitochondrial DNA Methylation on Markers of Heart Rate Variability. *Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease*. 5 (4), .

Byun, H.-M. (2019) Platelet mitochondrial DNA methylation: Markers of cardiovascular disease predisposition in overweight and obese individuals. *Nutrition Bulletin*. 0 (0), .

Byun, H.-M. & Baccarelli, A.A. (2014) Environmental exposure and mitochondrial epigenetics: study design and analytical challenges. *Human genetics*. 133 (3), 247–257.

Byun, H.-M. & Barrow, T.M. (2015a) Analysis of pollutant-induced changes in mitochondrial DNA methylation. *Methods in molecular biology (Clifton, N.J.)*. 1265271–283.

Byun, H.-M. & Barrow, T.M. (2015b) Analysis of pollutant-induced changes in mitochondrial DNA methylation. *Methods in Molecular Biology (Clifton, N.J.)*. 1265271–283.

Byun, H.-M., Colicino, E., Trevisi, L., Fan, T., Christiani, D.C. & Baccarelli, A.A. (2016) Effects of Air Pollution and Blood Mitochondrial DNA Methylation on Markers of Heart Rate Variability. *Journal of the American Heart Association*. 5 (4), .

Byun, Panni, T., Motta, V., Hou, L., Nordio, F., Apostoli, P., Bertazzi, P.A. & Baccarelli, A.A. (2013) Effects of airborne pollutants on mitochondrial DNA Methylation. *Particle and Fibre Toxicology*. 1018.

Calabrese, E.J., Mattson, M.P. & Calabrese, V. (2010) Resveratrol commonly displays hormesis: occurrence and biomedical significance. *Human & Experimental Toxicology*. 29 (12), 980–1015.

Calle, E.E., Thun, M.J., Petrelli, J.M., Rodriguez, C. & Heath, C.W. (1999) Body-Mass Index and Mortality in a Prospective Cohort of U.S. Adults. *New England Journal of Medicine*. 341 (15), 1097–1105.

C. Alva-Ensastegui, J., Palomar-Pardavé, M., Romero-Romo, M. & T. Ramírez-Silva, M. (2018) Quercetin spectrofluorometric quantification in aqueous media using different surfactants as fluorescence promoters. *RSC Advances*. 8 (20), 10980–10986.

Camacho, S. & Ruppel, A. (2017) Is the calorie concept a real solution to the obesity epidemic? *Global Health Action*. 10 (1), .

Caple, F., Williams, E.A., Spiers, A., Tyson, J., Burtle, B., Daly, A.K., Mathers, J.C. & Hesketh, J.E. (2010) Inter-individual variation in DNA damage and base excision repair in young, healthy non-smokers: effects of dietary supplementation and genotype. *The British Journal of Nutrition*. 103 (11), 1585–1593.

Carlos-Reyes, Á., López-González, J.S., Meneses-Flores, M., Gallardo-Rincón, D., Ruíz-García, E., Marchat, L.A., Astudillo-de la Vega, H., Hernández de la Cruz, O.N. & López-Camarillo, C. (2019) Dietary Compounds as Epigenetic Modulating Agents in Cancer. *Frontiers in Genetics*. 10.

Carnevale, R., Loffredo, L., Pignatelli, P., Nocella, C., Bartimoccia, S., Di Santo, S., Martino, F., Catasca, E., Perri, L. & Violi, F. (2012) Dark chocolate inhibits platelet isoprostanes via NOX2 down-regulation in smokers. *Journal of Thrombosis and Haemostasis*. 10 (1), 125–132.

Chen, H., Dzitoyeva, S. & Manev, H. (2012) Effect of valproic acid on mitochondrial epigenetics. *European journal of pharmacology*. 690 (1–3), 51–59.

Chen, Z., Newgard, C.B., Kim, J.S., IIkayeva, O., Alderete, T.L., Thomas, D.C., Berhane, K., Breton, C., Chatzi, L., Bastain, T.M., McConnell, R., Avol, E., Lurmann, F., Muehlbauer, M.J., Hauser, E.R. & Gilliland, F.D. (2019) Near-roadway air pollution exposure and altered fatty acid oxidation among adolescents and young adults – The interplay with obesity. *Environment International*. 130104935.

Cheng, Z. & Almeida, F., A. (2014) *Mitochondrial alteration in type 2 diabetes and obesity: An epigenetic link: Cell Cycle: Vol 13, No 6*. [Online] [online]. Available from: http://www.tandfonline.com/doi/abs/10.4161/cc.28189 (Accessed 28 February 2017).

Cheng, Z., Schmelz, E.M., Liu, D. & Hulver, M.W. (2014) Targeting mitochondrial alterations to prevent type 2 diabetes--evidence from studies of dietary redox-active compounds. *Molecular Nutrition & Food Research*. 58 (8), 1739–1749.

Chew, S., Kolosowska, N., Saveleva, L., Malm, T. & Kanninen, K.M. (2020) Impairment of mitochondrial function by particulate matter: Implications for the brain. *Neurochemistry International*. 135104694.

Chignell, C.F., Bilskj, P., Reszka, K.J., Motten, A.G., Sik, R.H. & Dahl, T.A. (1994) Spectral and Photochemical Properties of Curcumin. *Photochemistry and Photobiology*. 59 (3), 295–302.

Chuengsamarn, S., Rattanamongkolgul, S., Phonrat, B., Tungtrongchitr, R. & Jirawatnotai, S. (2014) Reduction of atherogenic risk in patients with type 2 diabetes by curcuminoid extract: a randomized controlled trial. *The Journal of Nutritional Biochemistry*. 25 (2), 144–150.

Clay Montier, L.L., Deng, J. & Bai, Y. (2009) Number matters: control of mammalian mitochondrial DNA copy number. *Journal of genetics and genomics = Yi chuan xue bao*. 36 (3), 125–131.

Comuzzie, A.G. & Allison, D.B. (1998) The Search for Human Obesity Genes. *Science*. 280 (5368), 1374–1377.

Conroy, R.M., Pyörälä, K., Fitzgerald, A.P., Sans, S., Menotti, A., De Backer, G., De Bacquer, D., Ducimetière, P., Jousilahti, P., Keil, U., Njølstad, I., Oganov, R.G., Thomsen, T., Tunstall-Pedoe, H., Tverdal, A., Wedel, H., Whincup, P., Wilhelmsen, L. & Graham, I.M. (2003) Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. *European Heart Journal*. 24 (11), 987–1003.

Corsi, S., Iodice, S., Vigna, L., Cayir, A., Mathers, J.C., Bollati, V. & Byun, H.-M. (2020) Platelet mitochondrial DNA methylation predicts future cardiovascular outcome in adults with overweight and obesity. *Clinical Epigenetics*. 12 (1), 29.

Cory, H., Passarelli, S., Szeto, J., Tamez, M. & Mattei, J. (2018) The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. *Frontiers in Nutrition*. 5.

Cosselman, K.E., Navas-Acien, A. & Kaufman, J.D. (2015) Environmental factors in cardiovascular disease. *Nature Reviews Cardiology*. 12 (11), 627–642.

Costa, L.G., Garrick, J.M., Roquè, P.J. & Pellacani, C. (2016) Mechanisms of Neuroprotection by Quercetin: Counteracting Oxidative Stress and More. *Oxidative Medicine and Cellular Longevity*. 2016.

Costantino, S., Paneni, F. & Cosentino, F. (2016) Ageing, metabolism and cardiovascular disease. *The Journal of Physiology*. 594 (8), 2061–2073.

Cowman, J., Dunne, E., Oglesby, I., Byrne, B., Ralph, A., Voisin, B., Müllers, S., Ricco, A.J. & Kenny, D. (2015) Age-related changes in platelet function are more profound in women than in men. *Scientific Reports*. 5.

Crider, K.S., Yang, T.P., Berry, R.J. & Bailey, L.B. (2012) Folate and DNA Methylation: A Review of Molecular Mechanisms and the Evidence for Folate's Role. *Advances in Nutrition: An International Review Journal*. 3 (1), 21–38.

CRUK (2016) *Overweight and obesity statistics*. [Online] [online]. Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/risk/overweight-and-obesity (Accessed 28 May 2019).

Cuyàs, E., Fernández-Arroyo, S., Verdura, S., García, R.Á.-F., Stursa, J., Werner, L., Blanco-González, E., Montes-Bayón, M., Joven, J., Viollet, B., Neuzil, J. & Menendez, J.A. (2017) Metformin regulates global DNA methylation via mitochondrial one-carbon metabolism. *Oncogene*.

D'Agostino, R.B., Vasan, R.S., Pencina, M.J., Wolf, P.A., Cobain, M., Massaro, J.M. & Kannel, W.B. (2008) General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation*. 117 (6), 743–753.

Danaei, G., Ding, E.L., Mozaffarian, D., Taylor, B., Rehm, J., Murray, C.J.L. & Ezzati, M. (2009) The preventable causes of death in the United States: comparative risk assessment of dietary, lifestyle, and metabolic risk factors. *PLoS medicine*. 6 (4), e1000058.

D'Aquila, P., Giordano, M., Montesanto, A., De Rango, F., Passarino, G. & Bellizzi, D. (2015) Age-and gender-related pattern of methylation in the MT-RNR1 gene. *Epigenomics*. 7 (5), 707–716.

Darvall, K.A.L., Sam, R.C., Silverman, S.H., Bradbury, A.W. & Adam, D.J. (2007) Obesity and Thrombosis. *European Journal of Vascular and Endovascular Surgery*. 33 (2), 223–233.

De Paepe, B. & Van Coster, R. (2017) A Critical Assessment of the Therapeutic Potential of Resveratrol Supplements for Treating Mitochondrial Disorders. *Nutrients*. 9 (9), .

Devall, M., Roubroeks, J., Mill, J., Weedon, M. & Lunnon, K. (2016a) Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neuroscience Letters*. 62547–55.

Devall, M., Roubroeks, J., Mill, J., Weedon, M. & Lunnon, K. (2016b) Epigenetic regulation of mitochondrial function in neurodegenerative disease: new insights from advances in genomic technologies. *Neuroscience letters*. 62547–55.

Devall, M., Smith, R.G., Jeffries, A., Hannon, E., Davies, M.N., Schalkwyk, L., Mill, J., Weedon, M. & Lunnon, K. (2017) Regional differences in mitochondrial DNA methylation in human post-mortem brain tissue. *Clinical Epigenetics*. 947.

Deverdun, J., Akbaraly, T.N., Charroud, C., Abdennour, M., Brickman, A.M., Chemouny, S., Steffener, J., Portet, F., Bonafe, A., Stern, Y., Ritchie, K., Molino, F., Le Bars, E. & Menjot de Champfleur, N. (2016) Mean arterial pressure change associated with cerebral blood flow in healthy older adults. *Neurobiology of Aging*. 4649–57.

Dick, K.J., Nelson, C.P., Tsaprouni, L., Sandling, J.K., Aïssi, D., Wahl, S., Meduri, E., Morange, P.-E., Gagnon, F., Grallert, H., Waldenberger, M., Peters, A., Erdmann, J., Hengstenberg, C., Cambien, F., Goodall, A.H., Ouwehand, W.H., Schunkert, H., Thompson, J.R., et al. (2014) DNA methylation and body-mass index: a genome-wide analysis. *Lancet (London, England)*. 383 (9933), 1990–1998.

van Dijk, S.J., Molloy, P.L., Varinli, H., Morrison, J.L., Muhlhausler, B.S., Buckley, M., Clark, S.J., McMillen, I.C., Noakes, M., Samaras, K. & Tellam, R.L. (2015) Epigenetics and human obesity. *International Journal of Obesity*. 39 (1), 85–97.

DiNicolantonio, J.J., Bhutani, J., OKeefe, J.H. & Crofts, C. (2017) Postprandial insulin assay as the earliest biomarker for diagnosing pre-diabetes, type 2 diabetes and increased cardiovascular risk. *Open Heart*. 4 (2), .

Diot, A., Morten, K. & Poulton, J. (2016) Mitophagy plays a central role in mitochondrial ageing. *Mammalian Genome*. 27381–395.

Dong, Z., Pu, L. & Cui, H. (2020) Mitoepigenetics and Its Emerging Roles in Cancer. *Frontiers in Cell and Developmental Biology*. 8.

Dor, Y. & Cedar, H. (2018) Principles of DNA methylation and their implications for biology and medicine. *The Lancet*. 392 (10149), 777–786.

Dostal, V. & Churchill, M.E.A. (2019) Cytosine methylation of mitochondrial DNA at CpG sequences impacts transcription factor A DNA binding and transcription. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*. 1862 (5), 598–607.

Dou, X., Boyd-Kirkup, J.D., McDermott, J., Zhang, X., Li, F., Rong, B., Zhang, R., Miao, B., Chen, P., Cheng, H., Xue, J., Bennett, D., Wong, J., Lan, F. & Han, J.-D.J. (2019) The strand-biased mitochondrial DNA methylome and its regulation by DNMT3A. *Genome Research*. 29 (10), 1622–1634.

D'Souza, A.R. & Minczuk, M. (2018) Mitochondrial transcription and translation: overview. *Essays In Biochemistry*. 62 (3), 309–320.

Dzitoyeva, S., Chen, H. & Manev, H. (2012a) Effect of aging on 5-hydroxymethylcytosine in brain mitochondria. *Neurobiology of Aging*. 33 (12), 2881–2891.

Dzitoyeva, S., Chen, H. & Manev, H. (2012b) Effect of aging on 5-hydroxymethylcytosine in brain mitochondria. *Neurobiology of aging*. 33 (12), 2881–2891.

EAPC (2012) *HeartScore - PMS*. [Online] [online]. Available from: https://heartscore.escardio.org/2012/calc.aspx?model=europelow (Accessed 12 February 2019).

Ekstrand, M.I., Falkenberg, M., Rantanen, A., Park, C.B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C.M. & Larsson, N.-G. (2004) Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Human Molecular Genetics*. 13 (9), 935–944.

Elagizi, A., Kachur, S., Lavie, C.J., Carbone, S., Pandey, A., Ortega, F.B. & Milani, R.V. (2018) An Overview and Update on Obesity and the Obesity Paradox in Cardiovascular Diseases. *Progress in Cardiovascular Diseases*. 61 (2), 142–150.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R.M., Serra-Majem, L., Pintó, X., Basora, J., Muñoz, M.A., Sorlí, J.V., Martínez, J.A., Fitó, M., Gea, A., et al. (2018) Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. *The New England Journal of Medicine*. 378 (25), e34.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R.M., Serra-Majem, L., Pintó, X., Basora, J., Muñoz, M.A., Sorlí, J.V., Martínez, J.A. & Martínez-González, M.A. (2013) Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. *New England Journal of Medicine*. 368 (14), 1279–1290.

Faghihzadeh, F., Adibi, P., Rafiei, R. & Hekmatdoost, A. (2014) Resveratrol supplementation improves inflammatory biomarkers in patients with nonalcoholic fatty liver disease. *Nutrition Research (New York, N.Y.)*. 34 (10), 837–843.

Falkenberg, M. (2018) Mitochondrial DNA replication in mammalian cells: overview of the pathway. *Essays In Biochemistry*. 62 (3), 287–296.

Fang, M., Chen, D. & Yang, C.S. (2007) Dietary polyphenols may affect DNA methylation. *The Journal of Nutrition*. 137 (1 Suppl), 223S-228S.

Feinberg, A.P. & Irizarry, R.A. (2010) Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease. *Proceedings of the National Academy of Sciences*. 107 (suppl 1), 1757–1764.

Felitti, V.J., Anda, R.F., Nordenberg, D., Williamson, D.F., Spitz, A.M., Edwards, V., Koss, M.P. & Marks, J.S. (2019) Relationship of Childhood Abuse and Household Dysfunction to Many of the Leading Causes of Death in Adults: The Adverse Childhood Experiences (ACE) Study. *American Journal of Preventive Medicine*. 56 (6), 774–786.

Feng, S., Xiong, L., Ji, Z., Cheng, W. & Yang, H. (2012) Correlation between increased ND2 expression and demethylated displacement loop of mtDNA in colorectal cancer. *Molecular Medicine Reports*. 6 (1), 125–130.

Ferretta, A., Gaballo, A., Tanzarella, P., Piccoli, C., Capitanio, N., Nico, B., Annese, T., Di Paola, M., Dell'Aquila, C., De Mari, M., Ferranini, E., Bonifati, V., Pacelli, C. & Cocco, T. (2014) Effect of resveratrol on mitochondrial function: Implications in parkin-associated familiar Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 1842 (7), 902–915.

Festi, D., Colecchia, A., Pini, S., Scaioli, E., Maffeis, C., Coccheri, S. & Petroni, M.L. (2009) Development and application of a simple and powerful tool for nutrition and lifestyle education for the Italian general population by general practitioners and family paediatricians. *Mediterranean Journal of Nutrition and Metabolism*. 2 (2), 139–144.

Fitzgerald, K., Hodges, R., Hanes, D., Stack, E., Cheishvili, D., Szyf, M., Henkel, J., Twedt, M., Giannopoulou, D., Herdell, J., Logan, S. & Bradley, R. (2020) Reversal of Epigenetic Age with Diet and Lifestyle in a Pilot Randomized Clinical Trial. *medRxiv*. 2020.07.07.20148098.

Fontana, G.A. & Gahlon, H.L. (2020) Mechanisms of replication and repair in mitochondrial DNA deletion formation. *Nucleic Acids Research*. 48 (20), 11244–11258.

Fontana, L., Partridge, L. & Longo, V.D. (2010) Extending healthy life span--from yeast to humans. *Science (New York, N.Y.)*. 328 (5976), 321–326.

Fraszczyk, E., Luijten, M., Spijkerman, A.M.W., Snieder, H., Wackers, P.F.K., Bloks, V.W., Nicoletti, C.F., Nonino, C.B., Crujeiras, A.B., Buurman, W.A., Greve, J.W., Rensen, S.S., Wolffenbuttel, B.H.R. & van Vliet-Ostaptchouk, J.V. (2020) The effects of bariatric surgery on clinical profile, DNA methylation, and ageing in severely obese patients. *Clinical Epigenetics*. 12.

Fritz, M.S. & MacKinnon, D.P. (2007) Required Sample Size to Detect the Mediated Effect. *Psychological science*. 18 (3), 233–239.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L. & Paul, C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences of the United States of America*. 89 (5), 1827–1831.

Fukui, H. & Moraes, C.T. (2009) Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. *Human Molecular Genetics*. 18 (6), 1028–1036.

Gabory, A., Attig, L. & Junien, C. (2011) Epigenetic mechanisms involved in developmental nutritional programming. *World Journal of Diabetes*. 2 (10), 164–175.

Gambini, J., Inglés, M., Olaso, G., Lopez-Grueso, R., Bonet-Costa, V., Gimeno-Mallench, L., Mas-Bargues, C., Abdelaziz, K.M., Gomez-Cabrera, M.C., Vina, J. & Borras, C. (2015) *Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and Humans*. [Online]

[online]. Available from: https://www.hindawi.com/journals/omcl/2015/837042/ (Accessed 20 December 2019).

Gao, X., Jia, M., Zhang, Y., Breitling, L.P. & Brenner, H. (2015) DNA methylation changes of whole blood cells in response to active smoking exposure in adults: a systematic review of DNA methylation studies. *Clinical Epigenetics*. 7113.

Garcia-Souza, L.F. & Oliveira, M.F. (2014) Mitochondria: Biological roles in platelet physiology and pathology. *The International Journal of Biochemistry & Cell Biology*. 50156–160.

Gautrey, H.E., van Otterdijk, S.D., Cordell, H.J., Newcastle 85+ Study Core Team, Mathers, J.C. & Strathdee, G. (2014) DNA methylation abnormalities at gene promoters are extensive and variable in the elderly and phenocopy cancer cells. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 28 (7), 3261–3272.

Gavriil, L., Detopoulou, M., Petsini, F., Antonopoulou, S. & Fragopoulou, E. (2019) Consumption of plant extract supplement reduces platelet activating factor-induced platelet aggregation and increases platelet activating factor catabolism: a randomised, double-blind and placebo-controlled trial. *British Journal of Nutrition*. 121 (09), 982–991.

Gaziano, T.A., Bitton, A., Anand, S., Abrahams-Gessel, S. & Murphy, A. (2010) Growing Epidemic of Coronary Heart Disease in Low- and Middle-Income Countries. *Current problems in cardiology*. 35 (2), 72–115.

Gensous, N., Garagnani, P., Santoro, A., Giuliani, C., Ostan, R., Fabbri, C., Milazzo, M., Gentilini, D., di Blasio, A.M., Pietruszka, B., Madej, D., Bialecka-Debek, A., Brzozowska, A., Franceschi, C. & Bacalini, M.G. (2020) One-year Mediterranean diet promotes epigenetic rejuvenation with country- and sex-specific effects: a pilot study from the NU-AGE project. *GeroScience*. 42 (2), 687–701.

Gentilini, D., Garagnani, P., Pisoni, S., Bacalini, M.G., Calzari, L., Mari, D., Vitale, G., Franceschi, C. & Di Blasio, A.M. (2015) Stochastic epigenetic mutations (DNA methylation) increase exponentially in human aging and correlate with X chromosome inactivation skewing in females. *Aging (Albany NY)*. 7 (8), 568–576.

Gerrits, A.J., Gitz, E., Koekman, C.A., Visseren, F.L., van Haeften, T.W. & Akkerman, J.W.N. (2012) Induction of insulin resistance by the adipokines resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 in human megakaryocytes. *Haematologica*. 97 (8), 1149–1157.

Gerszten, R.E. & Wang, T.J. (2008) The search for new cardiovascular biomarkers. *Nature*. 451 (7181), 949–952.

Ghosh, S., Sengupta, S. & Scaria, V. (2014) Comparative analysis of human mitochondrial methylomes shows distinct patterns of epigenetic regulation in mitochondria. *Mitochondrion*. 18 (Supplement C), 58–62.

Ghosh, S., Sengupta, S. & Scaria, V. (2016) Hydroxymethyl cytosine marks in the human mitochondrial genome are dynamic in nature. *Mitochondrion*. 2725–31.

Ghosh, S., Singh, K.K., Sengupta, S. & Scaria, V. (2015) Mitoepigenetics: The different shades of grey. *Mitochondrion*. 25 (Supplement C), 60–66.

Goldberg, A.D., Allis, C.D. & Bernstein, E. (2007) Epigenetics: A Landscape Takes Shape. *Cell*. 128 (4), 635–638.

Greaves, L.C., Barron, M.J., Plusa, S., Kirkwood, T.B., Mathers, J.C., Taylor, R.W. & Turnbull, D.M. (2010) Defects in multiple complexes of the respiratory chain are present in ageing human colonic crypts. *Experimental Gerontology*. 45 (7–8), 573–579.

Greaves, L.C., Nooteboom, M., Elson, J.L., Tuppen, H.A.L., Taylor, G.A., Commane, D.M., Arasaradnam, R.P., Khrapko, K., Taylor, R.W., Kirkwood, T.B.L., Mathers, J.C. & Turnbull, D.M. (2014) Clonal expansion of early to mid-life mitochondrial DNA point mutations drives mitochondrial dysfunction during human ageing. *PLoS genetics*. 10 (9), e1004620.

Green, M.A., Strong, M., Razak, F., Subramanian, S.V., Relton, C. & Bissell, P. (2016) Who are the obese? A cluster analysis exploring subgroups of the obese. *Journal of Public Health (Oxford, England)*. 38 (2), 258–264.

Gresele, P., Pignatelli, P., Guglielmini, G., Carnevale, R., Mezzasoma, A.M., Ghiselli, A., Momi, S. & Violi, F. (2008) Resveratrol, at Concentrations Attainable with Moderate Wine Consumption, Stimulates Human Platelet Nitric Oxide Production. *The Journal of Nutrition*. 138 (9), 1602–1608.

Grover, S.A., Kaouache, M., Rempel, P., Joseph, L., Dawes, M., Lau, D.C.W. & Lowensteyn, I. (2015) Years of life lost and healthy life-years lost from diabetes and cardiovascular disease in overweight and obese people: a modelling study. *The Lancet. Diabetes & Endocrinology*. 3 (2), 114–122.

Gu, S., Tian, Y., Chlenski, A., Salwen, H.R., Lu, Z., Raj, J.U. & Yang, Q. (2012) Valproic acid shows a potent antitumor effect with alteration of DNA methylation in neuroblastoma. *Anti-Cancer Drugs*. 23 (10), 1054–1066.

Gueguen, N., Desquiret-Dumas, V., Leman, G., Chupin, S., Baron, S., Nivet-Antoine, V., Vessières, E., Ayer, A., Henrion, D., Lenaers, G., Reynier, P. & Procaccio, V. (2015) Resveratrol Directly Binds to Mitochondrial Complex I and Increases Oxidative Stress in Brain Mitochondria of Aged Mice. *PLoS ONE*. 10 (12), .

Gupta, S.C., Patchva, S. & Aggarwal, B.B. (2012) Therapeutic Roles of Curcumin: Lessons Learned from Clinical Trials. *The AAPS Journal*. 15 (1), 195–218.

Gurney, D., Lip, G.Y.H. & Blann, A.D. (2002) A reliable plasma marker of platelet activation: Does it exist? *American Journal of Hematology*. 70 (2), 139–144.

Gustafsson, C.M., Falkenberg, M. & Larsson, N.-G. (2016) Maintenance and Expression of Mammalian Mitochondrial DNA. *Annual Review of Biochemistry*. 85 (1), 133–160.

de Haan, G. & Lazare, S.S. (2018) Aging of hematopoietic stem cells. *Blood*. 131 (5), 479–487.

Hall, K.D., Ayuketah, A., Brychta, R., Cai, H., Cassimatis, T., Chen, K.Y., Chung, S.T., Costa, E., Courville, A., Darcey, V., Fletcher, L.A., Forde, C.G., Gharib, A.M., Guo, J., Howard, R., Joseph, P.V., McGehee, S., Ouwerkerk, R., Raisinger, K., et al. (2019) Ultra-Processed Diets Cause Excess Calorie Intake and Weight Gain: An Inpatient Randomized Controlled Trial of Ad Libitum Food Intake. *Cell Metabolism*.

Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., Klotzle, B., Bibikova, M., Fan, J.-B., Gao, Y., Deconde, R., Chen, M., Rajapakse, I., Friend, S., Ideker, T. & Zhang, K. (2013) Genome-wide methylation profiles reveal quantitative views of human aging rates. *Molecular Cell*. 49 (2), 359–367.

Harker, L.A., Roskos, L.K., Marzec, U.M., Carter, R.A., Cherry, J.K., Sundell, B., Cheung, E.N., Terry, D. & Sheridan, W. (2000) Effects of megakaryocyte growth and development factor on platelet production, platelet life span, and platelet function in healthy human volunteers. *Blood*. 95 (8), 2514–2522.

Hayatsu, H. (2008) Discovery of bisulfite-mediated cytosine conversion to uracil, the key reaction for DNA methylation analysis — A personal account. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*. 84 (8), 321–330.

Hazkani-Covo, E., Zeller, R.M. & Martin, W. (2010) Molecular Poltergeists: Mitochondrial DNA Copies (numts) in Sequenced Nuclear Genomes. *PLoS Genetics*. 6 (2), .

Ho, Y.-H. & Méndez-Ferrer, S. (2020) Microenvironmental contributions to hematopoietic stem cell aging. *Haematologica*. 105 (1), 38–46.

van Holten, T.C., Waanders, L.F., de Groot, P.G., Vissers, J., Hoefer, I.E., Pasterkamp, G., Prins, M.W.J. & Roest, M. (2013) Circulating biomarkers for predicting cardiovascular disease risk; a systematic review and comprehensive overview of meta-analyses. *PloS One*. 8 (4), e62080.

Hong, E.E., Okitsu, C.Y., Smith, A.D. & Hsieh, C.-L. (2013) Regionally Specific and Genome-Wide Analyses Conclusively Demonstrate the Absence of CpG Methylation in Human Mitochondrial DNA. *Molecular and Cellular Biology*. 33 (14), 2683–2690.

Horvath, S. (2013) DNA methylation age of human tissues and cell types. *Genome Biology*. 14 (10), R115.

Horvath, S., Erhart, W., Brosch, M., Ammerpohl, O., von Schönfels, W., Ahrens, M., Heits, N., Bell, J.T., Tsai, P.-C., Spector, T.D., Deloukas, P., Siebert, R., Sipos, B., Becker, T., Röcken, C., Schafmayer, C. & Hampe, J. (2014) Obesity accelerates epigenetic aging of human liver. *Proceedings of the National Academy of Sciences of the United States of America*. 111 (43), 15538–15543.

Horvath, S., Pirazzini, C., Bacalini, M.G., Gentilini, D., Di Blasio, A.M., Delledonne, M., Mari, D., Arosio, B., Monti, D., Passarino, G., De Rango, F., D'Aquila, P., Giuliani, C., Marasco, E., Collino, S., Descombes, P., Garagnani, P. & Franceschi, C. (2015) Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging*. 7 (12), 1159–1170.

Ho-Tin-Noé, B., Demers, M. & Wagner, D.D. (2011) How platelets safeguard vascular integrity. *Journal of thrombosis and haemostasis : JTH*. 9 (Suppl 1), 56–65.

Hotoleanu, C. (2020) Association between obesity and venous thromboembolism. *Medicine and Pharmacy Reports*. 93 (2), 162–168.

Hubbard, G.P., Wolffram, S., Lovegrove, J.A. & Gibbins, J.M. (2004a) Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *Journal of thrombosis and haemostasis: JTH*. 2 (12), 2138–2145.

Hubbard, G.P., Wolffram, S., Lovegrove, J.A. & Gibbins, J.M. (2004b) Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *Journal of Thrombosis and Haemostasis*. 2 (12), 2138–2145.

Hudson, G., Gomez-Duran, A., Wilson, I.J. & Chinnery, P.F. (2014) Recent Mitochondrial DNA Mutations Increase the Risk of Developing Common Late-Onset Human Diseases. *PLOS Genetics*. 10 (5), e1004369.

Huo, Y. & Ley, K.F. (2004) Role of Platelets in the Development of Atherosclerosis. *Trends in Cardiovascular Medicine*. 14 (1), 18–22.

Iacobazzi, V., Castegna, A., Infantino, V. & Andria, G. (2013) Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool. *Molecular Genetics and Metabolism*. 110 (1–2), 25–34.

Infantino, V., Castegna, A., Iacobazzi, F., Spera, I., Scala, I., Andria, G. & Iacobazzi, V. (2011) Impairment of methyl cycle affects mitochondrial methyl availability and glutathione level in Down's syndrome. *Molecular Genetics and Metabolism*. 102 (3), 378–382.

Ioachimescu, A.G., Brennan, D.M., Hoar, B.M., Hazen, S.L. & Hoogwerf, B.J. (2008) Serum uric acid is an independent predictor of all-cause mortality in patients at high risk of cardiovascular disease: a preventive cardiology information system (PreCIS) database cohort study. *Arthritis and Rheumatism*. 58 (2), 623–630.

Iyer, K.S. & Dayal, S. (2020) Modulators of platelet function in aging. *Platelets*. 31 (4), 474–482.

Jaacks, L.M., Vandevijvere, S., Pan, A., McGowan, C.J., Wallace, C., Imamura, F., Mozaffarian, D., Swinburn, B. & Ezzati, M. (2019) The obesity transition: stages of the global epidemic. *The Lancet Diabetes & Endocrinology*. 7 (3), 231–240.

Jaenisch, R. & Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics*. 33245–254.

Jang, H., Mason, J.B. & Choi, S.-W. (2005) Genetic and epigenetic interactions between folate and aging in carcinogenesis. *The Journal of Nutrition*. 135 (12 Suppl), 2967S-2971S.

Janssen, B.G., Byun, H.-M., Gyselaers, W., Lefebvre, W., Baccarelli, A.A. & Nawrot, T.S. (2015) Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. *Epigenetics*. 10 (6), 536–544.

Janssen, B.G., Byun, H.-M., Roels, H.A., Gyselaers, W., Penders, J., Baccarelli, A.A. & Nawrot, T.S. (2017) Regulating role of fetal thyroid hormones on placental mitochondrial DNA methylation: epidemiological evidence from the ENVIRONAGE birth cohort study. *Clinical Epigenetics*. 9.

Janssen, B.G., Gyselaers, W., Byun, H.-M., Roels, H.A., Cuypers, A., Baccarelli, A.A. & Nawrot, T.S. (2017) Placental mitochondrial DNA and CYP1A1 gene methylation as molecular signatures for tobacco smoke exposure in pregnant women and the relevance for birth weight. *Journal of Translational Medicine*. 15.

Janssen, I., Katzmarzyk, P.T. & Ross, R. (2004) Waist circumference and not body mass index explains obesity-related health risk. *The American Journal of Clinical Nutrition*. 79 (3), 379–384.

Jayaprakash, A.D., Benson, E.K., Gone, S., Liang, R., Shim, J., Lambertini, L., Toloue, M.M., Wigler, M., Aaronson, S.A. & Sachidanandam, R. (2015) Stable heteroplasmy at the single-cell level is facilitated by intercellular exchange of mtDNA. *Nucleic Acids Research*. 43 (4), 2177–2187.

Ježková, J., Ďurovcová, V., Wenchich, L., Hansíková, H., Zeman, J., Hána, V., Marek, J., Lacinová, Z., Haluzík, M. & Kršek, M. (2019) The relationship of mitochondrial dysfunction and the development of insulin resistance in Cushing's syndrome. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*. 121459–1471.

Jia, L., Li, J., He, B., Jia, Y., Niu, Y., Wang, C. & Zhao, R. (2016) Abnormally activated one-carbon metabolic pathway is associated with mtDNA hypermethylation and mitochondrial malfunction in the oocytes of polycystic gilt ovaries. *Scientific Reports*. 619436.

Jia, Y., Li, R., Cong, R., Yang, X., Sun, Q., Parvizi, N. & Zhao, R. (2013) Maternal Low-Protein Diet Affects Epigenetic Regulation of Hepatic Mitochondrial DNA Transcription in a Sex-Specific Manner in Newborn Piglets Associated with GR Binding to Its Promoter. *PLOS ONE*. 8 (5), e63855.

Joehanes, R., Just, A.C., Marioni, R.E., Pilling, L.C., Reynolds, L.M., Mandaviya, P.R., Guan, W., Xu, T., Elks, C.E., Aslibekyan, S., Moreno-Macias, H., Smith, J.A., Brody, J.A., Dhingra, R., Yousefi, P., Pankow, J.S., Kunze, S., Shah, S.H., McRae, A.F., et al. (2016) Epigenetic Signatures of Cigarette Smoking. *Circulation. Cardiovascular Genetics*. 9 (5), 436–447.

Jones, C.I. (2016) Platelet function and ageing. *Mammalian Genome*. 27358–366.

Justice, A.E., Winkler, T.W., Feitosa, M.F., Graff, M., Fisher, V.A., Young, K., Barata, L., Deng, X., Czajkowski, J., Hadley, D., Ngwa, J.S., Ahluwalia, T.S., Chu, A.Y., Heard-Costa, N.L., Lim, E., Perez, J., Eicher, J.D., Kutalik, Z., Xue, L., et al. (2017) Genome-wide meta-analysis of 241,258 adults accounting for smoking behaviour identifies novel loci for obesity traits. *Nature Communications*. 814977.

Kang, D., Kim, S.H. & Hamasaki, N. (2007) Mitochondrial transcription factor A (TFAM): Roles in maintenance of mtDNA and cellular functions. *Mitochondrion*. 7 (1), 39–44.

Karelis, A.D., Faraj, M., Bastard, J.-P., St-Pierre, D.H., Brochu, M., Prud'homme, D. & Rabasa-Lhoret, R. (2005) The metabolically healthy but obese individual presents a favorable inflammation profile. *The Journal of Clinical Endocrinology and Metabolism*. 90 (7), 4145–4150.

Karymov, M.A., Tomschik, M., Leuba, S.H., Caiafa, P. & Zlatanova, J. (2001) DNA methylation-dependent chromatin fiber compaction in vivo and in vitro: requirement for linker histone. *The FASEB Journal*. 15 (14), 2631–2641.

Kazak, Reyes, A. & Holt, I.J. (2012) Minimizing the damage: repair pathways keep mitochondrial DNA intact. *Nature Reviews Molecular Cell Biology*. 13 (10), 659–671.

Kazmi, N., Elliott, H.R., Burrows, K., Tillin, T., Hughes, A.D., Chaturvedi, N., Gaunt, T.R. & Relton, C.L. (2020) Associations between high blood pressure and DNA methylation. *PLOS ONE*. 15 (1), e0227728.

Keating, F.K., Schneider, D.J., Savage, P.D., Bunn, J.Y., Harvey-Berino, J., Ludlow, M., Toth, M.J. & Ades, P.A. (2013) Effect of exercise training and weight loss on platelet reactivity in overweight patients with coronary artery disease. *Journal of Cardiopulmonary Rehabilitation and Prevention*. 33 (6), 371–377.

Khan, S.S., Ning, H., Wilkins, J.T., Allen, N., Carnethon, M., Berry, J.D., Sweis, R.N. & Lloyd-Jones, D.M. (2018) Association of Body Mass Index With Lifetime Risk of Cardiovascular Disease and Compression of Morbidity. *JAMA Cardiology*. 3 (4), 280–287.

Kim, K., Friso, S. & Choi, S.-W. (2009) DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. *The Journal of nutritional biochemistry*. 20 (12), 917–926.

Kim, K. & Park, K.-I. (2019) *A Review of Antiplatelet Activity of Traditional Medicinal Herbs on Integrative Medicine Studies*. [Online] [online]. Available from: https://www.hindawi.com/journals/ecam/2019/7125162/ (Accessed 3 February 2020).

Kim, M., Long, T.I., Arakawa, K., Wang, R., Yu, M.C. & Laird, P.W. (2010) DNA Methylation as a Biomarker for Cardiovascular Disease Risk. *PLOS ONE*. 5 (3), e9692.

Kim, Y. & Je, Y. (2017) Flavonoid intake and mortality from cardiovascular disease and all causes: A meta-analysis of prospective cohort studies. *Clinical nutrition ESPEN*. 2068–77.

Koopman, J.J.E. & Kuipers, R.S. (2017) From arterial ageing to cardiovascular disease. *The Lancet*. 389 (10080), 1676–1678.

Korhonen, J.A., Gaspari, M. & Falkenberg, M. (2003) TWINKLE Has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *The Journal of Biological Chemistry*. 278 (49), 48627–48632.

Kornblith, L.Z., Howard, B., Kunitake, R., Redick, B., Nelson, M., Cohen, M. & Callcut, R. (2015) Obesity and clotting: Body mass index independently contributes to

hypercoagulability after injury. *The Journal of Trauma and Acute Care Surgery*. 78 (1), 30–6; discussion 37.

Krueger, P.M. & Reither, E.N. (2015) Mind the gap: race/ethnic and socioeconomic disparities in obesity. *Current Diabetes Reports*. 15 (11), 95.

Lambertini, L. & Byun, H.-M. (2016) Mitochondrial Epigenetics and Environmental Exposure. *Current Environmental Health Reports*. 3 (3), 214–224.

Landsberg, L., Aronne, L.J., Beilin, L.J., Burke, V., Igel, L.I., Lloyd-Jones, D. & Sowers, J. (2013) Obesity-related hypertension: pathogenesis, cardiovascular risk, and treatment: a position paper of The Obesity Society and the American Society of Hypertension. *Journal of Clinical Hypertension (Greenwich, Conn.)*. 15 (1), 14–33.

Lannan, K.L., Refaai, M.A., Ture, S.K., Morrell, C.N., Blumberg, N., Phipps, R.P. & Spinelli, S.L. (2016) Resveratrol preserves the function of human platelets stored for transfusion. *British journal of haematology*. 172 (5), 794–806.

Larasati, Y.A., Yoneda-Kato, N., Nakamae, I., Yokoyama, T., Meiyanto, E. & Kato, J. (2018) Curcumin targets multiple enzymes involved in the ROS metabolic pathway to suppress tumor cell growth. *Scientific Reports*. 8.

Larson, A., Witman, M.A.H., Guo, Y., Ives, S., Richardson, R.S., Bruno, R.S., Jalili, T. & Symons, J.D. (2012) Acute, quercetin-induced reductions in blood pressure in hypertensive individuals are not secondary to lower plasma angiotensin-converting enzyme activity or endothelin-1: nitric oxide. *Nutrition Research*. 32 (8), 557–564.

Lee, C., Zeng, J., Drew, B.G., Sallam, T., Martin-Montalvo, A., Wan, J., Kim, S.-J., Mehta, H., Hevener, A.L., de Cabo, R. & Cohen, P. (2015) The Mitochondrial-Derived Peptide MOTS-c Promotes Metabolic Homeostasis and Reduces Obesity and Insulin Resistance. *Cell Metabolism*. 21 (3), 443–454.

Lee, W.J., Shim, J.-Y. & Zhu, B.T. (2005a) Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. *Molecular Pharmacology*. 68 (4), 1018–1030.

Lee, W.J., Shim, J.-Y. & Zhu, B.T. (2005b) Mechanisms for the Inhibition of DNA Methyltransferases by Tea Catechins and Bioflavonoids. *Molecular Pharmacology*. 68 (4), 1018–1030.

Leeksma, C.H.W. & Cohen, J.A. (1955) Determination of the Life of Human Blood Platelets using Labelled Di iso propylfluorophosphonate. *Nature*. 175 (4456), 552–553.

Leenen, F.A.D., Muller, C.P. & Turner, J.D. (2016a) DNA methylation: conducting the orchestra from exposure to phenotype? *Clinical Epigenetics*. 8 (1), .

Leenen, F.A.D., Muller, C.P. & Turner, J.D. (2016b) DNA methylation: conducting the orchestra from exposure to phenotype? *Clinical Epigenetics*. 8 (1), .

Leni, Z., Künzi, L. & Geiser, M. (2020) Air pollution causing oxidative stress. *Current Opinion in Toxicology*. 20–211–8.

Levine, M.E., Lu, A.T., Quach, A., Chen, B.H., Assimes, T.L., Bandinelli, S., Hou, L., Baccarelli, A.A., Stewart, J.D., Li, Y., Whitsel, E.A., Wilson, J.G., Reiner, A.P., Aviv, A., Lohman, K., Liu, Y., Ferrucci, L. & Horvath, S. (2018) An epigenetic biomarker of aging for lifespan and healthspan. *Aging*. 10 (4), 573–591.

Leytin, V., Allen, D.J., Mutlu, A., Gyulkhandanyan, A.V., Mykhaylov, S. & Freedman, J. (2009) Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Laboratory Investigation*. 89 (4), 374–384.

Li, H., Sureda, A., Devkota, H.P., Pittalà, V., Barreca, D., Silva, A.S., Tewari, D., Xu, S. & Nabavi, S.M. (2019) Curcumin, the golden spice in treating cardiovascular diseases. *Biotechnology Advances*.

Liao, K., Yan, J., Mai, K. & Ai, Q. (2016) Dietary lipid concentration affects liver mitochondrial DNA copy number, gene expression and DNA methylation in large yellow croaker (Larimichthys crocea). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 19325–32.

Liao, K., Yan, J., Mai, K. & Ai, Q. (2015) Dietary Olive and Perilla Oils Affect Liver Mitochondrial DNA Methylation in Large Yellow Croakers. *The Journal of Nutrition*. 145 (11), 2479–2485.

Lievens, D. & von Hundelshausen, P. (2011) Platelets in atherosclerosis. *Thrombosis and Haemostasis*. 106 (5), 827–838.

Lind, L., Ingelsson, E., Sundström, J., Siegbahn, A. & Lampa, E. (2018) Methylation-based estimated biological age and cardiovascular disease. *European Journal of Clinical Investigation*. 48 (2), .

Link, A., Balaguer, F., Shen, Y., Lozano, J.J., Leung, H.-C.E., Boland, C.R. & Goel, A. (2013) Curcumin Modulates DNA Methylation in Colorectal Cancer Cells. *PLoS ONE*. 8 (2), .

Liu, B., Du, Q., Chen, L., Fu, G., Li, S., Fu, L., Zhang, X., Ma, C. & Bin, C. (2016) CpG methylation patterns of human mitochondrial DNA. *Scientific Reports*. 6srep23421.

Liu, Z., Xie, Z., Jones, W., Pavlovicz, R.E., Liu, S., Yu, J., Li, P., Lin, J., Fuchs, J.R., Marcucci, G., Li, C. & Chan, K.K. (2009) Curcumin is a potent DNA hypomethylation agent. *Bioorganic & Medicinal Chemistry Letters*. 19 (3), 706–709.

Liyanage, T., Ninomiya, T., Wang, A., Neal, B., Jun, M., Wong, M.G., Jardine, M., Hillis, G.S. & Perkovic, V. (2016) Effects of the Mediterranean Diet on Cardiovascular Outcomes—A Systematic Review and Meta-Analysis. *PLoS ONE*. 11 (8), .

Locke, A.E., Kahali, B., Berndt, S.I., Justice, A.E., Pers, T.H., Day, F.R., Powell, C., Vedantam, S., Buchkovich, M.L., Yang, J., Croteau-Chonka, D.C., Esko, T., Fall, T., Ferreira, T., Gustafsson, S., Kutalik, Z., Luan, J., Mägi, R., Randall, J.C., et al. (2015) Genetic studies of body mass index yield new insights for obesity biology. *Nature*. 518 (7538), 197–206.

Loos, R.J.F. & Kilpeläinen, T.O. (2018) Genes that make you fat, but keep you healthy. *Journal of Internal Medicine*.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M. & Kroemer, G. (2013) The Hallmarks of Aging. *Cell*. 153 (6), 1194–1217.

de Lorgeril, M., Martin Jean-Louis, Monjaud Isabelle, Delaye Jacques, & Mamelle Nicole (1999) Mediterranean Diet, Traditional Risk Factors, and the Rate of Cardiovascular Complications After Myocardial Infarction. *Circulation*. 99 (6), 779–785.

Lott, M.T., Leipzig, J.N., Derbeneva, O., Xie, H.M., Chalkia, D., Sarmady, M., Procaccio, V. & Wallace, D.C. (2013) mtDNA Variation and Analysis Using Mitomap and Mitomaster. *Current Protocols in Bioinformatics*. 441.23.1-26.

Lu, A.T., Quach, A., Wilson, J.G., Reiner, A.P., Aviv, A., Raj, K., Hou, L., Baccarelli, A.A., Li, Y., Stewart, J.D., Whitsel, E.A., Assimes, T.L., Ferrucci, L. & Horvath, S. (2019) DNA methylation GrimAge strongly predicts lifespan and healthspan. *Aging*. 11 (2), 303–327.

Lu, A.T., Seeboth, A., Tsai, P.-C., Sun, D., Quach, A., Reiner, A.P., Kooperberg, C., Ferrucci, L., Hou, L., Baccarelli, A.A., Li, Y., Harris, S.E., Corley, J., Taylor, A., Deary, I.J., Stewart, J.D., Whitsel, E.A., Assimes, T.L., Chen, W., et al. (2019) DNA methylation-based estimator of telomere length. *Aging*. 11 (16), 5895–5923.

Lubecka, K., Kurzava, L., Flower, K., Buvala, H., Zhang, H., Teegarden, D., Camarillo, I., Suderman, M., Kuang, S., Andrisani, O., Flanagan, J.M. & Stefanska, B. (2016) Stilbenoids remodel the DNA methylation patterns in breast cancer cells and inhibit oncogenic NOTCH signaling through epigenetic regulation of MAML2 transcriptional activity. *Carcinogenesis*. 37 (7), 656–668.

Lyn, R., Heath, E. & Dubhashi, J. (2019) Global Implementation of Obesity Prevention Policies: a Review of Progress, Politics, and the Path Forward. *Current Obesity Reports*. 8 (4), 504–516.

Ma, J., Rebholz, C.M., Braun, K.V.E., Reynolds, L.M., Aslibekyan, S., Xia, R., Biligowda, N.G., Huan, T., Liu, C., Mendelson, M.M., Joehanes, R., Hu, E.A., Vitolins, M.Z., Wood, A.C., Lohman, K., Ochoa-Rosales, C., Meurs, J. van, Uitterlinden, A., Liu, Y., et al. (2020) Whole Blood DNA Methylation Signatures of Diet Are Associated with Cardiovascular Disease Risk Factors and All-cause Mortality. *Circulation: Genomic and Precision Medicine*.

Maierhofer, A., Flunkert, J., Oshima, J., Martin, G.M., Haaf, T. & Horvath, S. (2017) Accelerated epigenetic aging in Werner syndrome. *Aging (Albany NY)*. 9 (4), 1143–1152.

Malara, A., Abbonante, V., Buduo, C.A.D., Tozzi, L., Currao, M. & Balduini, A. (2015) The secret life of a megakaryocyte: emerging roles in bone marrow homeostasis control. *Cellular and molecular life sciences : CMLS*. 72 (8), 1517–1536.

Marcucci, R., Gori, A.M., Paniccia, R., Giusti, B., Valente, S., Giglioli, C., Buonamici, P., Antoniucci, D., Abbate, R. & Gensini, G.F. (2009) Cardiovascular Death and Nonfatal Myocardial Infarction in Acute Coronary Syndrome Patients Receiving

Coronary Stenting Are Predicted by Residual Platelet Reactivity to ADP Detected by a Point-of-Care Assay. *Circulation*. 119 (2), 237–242.

Marioni, R.E., Shah, S., McRae, A.F., Chen, B.H., Colicino, E., Harris, S.E., Gibson, J., Henders, A.K., Redmond, P., Cox, S.R., Pattie, A., Corley, J., Murphy, L., Martin, N.G., Montgomery, G.W., Feinberg, A.P., Fallin, M.D., Multhaup, M.L., Jaffe, A.E., et al. (2015) DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biology*. 1625.

Martínez, J.A., Milagro, F.I., Claycombe, K.J. & Schalinske, K.L. (2014) Epigenetics in Adipose Tissue, Obesity, Weight Loss, and Diabetes. *Advances in Nutrition: An International Review Journal*. 5 (1), 71–81.

Martínez-González, M.Á., Corella, D., Salas-Salvadó, J., Ros, E., Covas, M.I., Fiol, M., Wärnberg, J., Arós, F., Ruíz-Gutiérrez, V., Lamuela-Raventós, R.M., Lapetra, J., Muñoz, M.Á., Martínez, J.A., Sáez, G., Serra-Majem, L., Pintó, X., Mitjavila, M.T., Tur, J.A., Portillo, M. del P., et al. (2012) Cohort Profile: Design and methods of the PREDIMED study. *International Journal of Epidemiology*. 41 (2), 377–385.

Martinez-Gonzalez, M.A. & Martín-Calvo, N. (2016) Mediterranean diet and life expectancy; beyond olive oil, fruits and vegetables. *Current opinion in clinical nutrition and metabolic care*. 19 (6), 401–407.

Mathers, J.C. & Byun, H.-M. (2016) 'Nutrition, Epigenetics and Aging', in *Nutrition, Epigenetics and Health*. [Online]. Southampton University, UK: Graham Burdge and Kare Lillycrop. pp. 102–132.

Mathers, J.C., Strathdee, G. & Relton, C.L. (2010) Induction of epigenetic alterations by dietary and other environmental factors. *Advances in Genetics*. 713–39.

Matsuda, S., Yasukawa, T., Sakaguchi, Y., Ichiyanagi, K., Unoki, M., Gotoh, K., Fukuda, K., Sasaki, H., Suzuki, T. & Kang, D. (2018) Accurate estimation of 5-methylcytosine in mammalian mitochondrial DNA. *Scientific Reports*. 8.

Maugeri, A., Mazzone, M.G., Giuliano, F., Vinciguerra, M., Basile, G., Barchitta, M. & Agodi, A. (2018) *Curcumin Modulates DNA Methyltransferase Functions in a Cellular Model of Diabetic Retinopathy*. [Online] [online]. Available from: https://www.hindawi.com/journals/omcl/2018/5407482/ (Accessed 4 July 2018).

Mawlood, S.K., Dennany, L., Watson, N., Dempster, J. & Pickard, B.S. (2016) Quantification of global mitochondrial DNA methylation levels and inverse correlation with age at two CpG sites. *Aging*. 8 (4), 636–641.

McClelland, M. & Ivarie, R. (1982) Asymmetrical distribution of CpG in an ‘average’ mammalian gene. *Nucleic Acids Research*. 10 (23), 7865–7877.

McKay, D.L., Eliasziw, M., Chen, C.Y.O. & Blumberg, J.B. (2018) A Pecan-Rich Diet Improves Cardiometabolic Risk Factors in Overweight and Obese Adults: A Randomized Controlled Trial. *Nutrients*. 10 (3), .

McKay, J.A., Waltham, K.J., Williams, E.A. & Mathers, J.C. (2011) Folate depletion during pregnancy and lactation reduces genomic DNA methylation in murine adult offspring. *Genes & Nutrition*. 6 (2), 189–196.

Mechta, M., Ingerslev, L.R., Fabre, O., Picard, M. & Barrès, R. (2017) Evidence Suggesting Absence of Mitochondrial DNA Methylation. *Frontiers in Genetics*. 8.

Meier, T., Gräfe, K., Senn, F., Sur, P., Stangl, G.I., Dawczynski, C., März, W., Kleber, M.E. & Lorkowski, S. (2019) Cardiovascular mortality attributable to dietary risk factors in 51 countries in the WHO European Region from 1990 to 2016: a systematic analysis of the Global Burden of Disease Study. *European Journal of Epidemiology*. 34 (1), 37–55.

Mejia-Ramirez, E. & Florian, M.C. (2020) Understanding intrinsic hematopoietic stem cell aging. *Haematologica*. 105 (1), 22–37.

Melchinger, H., Jain, K., Tyagi, T. & Hwa, J. (2019) Role of Platelet Mitochondria: Life in a Nucleus-Free Zone. *Frontiers in Cardiovascular Medicine*. 6.

Mendelson, M.M., Marioni, R.E., Joehanes, R., Liu, C., Hedman, Å.K., Aslibekyan, S., Demerath, E.W., Guan, W., Zhi, D., Yao, C., Huan, T., Willinger, C., Chen, B., Courchesne, P., Multhaup, M., Irvin, M.R., Cohain, A., Schadt, E.E., Grove, M.L., et al. (2017) Association of Body Mass Index with DNA Methylation and Gene Expression in Blood Cells and Relations to Cardiometabolic Disease: A Mendelian Randomization Approach. *PLOS Medicine*. 14 (1), e1002215.

Méndez-del Villar, M., González-Ortiz, M., Martínez-Abundis, E., Pérez-Rubio, K.G. & Lizárraga-Valdez, R. (2014) Effect of resveratrol administration on metabolic syndrome, insulin sensitivity, and insulin secretion. *Metabolic Syndrome and Related Disorders*. 12 (10), 497–501.

Menga, A., Palmieri, E.M., Cianciulli, A., Infantino, V., Mazzone, M., Scilimati, A., Palmieri, F., Castegna, A. & Iacobazzi, V. (2017) SLC25A26 overexpression impairs cell function via mtDNA hypermethylation and rewiring of methyl metabolism. *The FEBS Journal*. 284 (6), 967–984.

Mentch, S.J., Merhmohamadi, M., Huang, L., Liu, X., Gupta, D., Mattocks, D., Gomez, P., Ables, G., Bamman, M.M., Thalacker-Mercer, A.E., Nichenametla, S. & Locasale, J.W. (2015) Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism. *Cell metabolism*. 22 (5), 861–873.

Mercer, T.R., Neph, S., Dinger, M.E., Crawford, J., Smith, M.A., Shearwood, A.-M.J., Haugen, E., Bracken, C.P., Rackham, O., Stamatoyannopoulos, J.A., Filipovska, A. & Mattick, J.S. (2011) The human mitochondrial transcriptome. *Cell*. 146 (4), 645–658.

Michelson, A.D. (2004) Platelet Function Testing in Cardiovascular Diseases. *Circulation*. 110 (19), e489–e493.

Milagro, F.I., Campión, J., Cordero, P., Goyenechea, E., Gómez-Uriz, A.M., Abete, I., Zulet, M.A. & Martínez, J.A. (2011) A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. *The FASEB Journal*. 25 (4), 1378–1389.

Milenkovic, D., Vanden Berghe, W., Boby, C., Leroux, C., Declerck, K., Szarc vel Szic, K., Heyninck, K., Laukens, K., Bizet, M., Defrance, M., Dedeurwaerder, S., Calonne, E., Fuks, F., Haegeman, G., Haenen, G.R.M.M., Bast, A. & Weseler, A.R. (2014) Dietary flavanols modulate the transcription of genes associated with cardiovascular pathology without changes in their DNA methylation state. *PloS One*. 9 (4), e95527.

Mirza, S., Sharma, G., Parshad, R., Gupta, S.D., Pandya, P. & Ralhan, R. (2013) Expression of DNA methyltransferases in breast cancer patients and to analyze the effect of natural compounds on DNA methyltransferases and associated proteins. *Journal of Breast Cancer*. 16 (1), 23–31.

Mishra, M. & Kowluru, R.A. (2015a) Epigenetic Modification of Mitochondrial DNA in the Development of Diabetic Retinopathy. *Investigative Ophthalmology & Visual Science*. 56 (9), 5133–5142.

Mishra, M. & Kowluru, R.A. (2015b) Epigenetic Modification of Mitochondrial DNA in the Development of Diabetic Retinopathy. *Investigative Ophthalmology & Visual Science*. 56 (9), 5133–5142.

Monasta, L., Abbafati, C., Logroscino, G., Remuzzi, G., Perico, N., Bikbov, B., Tamburlini, G., Beghi, E., Traini, E., Redford, S.B., Ariani, F., Borzì, A.M., Bosetti, C., Carreras, G., Caso, V., Castelpietra, G., Cirillo, M., Conti, S., Cortesi, P.A., et al. (2019) Italy's health performance, 1990–2017: findings from the Global Burden of Disease Study 2017. *The Lancet Public Health*. 4 (12), e645–e657.

Moore, L.D., Le, T. & Fan, G. (2013) DNA methylation and its basic function. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*. 38 (1), 23–38.

Morange, P.-E. & Alessi, M.-C. (2013) Thrombosis in central obesity and metabolic syndrome: mechanisms and epidemiology. *Thrombosis and Haemostasis*. 110 (4), 669–680.

Morris, M.J., Hesson, L.B. & Youngson, N.A. (2020) Non-CpG methylation biases bisulphite PCR towards low or unmethylated mitochondrial DNA: recommendations for the field. *Environmental Epigenetics*. 6 (1), .

Mosawy, S., Jackson, D.E., Woodman, O.L. & Linden, M.D. (2013) Inhibition of platelet-mediated arterial thrombosis and platelet granule exocytosis by 3',4'-dihydroxyflavonol and quercetin. *Platelets*. 24 (8), 594–604.

Mozaffarian, D. (2016) Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity. *Circulation*. 133 (2), 187–225.

Mposhi, A., Van der Wijst, M.G., Faber, K.N. & Rots, M.G. (2017) Regulation of mitochondrial gene expression, the epigenetic enigma. *Frontiers in Bioscience (Landmark Edition)*. 221099–1113.

Must, A., Spadano, J., Coakley, E.H., Field, A.E., Colditz, G. & Dietz, W.H. (1999) The disease burden associated with overweight and obesity. *JAMA*. 282 (16), 1523–1529.

Naghavi, M., Abajobir, A.A., Abbafati, C., Abbas, K.M., Abd-Allah, F., Abera, S.F., Aboyans, V., Adetokunboh, O., Afshin, A., Agrawal, A., Ahmadi, A., Ahmed, M.B., Aichour, A.N., Aichour, M.T.E., Aichour, I., Aiyar, S., Alahdab, F., Al-Aly, Z., Alam, K., et al. (2017) Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*. 390 (10100), 1151–1210.

Nansseu, J.R.N. & Noubiap, J.J.N. (2015) Aspirin for primary prevention of cardiovascular disease. *Thrombosis Journal*. 13 (1), 38.

Naoi, M., Wu, Y., Shamoto-Nagai, M. & Maruyama, W. (2019) Mitochondria in Neuroprotection by Phytochemicals: Bioactive Polyphenols Modulate Mitochondrial Apoptosis System, Function and Structure. *International Journal of Molecular Sciences*. 20 (10), .

Nass, M.M. (1973) Differential methylation of mitochondrial and nuclear DNA in cultured mouse, hamster and virus-transformed hamster cells. In vivo and in vitro methylation. *Journal of Molecular Biology*. 80 (1), 155–175.

Naveiras, O., Nardi, V., Wenzel, P.L., Fahey, F. & Daley, G.Q. (2009) Bone marrow adipocytes as negative regulators of the hematopoietic microenvironment. *Nature*. 460 (7252), 259–263.

NCBI (2019) *The Genetic codes*. [Online] [online]. Available from: https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?#SG2 (Accessed 18 August 2020).

NCD-RisC, N.R.F.C. (2016) Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *The Lancet*. 387 (10026), 1377–1396.

NCMP (2018) *National Child Measurement Programme, England - 2017/18 School Year [PAS]*. [Online] [online]. Available from: https://digital.nhs.uk/data-and-information/publications/statistical/national-child-measurement-programme/2017-18-school-year (Accessed 28 May 2019).

Neeland, I.J., Poirier, P. & Després, J.-P. (2018) Cardiovascular and Metabolic Heterogeneity of Obesity. *Circulation*. 137 (13), 1391–1406.

Nelson, K.M., Dahlin, J.L., Bisson, J., Graham, J., Pauli, G.F. & Walters, M.A. (2017) The Essential Medicinal Chemistry of Curcumin. *Journal of Medicinal Chemistry*. 60 (5), 1620–1637.

Nevalainen, T., Kananen, L., Marttila, S., Jylhävä, J., Mononen, N., Kähönen, M., Raitakari, O.T., Hervonen, A., Jylhä, M., Lehtimäki, T. & Hurme, M. (2017) Obesity accelerates epigenetic aging in middle-aged but not in elderly individuals. *Clinical Epigenetics*. 920.

Nicholls, T.J. & Minczuk, M. (2014) In D-loop: 40years of mitochondrial 7S DNA. *Experimental Gerontology*. 56175–181.

Nicholls, T.J., Nadalutti, C.A., Motori, E., Sommerville, E.W., Gorman, G.S., Basu, S., Hoberg, E., Turnbull, D.M., Chinnery, P.F., Larsson, N.-G., Larsson, E., Falkenberg, M., Taylor, R.W., Griffith, J.D. & Gustafsson, C.M. (2018) Topoisomerase 3α Is Required for Decatenation and Segregation of Human mtDNA. *Molecular Cell*. 69 (1), 9-23.e6.

Nooteboom, M., Johnson, R., Taylor, R.W., Wright, N.A., Lightowlers, R.N., Kirkwood, T.B.L., Mathers, J.C., Turnbull, D.M. & Greaves, L.C. (2010) Age-associated mitochondrial DNA mutations lead to small but significant changes in cell proliferation and apoptosis in human colonic crypts. *Aging Cell*. 9 (1), 96–99.

North, B.J. & Sinclair, D.A. (2012) The Intersection Between Aging and Cardiovascular Disease. *Circulation Research*. 110 (8), 1097–1108.

Novotny, J.A., Baer, D.J., Khoo, C., Gebauer, S.K. & Charron, C.S. (2015) Cranberry juice consumption lowers markers of cardiometabolic risk, including blood pressure and circulating C-reactive protein, triglyceride, and glucose concentrations in adults. *The Journal of Nutrition*. 145 (6), 1185–1193.

Obeid, R. (2013) The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. *Nutrients*. 5 (9), 3481–3495.

Ogura, M., Morishima, Y., Ohno, R., Kato, Y., Hirabayashi, N., Nagura, H. & Saito, H. (1985) Establishment of a novel human megakaryoblastic leukemia cell line, MEG-01, with positive Philadelphia chromosome. *Blood*. 66 (6), 1384–1392.

Okada, A.K., Teranishi, K., Lobo, F., Isas, J.M., Xiao, J., Yen, K., Cohen, P. & Langen, R. (2017) The Mitochondrial-Derived Peptides, HumaninS14G and Small Humanin-like Peptide 2, Exhibit Chaperone-like Activity. *Scientific Reports*. 7 (1), 7802.

Ormazabal, A., Casado, M., Molero-Luis, M., Montoya, J., Rahman, S., Aylett, S.-B., Hargreaves, I., Heales, S. & Artuch, R. (2015) Can folic acid have a role in mitochondrial disorders? *Drug Discovery Today*. 20 (11), 1349–1354.

Osellame, L.D., Blacker, T.S. & Duchen, M.R. (2012) Cellular and molecular mechanisms of mitochondrial function. *Best Practice & Research. Clinical Endocrinology & Metabolism*. 26 (6), 711–723.

Owa, C., Poulin, M., Yan, L. & Shioda, T. (2018) Technical adequacy of bisulfite sequencing and pyrosequencing for detection of mitochondrial DNA methylation: Sources and avoidance of false-positive detection. *PLOS ONE*. 13 (2), e0192722.

Palmfeldt, J. & Bross, P. (2017) Proteomics of human mitochondria. *Mitochondrion*. 332–14.

Pandey, K.B. & Rizvi, S.I. (2009a) Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*. 2 (5), 270–278.

Pandey, K.B. & Rizvi, S.I. (2009b) Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*. 2 (5), 270–278.

Papadaki, A., Johnson, L., Toumpakari, Z., England, C., Rai, M., Toms, S., Penfold, C., Zazpe, I., Martínez-González, M.A. & Feder, G. (2018) Validation of the English

Version of the 14-Item Mediterranean Diet Adherence Screener of the PREDIMED Study, in People at High Cardiovascular Risk in the UK. *Nutrients*. 10 (2), .

Patel, S.R., Hartwig, J.H. & Italiano, J.E. (2005) The biogenesis of platelets from megakaryocyte proplatelets. *Journal of Clinical Investigation*. 115 (12), 3348–3354.

Patil, V., Cuenin, C., Chung, F., Aguilera, J.R.R., Fernandez-Jimenez, N., Romero-Garmendia, I., Bilbao, J.R., Cahais, V., Rothwell, J. & Herceg, Z. (2019) Human mitochondrial DNA is extensively methylated in a non-CpG context. *Nucleic Acids Research*. 47 (19), 10072–10085.

Patti, M.-E. & Kahn, B.B. (2004) Nutrient sensor links obesity with diabetes risk. *Nature Medicine*. 10 (10), 1049–1050.

Pawlaczyk, I., Czerchawski, L., Kuliczkowski, W., Karolko, B., Pilecki, W., Witkiewicz, W. & Gancarz, R. (2011) Anticoagulant and anti-platelet activity of polyphenolic-polysaccharide preparation isolated from the medicinal plant Erigeron canadensis L. *Thrombosis Research*. 127 (4), 328–340.

Perk, J., De Backer, G., Gohlke, H., Graham, I., Reiner, Z., Verschuren, M., Albus, C., Benlian, P., Boysen, G., Cifkova, R., Deaton, C., Ebrahim, S., Fisher, M., Germano, G., Hobbs, R., Hoes, A., Karadeniz, S., Mezzani, A., Prescott, E., et al. (2012) European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). *European Heart Journal*. 33 (13), 1635–1701.

Perna, L., Zhang, Y., Mons, U., Holleczek, B., Saum, K.-U. & Brenner, H. (2016) Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clinical Epigenetics*. 864.

Pfanner, N., Warscheid, B. & Wiedemann, N. (2019) Mitochondrial proteins: from biogenesis to functional networks. *Nature Reviews Molecular Cell Biology*. 20 (5), 267–284.

Pichaud, N., Bérubé, R., Côté, G., Belzile, C., Dufresne, F., Morrow, G., Tanguay, R.M., Rand, D.M. & Blier, P.U. (2019) Age Dependent Dysfunction of Mitochondrial and ROS Metabolism Induced by Mitonuclear Mismatch. *Frontiers in Genetics*. 10.

Pirola, C.J., Gianotti, T.F., Burgueño, A.L., Rey-Funes, M., Loidl, C.F., Mallardi, P., Martino, J.S., Castaño, G.O. & Sookoian, S. (2013) Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut*. 62 (9), 1356–1363.

Podlesniy, P., Puigròs, M., Serra, N., Fernández-Santiago, R., Ezquerra, M., Tolosa, E. & Trullas, R. (2019) Accumulation of mitochondrial 7S DNA in idiopathic and LRRK2 associated Parkinson's disease. *EBioMedicine*. 48554–567.

Poirier, P., Giles, T.D., Bray, G.A., Hong, Y., Stern, J.S., Pi-Sunyer, F.X. & Eckel, R.H. (2006) Obesity and Cardiovascular Disease: Pathophysiology, Evaluation, and Effect of Weight Loss. *Circulation*. 113 (6), 898–918.

Ponterio, E. & Gnessi, L. (2015) Adenovirus 36 and Obesity: An Overview. *Viruses*. 7 (7), 3719–3740.

Preis, S.R., Pencina, M.J., D'Agostino, R.B., Meigs, J.B., Vasan, R.S. & Fox, C.S. (2013) Neck Circumference and the Development of Cardiovascular Disease Risk Factors in the Framingham Heart Study. *Diabetes Care*. 36 (1), e3–e3.

Priyadarsini, K.I. (2014) The Chemistry of Curcumin: From Extraction to Therapeutic Agent. *Molecules*. 19 (12), 20091–20112.

Prospective Studies Collaboration (2009) Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *The Lancet*. 373 (9669), 1083–1096.

Pufulete, M., Al-Ghnaniem, R., Khushal, A., Appleby, P., Harris, N., Gout, S., Emery, P.W. & Sanders, T. a. B. (2005) Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. *Gut*. 54 (5), 648–653.

Qi, L. & Cho, Y.A. (2008) Gene-environment interaction and obesity. *Nutrition reviews*. 66 (12), 684–694.

Quach, A., Levine, M.E., Tanaka, T., Lu, A.T., Chen, B.H., Ferrucci, L., Ritz, B., Bandinelli, S., Neuhouser, M.L., Beasley, J.M., Snetselaar, L., Wallace, R.B., Tsao, P.S., Absher, D., Assimes, T.L., Stewart, J.D., Li, Y., Hou, L., Baccarelli, A.A., et al. (2017) Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. *Aging (Albany NY)*. 9 (2), 419–437.

Rangel-Huerta, O.D., Pastor-Villaescusa, B., Aguilera, C.M. & Gil, A. (2015) A Systematic Review of the Efficacy of Bioactive Compounds in Cardiovascular Disease: Phenolic Compounds. *Nutrients*. 7 (7), 5177–5216.

Reaven, G.M. (2011) Insulin Resistance: the Link Between Obesity and Cardiovascular Disease. *Medical Clinics of North America*. 95 (5), 875–892.

Reczek, C.R. & Chandel, N.S. (2015) ROS-dependent signal transduction. *Current Opinion in Cell Biology*. 338–13.

Ricci, M.A., Manzo, C., García-Parajo, M.F., Lakadamyali, M. & Cosma, M.P. (2015) Chromatin fibers are formed by heterogeneous groups of nucleosomes in vivo. *Cell*. 160 (6), 1145–1158.

Ridker, P.M., Cannon, C.P., Morrow, D., Rifai, N., Rose, L.M., McCabe, C.H., Pfeffer, M.A., Braunwald, E., & Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI 22) Investigators (2005) C-reactive protein levels and outcomes after statin therapy. *The New England Journal of Medicine*. 352 (1), 20–28.

Ro, S., Ma, H.-Y., Park, C., Ortogero, N., Song, R., Hennig, G.W., Zheng, H., Lin, Y.-M., Moro, L., Hsieh, J.-T. & Yan, W. (2013) The mitochondrial genome encodes abundant small noncoding RNAs. *Cell Research*. 23 (6), 759–774.

Roetker Nicholas S., Pankow James S., Bressler Jan, Morrison Alanna C., & Boerwinkle Eric (2018) Prospective Study of Epigenetic Age Acceleration and Incidence of Cardiovascular Disease Outcomes in the ARIC Study (Atherosclerosis Risk in Communities). *Circulation: Genomic and Precision Medicine*. 11 (3), e001937.

Rönn, T., Poulsen, P., Hansson, O., Holmkvist, J., Almgren, P., Nilsson, P., Tuomi, T., Isomaa, B., Groop, L., Vaag, A. & Ling, C. (2008) Age influences DNA methylation and gene expression of COX7A1 in human skeletal muscle. *Diabetologia*. 51 (7), 1159–1168.

Rönn, T., Volkov, P., Gillberg, L., Kokosar, M., Perfilyev, A., Jacobsen, A.L., Jørgensen, S.W., Brøns, C., Jansson, P.-A., Eriksson, K.-F., Pedersen, O., Hansen, T., Groop, L., Stener-Victorin, E., Vaag, A., Nilsson, E. & Ling, C. (2015) Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. *Human Molecular Genetics*. 24 (13), 3792–3813.

Rosa-Garrido, M., Chapski, D.J. & Vondriska, T.M. (2018) Epigenomes in Cardiovascular Disease. *Circulation Research*. 122 (11), 1586–1607.

Rothwell, P.M., Cook, N.R., Gaziano, J.M., Price, J.F., Belch, J.F.F., Roncaglioni, M.C., Morimoto, T. & Mehta, Z. (2018) Effects of aspirin on risks of vascular events and cancer according to bodyweight and dose: analysis of individual patient data from randomised trials. *The Lancet*. 392 (10145), 387–399.

Royo, J.L., Hidalgo, M. & Ruiz, A. (2007) Pyrosequencing protocol using a universal biotinylated primer for mutation detection and SNP genotyping. *Nature Protocols*. 2 (7), 1734–1739.

Sae-Lee, C., Corsi, S., Barrow, T.M., Kuhnle, G.G.C., Bollati, V., Mathers, J.C. & Byun, H.-M. (2018) Dietary Intervention Modifies DNA Methylation Age Assessed by the Epigenetic Clock. *Molecular Nutrition & Food Research*. 62 (23), 1800092.

Saini, S.K., Mangalhara, K.C., Prakasam, G. & Bamezai, R.N.K. (2017) DNA Methyltransferase1 (DNMT1) Isoform3 methylates mitochondrial genome and modulates its biology. *Scientific Reports*. 7 (1), 1525.

Salbaum, J.M. & Kappen, C. (2012) Genetic and Epigenomic Footprints of Folate. *Progress in molecular biology and translational science*. 108129–158.

Samad, F. & Ruf, W. (2013) Inflammation, obesity, and thrombosis. *Blood*. 122 (20), 3415–3422.

Sandoval-Acuña, C., Ferreira, J. & Speisky, H. (2014) Polyphenols and mitochondria: An update on their increasingly emerging ROS-scavenging independent actions. *Archives of Biochemistry and Biophysics*. 55975–90.

Santhakumar, A.B., Kundur, A.R., Fanning, K., Netzel, M., Stanley, R. & Singh, I. (2015) Consumption of anthocyanin-rich Queen Garnet plum juice reduces platelet activation related thrombogenesis in healthy volunteers. *Journal of Functional Foods*. 1211–22.

Santilli, F., Vazzana, N., Liani, R., Guagnano, M.T. & Davì, G. (2012) Platelet activation in obesity and metabolic syndrome. *Obesity Reviews: An Official Journal of the International Association for the Study of Obesity*. 13 (1), 27–42.

Satoh, M. & Kuroiwa, T. (1991) Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. *Experimental Cell Research*. 196 (1), 137–140.

Saxena, P., Prakash, A. & Nigam, A. (2011) Efficacy of 2-hour post glucose insulin levels in predicting insulin resistance in polycystic ovarian syndrome with infertility. *Journal of Human Reproductive Sciences*. 4 (1), 20–22.

Scholes, S., Mindell, J. & Neave, A. (2018) *HSE17-CVD*. LONDON: NATCEN SOCIAL RESEARCH.

Schröder, H., Fitó, M., Estruch, R., Martínez-González, M.A., Corella, D., Salas-Salvadó, J., Lamuela-Raventós, R., Ros, E., Salaverría, I., Fiol, M., Lapetra, J., Vinyoles, E., Gómez-Gracia, E., Lahoz, C., Serra-Majem, L., Pintó, X., Ruiz-Gutierrez, V. & Covas, M.-I. (2011) A Short Screener Is Valid for Assessing Mediterranean Diet Adherence among Older Spanish Men and Women. *The Journal of Nutrition*. 141 (6), 1140–1145.

Seo, A.Y., Joseph, A.-M., Dutta, D., Hwang, J.C.Y., Aris, J.P. & Leeuwenburgh, C. (2010) New insights into the role of mitochondria in aging: mitochondrial dynamics and more. *Journal of Cell Science*. 123 (Pt 15), 2533–2542.

Seo, M.-J., Lee, Y.-J., Hwang, J.-H., Kim, K.-J. & Lee, B.-Y. (2015) The inhibitory effects of quercetin on obesity and obesity-induced inflammation by regulation of MAPK signaling. *The Journal of Nutritional Biochemistry*. 26 (11), 1308–1316.

Serban, M.-C., Sahebkar, A., Zanchetti, A., Mikhailidis, D.P., Howard, G., Antal, D., Andrica, F., Ahmed, A., Aronow, W.S., Muntner, P., Lip, G.Y.H., Graham, I., Wong, N., Rysz, J., Banach, M., & Lipid and Blood Pressure Meta-analysis Collaboration (LBPMC) Group (2016) Effects of Quercetin on Blood Pressure: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Journal of the American Heart Association*. 5 (7), .

Shaughnessy, D.T., McAllister, K., Worth, L., Haugen, A.C., Meyer, J.N., Domann, F.E., Houten, B.V., Mostoslavsky, R., Bultman, S.J., Baccarelli, A.A., Begley, T.J., Sobol, R.W. & Hirschey, M.D. (2014) Mitochondria, Energetics, Epigenetics, and Cellular Responses to Stress. *Environmental Health Perspectives (Online); Research Triangle Park.* 122 (12), .

Shen, M.Y., Hsiao, G., Liu, C.L., Fong, T.H., Lin, K.H., Chou, D.S. & Sheu, J.R. (2007) Inhibitory mechanisms of resveratrol in platelet activation: pivotal roles of p38 MAPK and NO/cyclic GMP. *British Journal of Haematology*. 139 (3), 475–485.

Shmookler Reis, R.J. & Goldstein, S. (1983) Mitochondrial DNA in mortal and immortal human cells. Genome number, integrity, and methylation. *The Journal of Biological Chemistry*. 258 (15), 9078–9085.

Shock, L.S., Thakkar, P.V., Peterson, E.J., Moran, R.G. & Taylor, S.M. (2011) DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in

mammalian mitochondria. *Proceedings of the National Academy of Sciences of the United States of America*. 108 (9), 3630–3635.

Song, S., Pursell, Z.F., Copeland, W.C., Longley, M.J., Kunkel, T.A. & Mathews, C.K. (2005) DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. *Proceedings of the National Academy of Sciences of the United States of America*. 102 (14), 4990–4995.

Springer, M. & Moco, S. (2019) Resveratrol and Its Human Metabolites—Effects on Metabolic Health and Obesity. *Nutrients*. 11 (1), .

Stanaway, J.D., Afshin, A., Gakidou, E., Lim, S.S., Abate, D., Abate, K.H., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R.S., Abebe, M., Abebe, Z., Abera, S.F., Abil, O.Z., Abraha, H.N., et al. (2018) Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*. 392 (10159), 1923–1994.

Stewart, J.B. & Chinnery, P.F. (2015) The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nature Reviews. Genetics*. 16 (9), 530–542.

Stoner, L., Lucero, A.A., Palmer, B.R., Jones, L.M., Young, J.M. & Faulkner, J. (2013) Inflammatory biomarkers for predicting cardiovascular disease. *Clinical Biochemistry*. 46 (15), 1353–1371.

Subramaniam, D., Thombre, R., Dhar, A. & Anant, S. (2014) DNA Methyltransferases: A Novel Target for Prevention and Therapy. *Frontiers in Oncology*. 4.

Sundaram, M.K., Hussain, A., Haque, S., Raina, R. & Afroze, N. (2019) Quercetin modifies 5′CpG promoter methylation and reactivates various tumor suppressor genes by modulating epigenetic marks in human cervical cancer cells. *Journal of Cellular Biochemistry*. 120 (10), 18357–18369.

Sureda, A., Bibiloni, M. del M., Julibert, A., Bouzas, C., Argelich, E., Llompart, I., Pons, A. & Tur, J.A. (2018) Adherence to the Mediterranean Diet and Inflammatory Markers. *Nutrients*. 10 (1), .

Tamminen, M., Lassila, R., Westerbacka, J., Vehkavaara, S. & Yki-Järvinen, H. (2003) Obesity is associated with impaired platelet-inhibitory effect of acetylsalicylic acid in nondiabetic subjects. *International Journal of Obesity*. 27 (8), 907–911.

Tangney, C. & Rasmussen, H.E. (2013) Polyphenols, Inflammation, and Cardiovascular Disease. *Current atherosclerosis reports*. 15 (5), 324.

Thomas, M.R. & Storey, R.F. (2015) The role of platelets in inflammation. *Thrombosis and Haemostasis*. 114 (3), 449–458.

Tong, H., Zhang, L., Gao, J., Wen, S., Zhou, H. & Feng, S. (2017) Methylation of mitochondrial DNA displacement loop region regulates mitochondrial copy number in colorectal cancer. *Molecular Medicine Reports*. 16 (4), 5347–5353.

Tost, J. & Gut, I.G. (2007) DNA methylation analysis by pyrosequencing. *Nature Protocols*. 2 (9), 2265–2275.

Tremblay, B.L., Guénard, F., Rudkowska, I., Lemieux, S., Couture, P. & Vohl, M.-C. (2017) Epigenetic changes in blood leukocytes following an omega-3 fatty acid supplementation. *Clinical Epigenetics*. 9 (1), 43.

Trichopoulou, A., Martínez-González, M.A., Tong, T.Y., Forouhi, N.G., Khandelwal, S., Prabhakaran, D., Mozaffarian, D. & de Lorgeril, M. (2014) Definitions and potential health benefits of the Mediterranean diet: views from experts around the world. *BMC Medicine*. 12.

Uhler, J.P. & Falkenberg, M. (2015) Primer removal during mammalian mitochondrial DNA replication. *DNA repair*. 3428–38.

The UniProt Consortium (2017) UniProt: the universal protein knowledgebase. *Nucleic Acids Research*. 45 (D1), D158–D169.

Upadhyay, R.K. (2015) Emerging risk biomarkers in cardiovascular diseases and disorders. *Journal of Lipids*. 2015971453.

Valeri, L. & VanderWeele, T.J. (2013) Mediation analysis allowing for exposure-mediator interactions and causal interpretation: theoretical assumptions and implementation with SAS and SPSS macros. *Psychological methods*. 18 (2), 137–150.

Vallance, T.M., Ravishankar, D., Albadawi, D.A.I., Osborn, H.M.I. & Vaiyapuri, S. (2019) Synthetic Flavonoids as Novel Modulators of Platelet Function and Thrombosis. *International Journal of Molecular Sciences*. 20 (12), .

Van Dijk, S.J., Tellam, R.L., Morrison, J.L., Muhlhausler, B.S. & Molloy, P.L. (2015) Recent developments on the role of epigenetics in obesity and metabolic disease. *Clinical Epigenetics*. 766.

Van Gaal, L.F., Mertens, I.L. & De Block, C.E. (2006) Mechanisms linking obesity with cardiovascular disease. *Nature*. 444 (7121), 875–880.

Vanyushin, B.F., Kiryanov, G.I., Kudryashova, I.B. & Belozersky, A.N. (1971) DNA-methylase in loach embryos (Misgurnus fossilis). *FEBS letters*. 15 (4), 313–316.

Vazhappilly, C.G., Ansari, S.A., Al-Jaleeli, R., Al-Azawi, A.M., Ramadan, W.S., Menon, V., Hodeify, R., Siddiqui, S.S., Merheb, M., Matar, R. & Radhakrishnan, R. (2019) Role of flavonoids in thrombotic, cardiovascular, and inflammatory diseases. *Inflammopharmacology*. 27 (5), 863–869.

Veglia, F., Baldassarre, D., de Faire, U., Kurl, S., Smit, A.J., Rauramaa, R., Giral, P., Amato, M., Di Minno, A., Ravani, A., Frigerio, B., Castelnuovo, S., Sansaro, D., Coggi, D., Bonomi, A., Tedesco, C.C., Mannarino, E., Humphries, S.E., Hamsten, A., et al. (2019) A priori-defined Mediterranean-like dietary pattern predicts cardiovascular events better in north Europe than in Mediterranean countries. *International Journal of Cardiology*. 28288–92.

Verdoia, M., Pergolini, P., Rolla, R., Nardin, M., Schaffer, A., Barbieri, L., Marino, P., Bellomo, G., Suryapranata, H. & De Luca, G. (2016) Advanced age and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. *Journal of thrombosis and haemostasis: JTH*. 14 (1), 57–64.

Vilahur, G., Ben-Aicha, S. & Badimon, L. (2017) New insights into the role of adipose tissue in thrombosis. *Cardiovascular Research*. 113 (9), 1046–1054.

Violi, F., Pastori Daniele, Pignatelli Pasquale, & Carnevale Roberto (2020) Nutrition, Thrombosis, and Cardiovascular Disease. *Circulation Research*. 126 (10), 1415–1442.

Vita, J.A. (2005) Polyphenols and cardiovascular disease: effects on endothelial and platelet function. *The American Journal of Clinical Nutrition*. 81 (1), 292S-297S.

Voelter-Mahlknecht, S. (2016) Epigenetic associations in relation to cardiovascular prevention and therapeutics. *Clinical Epigenetics*. 84.

Vos, S., Nawrot, T.S., Martens, D.S., Byun, H.-M. & Janssen, B.G. (2020) Mitochondrial DNA methylation in placental tissue: a proof of concept study by means of prenatal environmental stressors. *Epigenetics*. 0 (0), 1–11.

Wahl, S., Drong, A., Lehne, B., Loh, M., Scott, W.R., Kunze, S., Tsai, P.-C., Ried, J.S., Zhang, W., Yang, Y., Tan, S., Fiorito, G., Franke, L., Guarrera, S., Kasela, S., Kriebel, J., Richmond, R.C., Adamo, M., Afzal, U., et al. (2017) Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. *Nature*. 541 (7635), 81–86.

Wallace, D.C. & Chalkia, D. (2013) Mitochondrial DNA Genetics and the Heteroplasmy Conundrum in Evolution and Disease. *Cold Spring Harbor Perspectives in Biology*. 5 (11), .

Wallace, R.G., Twomey, L.C., Custaud, M.-A., Moyna, N., Cummins, P.M., Mangone, M. & Murphy, R.P. (2016) Potential Diagnostic and Prognostic Biomarkers of Epigenetic Drift within the Cardiovascular Compartment. *BioMed Research International*. 2016.

Wang, H., Leng, Y. & Gong, Y. (2018) Bone Marrow Fat and Hematopoiesis. *Frontiers in Endocrinology*. 9.

Wang, J., Tan, G.-J., Han, L.-N., Bai, Y.-Y., He, M. & Liu, H.-B. (2017) Novel biomarkers for cardiovascular risk prediction. *Journal of geriatric cardiology: JGC*. 14 (2), 135–150.

Wang, J.G., Staessen, J.A., Fagard, R.H., Birkenhäger, W.H., Gong, L. & Liu, L. (2001) Prognostic significance of serum creatinine and uric acid in older Chinese patients with isolated systolic hypertension. *Hypertension (Dallas, Tex.: 1979)*. 37 (4), 1069–1074.

Wang, L., Wu, Q., Fan, Z., Xie, R., Wang, Z. & Lu, Y. (2017) Platelet mitochondrial dysfunction and the correlation with human diseases. *Biochemical Society Transactions*. 45 (6), 1213–1223.

Wang, Z., Cai, F., Chen, X., Luo, M., Hu, L. & Lu, Y. (2013) The Role of Mitochondria-Derived Reactive Oxygen Species in Hyperthermia-Induced Platelet Apoptosis. *PLoS ONE*. 8 (9), .

Wang, Z. & Nakayama, T. (2010) Inflammation, a Link between Obesity and Cardiovascular Disease. *Mediators of Inflammation*. 2010e535918.

Waseem, M. & Parvez, S. (2016) Neuroprotective activities of curcumin and quercetin with potential relevance to mitochondrial dysfunction induced by oxaliplatin. *Protoplasma*. 253 (2), 417–430.

Westerman, K., Fernández-Sanlés Alba, Patil Prasad, Sebastiani Paola, Jacques Paul, Starr John M., J. Deary Ian, Liu Qing, Liu Simin, Elosua Roberto, DeMeo Dawn L., & Ordovás José M. (2020) Epigenomic Assessment of Cardiovascular Disease Risk and Interactions With Traditional Risk Metrics. *Journal of the American Heart Association*. 9 (8), e015299.

Westerman, K.E. & Ordovás, J.M. (2020) DNA methylation and incident cardiovascular disease. *Current Opinion in Clinical Nutrition and Metabolic Care*. 23 (4), 236–240.

WHO (2014) *Global status report on noncommunicable diseases 2014*.

WHO (2007) *ICD-9-CM*. [Online] [online]. Available from: https://www.cdc.gov/nchs/icd/icd9cm.htm (Accessed 12 February 2019).

Widmer, R.J., Freund, M.A., Flammer, A.J., Sexton, J., Lennon, R., Romani, A., Mulinacci, N., Vinceri, F.F., Lerman, L.O. & Lerman, A. (2013) Beneficial effects of polyphenol-rich olive oil in patients with early atherosclerosis. *European Journal of Nutrition*. 52 (3), 1223–1231.

Wiesner, R.J., Rüegg, J.C. & Morano, I. (1992) Counting target molecules by exponential polymerase chain reaction: Copy number of mitochondrial DNA in rat tissues. *Biochemical and Biophysical Research Communications*. 183 (2), 553–559.

Wijst, M.G.P. van der, Tilburg, A.Y. van, Ruiters, M.H.J. & Rots, M.G. (2017) Experimental mitochondria-targeted DNA methylation identifies GpC methylation, not CpG methylation, as potential regulator of mitochondrial gene expression. *Scientific Reports*. 7 (1), 177.

Willoughby, S., Holmes, A. & Loscalzo, J. (2002) Platelets and Cardiovascular Disease. *European Journal of Cardiovascular Nursing*. 1 (4), 273–288.

Wolters, J.E.J., van Breda, S.G.J., Caiment, F., Claessen, S.M., de Kok, T.M.C.M. & Kleinjans, J.C.S. (2017) Nuclear and Mitochondrial DNA Methylation Patterns Induced by Valproic Acid in Human Hepatocytes. *Chemical Research in Toxicology*. 30 (10), 1847–1854.

Wood dos Santos, T., Cristina Pereira, Q., Teixeira, L., Gambero, A., A. Villena, J. & Lima Ribeiro, M. (2018) Effects of Polyphenols on Thermogenesis and Mitochondrial Biogenesis. *International Journal of Molecular Sciences*. 19 (9), .

World Health Organization (2016) *WHO | Cardiovascular diseases (CVDs). Fact Sheet.* [Online] [online]. Available from: http://www.who.int/mediacentre/factsheets/fs317/en/ (Accessed 29 April 2017).

Wright, S.M. & Aronne, L.J. (2012) Causes of obesity. *Abdominal Radiology*. 37 (5), 730–732.

Wyss-Coray, T. (2016) Ageing, neurodegeneration and brain rejuvenation. *Nature*. 539 (7628), 180–186.

Xia, N., Daiber, A., Förstermann, U. & Li, H. (2017) Antioxidant effects of resveratrol in the cardiovascular system. *British Journal of Pharmacology*. 174 (12), 1633–1646.

Xu, Y., Li, H., Hedmer, M., Hossain, M.B., Tinnerberg, H., Broberg, K. & Albin, M. (2017) Occupational exposure to particles and mitochondrial DNA - relevance for blood pressure. *Environmental Health*. 16.

Yamazaki, M., Munetsuna, E., Yamada, H., Ando, Y., Mizuno, G., Murase, Y., Kondo, K., Ishikawa, H., Teradaira, R., Suzuki, K. & Ohashi, K. (2016) Fructose consumption induces hypomethylation of hepatic mitochondrial DNA in rats. *Life Sciences*. 149146–152.

Yang, Y., Wang, X., Zhang, L., An, H. & Zao, Z. (2011) Inhibitory effects of resveratrol on platelet activation induced by thromboxane a(2) receptor agonist in human platelets. *The American Journal of Chinese Medicine*. 39 (1), 145–159.

Yu, E., Mercer, J. & Bennett, M. (2012) Mitochondria in vascular disease. *Cardiovascular Research*. 95 (2), 173–182.

Yusuf, S., Hawken, S., Ôunpuu, S., Bautista, L., Franzosi, M.G., Commerford, P., Lang, C.C., Rumboldt, Z., Onen, C.L., Lisheng, L., Tanomsup, S., Wangai, P., Razak, F., Sharma, A.M. & Anand, S.S. (2005) Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study. *The Lancet*. 366 (9497), 1640–1649.

Yu-Wai-Man, P., Lai-Cheong, J., Borthwick, G.M., He, L., Taylor, G.A., Greaves, L.C., Taylor, R.W., Griffiths, P.G. & Turnbull, D.M. (2010) Somatic Mitochondrial DNA Deletions Accumulate to High Levels in Aging Human Extraocular Muscles. *Investigative Ophthalmology & Visual Science*. 51 (7), 3347–3353.

Zaimi, I., Pei, D., Koestler, D.C., Marsit, C.J., Vivo, I.D., Tworoger, S.S., Shields, A.E., Kelsey, K.T. & Michaud, D.S. (2018) Variation in DNA methylation of human blood over a 1-year period using the Illumina MethylationEPIC array. *Epigenetics*. 13 (10–11), 1056–1071.

Zamora-Mendoza, R., Rosas-Vargas, H., Ramos-Cervantes, M.T., Garcia-Zuniga, P., Perez-Lorenzana, H., Mendoza-Lorenzo, P., Perez-Ortiz, A.C., Estrada-Mena, F.J., Miliar-Garcia, A., Lara-Padilla, E., Ceballos, G., Rodriguez, A., Villarreal, F. & Ramirez-Sanchez, I. (2018) Dysregulation of mitochondrial function and biogenesis modulators in adipose tissue of obese children. *International Journal of Obesity*. 42 (4), 618–624.

Zampieri, M., Ciccarone, F., Calabrese, R., Franceschi, C., Bürkle, A. & Caiafa, P. (2015) Reconfiguration of DNA methylation in aging. *Mechanisms of Ageing and Development*. 15160–70.

Zhang, N. (2015) Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. *Animal Nutrition*. 1 (3), 144–151.

Zhang, W., Song, M., Qu, J. & Liu, G.-H. (2018) Epigenetic Modifications in Cardiovascular Aging and Diseases. *Circulation Research*. 123 (7), 773–786.

Zhang, Y., Wilson, R., Heiss, J., Breitling, L.P., Saum, K.-U., Schöttker, B., Holleczek, B., Waldenberger, M., Peters, A. & Brenner, H. (2017) DNA methylation signatures in peripheral blood strongly predict all-cause mortality. *Nature Communications*. 814617.

Zhang, Y. & Zhou, C. (2019) Formation and biological consequences of 5-Formylcytosine in genomic DNA. *DNA Repair*. 81102649.

Zhao, Y., Chen, B., Shen, J., Wan, L., Zhu, Y., Yi, T. & Xiao, Z. (2017a) The Beneficial Effects of Quercetin, Curcumin, and Resveratrol in Obesity. *Oxidative Medicine and Cellular Longevity*. 2017.

Zhao, Y., Chen, B., Shen, J., Wan, L., Zhu, Y., Yi, T. & Xiao, Z. (2017b) The Beneficial Effects of Quercetin, Curcumin, and Resveratrol in Obesity. *Oxidative Medicine and Cellular Longevity*. 2017.

Zharikov, S. & Shiva, S. (2013) Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease. *Biochemical Society Transactions*. 41 (1), 118–123.

Zheng, L.D., Linarelli, L.E., Brooke, J., Smith, C., Wall, S.S., Greenawald, M.H., Seidel, R.W., Estabrooks, P.A., Almeida, F.A. & Cheng, Z. (2016) Mitochondrial Epigenetic Changes Link to Increased Diabetes Risk and Early-Stage Prediabetes Indicator. *Oxidative Medicine and Cellular Longevity*. 2016e5290638.

Zheng, L.D., Linarelli, L.E., Liu, L., Wall, S.S., Greenawald, M.H., Seidel, R.W., Estabrooks, P.A., Almeida, F.A. & Cheng, Z. (2015) Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. *Clinical Epigenetics*. 7 (1), .

Zheng, Y., Joyce, B.T., Colicino, E., Liu, L., Zhang, W., Dai, Q., Shrubsole, M.J., Kibbe, W.A., Gao, T., Zhang, Z., Jafari, N., Vokonas, P., Schwartz, J., Baccarelli, A.A. & Hou, L. (2016) Blood Epigenetic Age may Predict Cancer Incidence and Mortality. *EBioMedicine*. 568–73.

Zhong, J., Agha, G. & Baccarelli, A.A. (2016) The Role of DNA Methylation in Cardiovascular Risk and Disease: Methodological Aspects, Study Design, and Data Analysis for Epidemiological Studies. *Circulation research*. 118 (1), 119–131.

Zhong, J., Trevisi, L., Urch, B., Lin, X., Speck, M., Coull, B.A., Liss, G., Thompson, A., Wu, S., Wilson, A., Koutrakis, P., Silverman, F., Gold, D.R. & Baccarelli, A.A. (2017) B-vitamin Supplementation Mitigates Effects of Fine Particles on Cardiac Autonomic

Dysfunction and Inflammation: A Pilot Human Intervention Trial. *Scientific Reports*. 745322.

Zhou, L., Xiao, X., Zhang, Q., Zheng, J. & Deng, M. (2019) Deciphering the Anti-obesity Benefits of Resveratrol: The "Gut Microbiota-Adipose Tissue" Axis. *Frontiers in Endocrinology*. 10.

Zsurka, G., Peeva, V., Kotlyar, A. & Kunz, W.S. (2018) Is There Still Any Role for Oxidative Stress in Mitochondrial DNA-Dependent Aging? *Genes*. 9 (4), 175.