UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN

"LIFE AND HEALTH SCIENCES – NUTRITION, FOOD AND HEALTH"

XXXV cycle

Investigation of the Role of Trimethylamine (TMA) and Trimethylamine-N-oxide (TMAO) as Inflammatory Mediators and their Impact on Human Health, with a Focus on Mitochondrial Dynamics. Evidence from both Observational Studies and *in vitro* Models.

PhD Student
Irene Petracci

Supervisor
Prof.ssa Rosita Gabbianelli

Co-supervisor
Dott.ssa Laura Bordoni

ABSTRACT

The primary aim of the Ph.D. project was to deepen the understanding of the impact of two food-derived metabolites, i.e., trimethylamine (TMA) and trimethylamine N-oxide (TMAO), on human health. TMA and TMAO derive from the metabolism of certain dietary precursors by the activity of gut bacteria and their circulating levels have been associated with chronic diseases. However, this topic has not been fully elucidated yet, and a mechanistic explanation of this association is still missing. Hence, the present work has investigated the impact of TMA and TMAO of human health from different perspectives, each of which corresponds to a section of the manuscript.

Since TMA and TMAO have been suggested to have an impact on mitochondrial dynamics, the first part of the project (Chapter 2) focused on the relationship between diet, TMA, TMAO, mitochondrial DNA copy number (mtDNAcn), and mitochondrial DNA methylation. Global DNA methylation was also investigated. Two hundred healthy subjects with extreme dietary patterns (healthy vs western diet) were recruited for the study. Their nutrients' intake and diets' quality (Healthy Eating Index) were assessed from their dietary records. Blood levels of TMA and TMAO, circulating levels of TMA precursors and their dietary intakes were also measured. MtDNAcn, nuclear DNA methylation in the long interspersed nuclear element 1 (LINE-1), and strand-specific D-loop methylation levels were assessed. The results showed that the TMAO/TMA ratio was negatively correlated with the D-loop methylation levels and positively correlated with mtDNAcn. Overall, these findings support the existence of a potential relationship between TMA metabolism and mitochondrial dynamics (and mtDNA), indicating a new avenue for further research.

The second part of the project (Chapter 3) was aimed to investigate the impact of several environmental exposures (including diet, physical activity, and smoking) on biological age. Indeed, lifestyle factors are known to affect DNA methylation throughout life, either accelerating or decelerating the aging process. Epigenetic clocks are used to estimate the biological age of an individual by measuring methylation patterns in specific areas of the genome. Interestingly, a new epigenetic clock based on only 6 CpGs may represent an easily accessible tool to measure the epigenetic age (EA) of an individual. Hence, this study aimed to validate the 6 CpG epigenetic clock comparing it with other biomarkers of aging (such as telomere length (TL) and methylation in the long interspersed nuclear element-1 (LINE-1)). Moreover, the impact of lifestyle-associated factors (one-carbon (1 C) metabolism-related nutrients' intake, circulating TMA and TMAO levels, body composition, physical activity, and smoking) on these molecular marks was also investigated.
Two hundred healthy participants having extreme dietary patterns (healthy vs western diet) were selected. Dietary intakes, circulating TMA and TMAO levels, body composition, physical activity

level and smoking habits were assessed. DNA was extracted from whole blood and used to measure epigenetic age (6CpG-EA), TL and LINE-1 methylation levels. The results showed that the 6CpG-EA was positively correlated with chronological age and negatively with TL and LINE-1 methylation. Despite no significant associations were detected with the overall diet quality (HEI), the 6CpG-EA was correlated with dietary intakes of the nutrients involved in the 1 C metabolism, especially in the western diet group, and with circulating TMA levels, especially in the healthy diet group. Overall, these results confirm that the 6CpG epigenetic clock is an easy tool to estimate biological age, in accordance with other molecular markers of aging, and suggest that the EA can be modulated not only by the micronutrients involved in the 1 C metabolism, but also by circulating levels of TMA.

Despite TMA is a well-known uremic toxin up to date most of the scientific attention has been conveyed onto the harmful effects of TMAO and its role in several complex diseases. However, recent studies have reproposed a potential effect of TMA in boosting the pro-inflammatory response. Thus, since colonic epithelial cells are the first target of TMA's effects (TMA is mainly produced in the colon), the third part of the project (Chapter 4) aimed to better elucidate the impact of excess intestinal TMA on intestinal epithelial cells, using colon adenocarcinoma Caco-2 cell line as an *in vitro* model. Given the prominent role of mitochondria in inflammation, the effect of TMA on mitochondrial dynamics was investigated, focusing on perturbation of ATP production and cellular membrane potential, as well as on the markers of mitochondrial damage (i.e., mtDNAcn, mt-cfDNA). Moreover, since a well-established link between inflammation and epigenetic perturbations exists, the effect of TMA on the expression levels and activity of DNA methyltransferases (DNMTs) and sirtuins (SIRTs), two key enzymes in the epigenetic landscape, was also examined. Lastly, a simplified model of intestinal epithelium was set up to preliminary evaluate the influence of TMA on intestinal permeability. Results showed that excess TMA in the intestinal environment may induce inflammation in intestinal cells (as demonstrated by the increased expression of the pro-inflammatory cytokines IL-6 and IL-1β) and perturb both epigenetic and mitochondrial homeostasis. Following the activated pro-inflammatory status, overwhelmed mitochondria may experience impaired mtDNA replication (as evidenced by the altered D-loop methylation and reduction of the mtDNAcn), which may compromise mitochondrial respiration (as proved by the downregulation of some respiratory chain components and decreased ATP content). Even though the intestinal permeability was not affected by TMA, this study certainly contributes to reinforcing the hypothesis that TMA (at least at high doses tested in the study) is not a harmless metabolite and it may have a contributing role in microbiota-induced intestinal diseases.

Alterations of TMAO metabolism and mitochondrial dynamics have been previously independently identified as risk factors for the development of cardiovascular disease (CVD). Thus, mitochondrial DNA copy number (mtDNAcn) and the circulating levels of TMAO have been suggested as promising biomarkers of cardiovascular events. Hence, this fourth part of the project (Chapter 5) was aimed to identify biomarkers that could be predictive of CVD. Circulating levels of TMA, TMAO, and mtDNAcn were investigated in the whole blood in a population of 389 coronary artery disease (CAD) patients and 151 healthy controls, in association with established risk factors for CVD (i.e., sex, age, hypertension, smoking, diabetes, glomerular filtration rate (GFR)) and troponin, a marker of acute myocardial injury. Results showed that mtDNAcn was significantly lower in CAD patients; it was correlated with GFR, hypertension, and TMA, but not with TMAO. Moreover, high TMA levels were associated with high TMAO levels only in CAD patients and not in controls, which corroborates the hypothesis that TMA levels are not linked *per se* to high TMAO levels, but a different metabolism of TMA and TMAO in the presence of cardiovascular disease may exist. Interestingly, a biomarker including mtDNAcn, sex, and hypertension (but neither TMA nor TMAO) emerged as a good predictor of CAD from this study. Hence, these findings recognize only mtDNAcn as a promising biomarker to monitor the exposure to risk factors and the efficacy of preventive interventions for personalized CAD risk reduction.

Several hypotheses have been proposed to explain the involvement of TMAO in the development of atherosclerosis and CVD risk. Among them, the induction of an inflammatory status has been proposed, with increased circulation of pro-inflammatory monocytes and the promotion of endothelial dysfunction. Nevertheless, contrasting evidence is emerging on TMAO role in CVD development and even a beneficial and protective role of TMAO has been hypothesized. Moreover, plasma TMA levels are recently gaining attention in cardiovascular health studies, since it seems that TMA may increase arterial blood pressure and exert toxic effects on vascular smooth muscle cells. Thus, this last section of the project (Chapter 6) was aimed to better elucidate the supposed pro-inflammatory actions of TMA and TMAO on circulating macrophages, using THP-1 monocytes as an *in vitro* model. Whether plasma TMA and TMAO may trigger the pro-inflammatory cascade was investigated and, considering the prominent role that mitochondria have in inflammation, the impact of TMA and TMAO on mitochondrial dynamics was also examined, focusing on perturbation of ATP production and cellular membrane potential, as well as on the markers of mitochondrial damage (i.e., mtDNAcn). Results revealed that both TMA and TMAO, were able to trigger inflammatory pathways in macrophages (as proved by the increased expression of the pro-inflammatory *IL-8*). However, TMA, more than TMAO, may disturb cellular homeostasis by altering mitochondrial dynamics as shown by the depletion of the intracellular ATP storage and by the upregulation of several components of the respiratory chain. Altogether, these findings contribute

to reinforcing the hypothesis that TMA might be more detrimental than its oxidized product (TMAO) in the context of vascular inflammation and that TMA, more that TMAO, may have a contributing role in CVD, even though the exact mechanisms remain to be fully elucidated.

RIASSUNTO

L'obiettivo primario del progetto di dottorato è stato quello di approfondire l'impatto sulla salute umana di due metaboliti di origine alimentare, ossia la trimetilammina (TMA) e l'ossido di trimetilammina (TMAO). TMA e TMAO derivano dal metabolismo di alcuni precursori alimentari ad opera del microbiota intestinale e i loro livelli ematici sono stati associati a malattie croniche. Tuttavia, ci sono delle opinioni contrastanti su questo argomento e ad oggi ancora manca una spiegazione meccanicistica su questa associazione. Pertanto, il presente lavoro ha voluto studiare l'impatto di TMA e TMAO sulla salute umana trattandolo da prospettive diverse, ed ognuna di esse corrisponde a una sezione di questo manoscritto.

Poiché è stato ipotizzato che gli effetti di TMA e TMAO siano mediati dal loro impatto sulle dinamiche mitocondriali, la prima parte del progetto (Capitolo 2) ha voluto approfondire la relazione tra dieta, TMA, TMAO, numero di copie del DNA mitocondriale (mtDNAcn) e metilazione del DNA mitocondriale. È stata presa in considerazione anche la metilazione globale del DNA. Per lo studio sono stati reclutati 200 soggetti sani con abitudini alimentari estreme (che seguivano una dieta sana o di tipo occidentale). Dai loro diari alimentari sono state estrapolate l'assunzione di nutrienti e la qualità delle loro diete (Healthy Eating Index). Sono stati misurati anche i livelli ematici di TMA e TMAO, i livelli circolanti dei precursori del TMA e il loro apporto nutrizionale. Sono stati valutati il mtDNAcn, i livelli di metilazione del DNA nucleare (nel retrotasposone LINE-1) e i livelli di metilazione dell'area D-loop nel DNA mitocondriale. Dai risultati è emerso che il rapporto TMAO/TMA correla negativamente con i livelli di metilazione del D-loop mitocondriale e positivamente con il mtDNAcn. Nel complesso, questi risultati supportano l'esistenza di una potenziale relazione tra il metabolismo del TMA e le dinamiche mitocondriali (e mtDNA), indicando una nuova strada per studi futuri.

Nella seconda parte del progetto (capitolo 3) è stato approfondito l'impatto che diversi fattori ambientali (tra cui dieta, attività fisica e fumo) possono avere sull'età biologica. Infatti, è noto che lo stile di vita influenza la metilazione del DNA, accelerando o rallentando il processo di invecchiamento. Gli orologi epigenetici sono strumenti che, misurando i livelli di metilazione in specifiche aree del genoma, possono essere utilizzati per stimare l'età biologica di un individuo. Di recente, è stato proposto un nuovo orologio epigenetico che, basandosi sull'analisi di sole 6 CpGs, può rappresentare uno strumento facilmente accessibile per misurare l'età epigenetica (EA) di un individuo. Pertanto, l'obiettivo principale di questo studio è stato quello di poter convalidare questo semplice orologio epigenetico basato su 6 CpGs, confrontandolo con altri biomarkers di invecchiamento, ossia la lunghezza dei telomeri (TL) e la metilazione a livello del retrotasposone

LINE-1 (usata come stima della metilazione globale del DNA). Inoltre, è stato approfondito anche l'impatto che alcuni fattori associati allo stile di vita (assunzione di nutrienti correlati al ciclo del carbonio (1C), livelli ematici di TMA e TMAO, composizione corporea, attività fisica e fumo) possono avere su questi biomarkers molecolari di invecchiamento.

Hanno partecipato allo studio duecento soggetti sani che seguivano stili alimentari estremi (dieta sana o di tipo occidentale). Sono stati valutati gli apporti nutrizionali, i livelli circolanti di TMA e TMAO, la composizione corporea, il livello di attività fisica e l'abitudine al fumo. Dal sangue intero è stato estratto Il DNA e utilizzato per misurare l'età epigenetica (6CpG-EA) e i livelli di metilazione di TL e LINE-1. Dai risultati è emerso che la 6CpG-EA è correlata positivamente con l'età cronologica e negativamente con la metilazione di TL e LINE-1. Nonostante non siano state rilevate associazioni significative con la qualità complessiva della dieta (HEI), la 6CpG-EA è stata correlata con l'assunzione di nutrienti coinvolti nel 1C (specialmente nel gruppo che seguiva una dieta di tipo occidentale) e con i livelli ematici di TMA specialmente nel gruppo che seguiva una dieta sana. Nel complesso, questi risultati confermano che l'orologio epigenetico basato su 6CpGs può essere usato per stimare l'età biologica di un individuo, in accordo con altri marcatori molecolari dell'invecchiamento, e suggeriscono che la 6CpG-EA può essere influenzata non solo dai micronutrienti coinvolti nel 1C, ma anche dai livelli ematici di TMA.

Nonostante il TMA sia una nota tossina uremica, ad oggi la maggior parte dell'attenzione scientifica è stata rivolta agli effetti dannosi del TMAO e al suo ruolo in diverse malattie "non comunicabili". Tuttavia, studi recenti hanno proposto che la specie nociva sia in realtà il TMA, piuttosto che il TMAO, e hanno ipotizzato una sua possibile implicazione nel favorire uno stato infiammatorio. Pertanto, poiché le cellule epiteliali del colon sono il primo bersaglio degli effetti del TMA (prodotto principalmente nel colon), la terza parte del progetto (capitolo 4) ha avuto l`obiettivo di chiarire l'impatto dell'eccesso di TMA intestinale sulle cellule epiteliali del colon, utilizzando cellule di adenocarcinoma Caco-2 come modello *in vitro*. Considerato il preminente ruolo dei mitocondri nell'infiammazione, è stato studiato l'effetto del TMA sulle dinamiche mitocondriali, in particolare sulla perturbazione della produzione di ATP e del potenziale della membrana cellulare, nonché sui biomarkers di danno mitocondriale (ad es. copie del DNA mitocondriale (mtDNAcn) e DNA mitocondriale circolante (mt-cfDNA)). Inoltre, poiché esiste un legame consolidato tra infiammazione e perturbazioni epigenetiche, è stato anche esaminato l'effetto de TMA sui livelli di espressione e sull'attività delle DNA metiltransferasi (DNMTs) e delle sirtuine (SIRTs), due enzimi chiave nel panorama epigenetico. Infine, è stato messo a punto un modello semplificato di epitelio intestinale per valutare a scopo preliminare l'influenza del TMA sulla permeabilità intestinale. I risultati hanno mostrato che l'eccesso di TMA nel lume intestinale può indurre infiammazione nelle cellule intestinali (come dimostrato dall'aumentata espressione delle citochine pro-infiammatorie

IL-6 e IL-1β) e perturbare l'omeostasi epigenetica e mitocondriale. A seguito dell'attivato stato pro-infiammatorio, la replicazione del mtDNA può risultare compromessa (come evidenziato dall'alterata metilazione del D-loop e dalla riduzione del mtDNAcn), e a sua volta può compromettere la respirazione mitocondriale (come dimostrato dalla diminuzione dell'espressione di alcuni componenti della catena respiratoria e del contenuto di ATP). Anche se la permeabilità intestinale non è risultata influenzata dal TMA, questo studio contribuisce certamente a rafforzare l'ipotesi che il TMA (almeno alle alte dosi testate nello studio) non sia un metabolita innocuo e che possa contribuire all'insorgenza delle malattie intestinali legate all'attività del microbiota.

Le alterazioni del metabolismo del TMAO e delle dinamiche mitocondriali sono state precedentemente identificate in modo indipendente come fattori di rischio per lo sviluppo di malattie cardiovascolari (CVD). Pertanto, il numero di copie del DNA mitocondriale (mtDNAcn) e i livelli ematici di TMAO sono stati suggeriti come promettenti biomarkers di eventi cardiovascolari. Perciò, questa quarta parte del progetto (capitolo 5) ha avuto lo scopo di identificare dei biomarkers predittivi di CVD. I livelli circolanti di TMA, TMAO e mtDNAcn sono stati studiati nel sangue intero in una popolazione di 389 pazienti con malattia coronarica (CAD) e 151 controlli sani, in associazione a noti fattori di rischio per CVD (cioè sesso, età, ipertensione, fumo, diabete, velocità di filtrazione glomerulare (GFR)) e troponina, un marker di danno miocardico acuto. Dai risultati è emerso che il mtDNAcn è significativamente più basso nei pazienti con CAD; inoltre è emersa una correlazione tra il mtDNAcn e GFR, ipertensione e TMA, ma nonTMAO. Inoltre, alti livelli di TMA sono stati associati ad alti livelli di TMAO solo nei pazienti con CAD e non nei controlli, il che avvalora l'ipotesi che i livelli ematici di TMA non siano di per sé legati a livelli elevati di TMAO, e che quindi si può verificare un'alterazione del metabolismo di TMA e TMAO in presenza di patologia cardiovascolare. Inoltre, dallo studio è emerso che il mtDNAcn, insieme a sesso e ipertensione (ma né TMA né TMAO), può essere considerato un buon predittore di CAD, utile anche per monitorare l'efficacia degli interventi preventivi per la riduzione personalizzata del rischio cardiovascolare.

Sono state proposte diverse ipotesi per spiegare il coinvolgimento del TMAO nella promozione dell'aterosclerosi e del rischio CVD. Tra questi, vi è l'induzione di uno stato infiammatorio, caratterizzato dall'aumento di monociti pro-infiammatori in circolo e da disfunzione dell'epitelio vascolare. Tuttavia, ci sono opinioni contrastanti sul ruolo del TMAO nello sviluppo di CVD, ed è stato addirittura ipotizzato un ruolo benefico e protettivo del TMAO nei confronti del danno vascolare. Perciò recentemente si è iniziato a porre l'attenzione sui livelli plasmatici di TMA, poiché sembra che il TMA possa aumentare la pressione arteriosa ed essere tossico per la muscolatura liscia dei vasi sanguigni. Pertanto, quest'ultima sezione del progetto (capitolo 6) ha avuto come

obiettivo quello di chiarire la presunta azione pro-infiammatoria di TMA e TMAO sui macrofagi circolanti, ed allo scopo sono stati utilizzati i monociti THP-1 come modello *in vitro*. Inoltre, considerando il ruolo di primo piano che i mitocondri hanno nel processo infiammatorio, è stato anche esaminato l'impatto di TMA e TMAO sulle dinamiche mitocondriali, in particolar modo sul potenziale di membrana, sulla produzione di ATP e sui marcatori di danno mitocondriale (mtDNAcn). Dai risultati è emerso che sia la TMA che il TMAO sono in grado di innescare una risposta infiammatoria nei macrofagi circolati (come dimostrato dall'aumentata espressione di *IL-8*). Tuttavia, il TMA, più del TMAO, può disturbare l'omeostasi cellulare alterando l'omeostasi mitocondriale come dimostrato dalla riduzione delle riserve intracellulari di ATP e dalla sovraregolazione di diversi componenti della catena respiratoria. Complessivamente, questi risultati contribuiscono a rafforzare l'ipotesi che il TMA potrebbe essere più dannoso del suo prodotto ossidato (TMAO) nel contesto dell'infiammazione vascolare e che il TMA, più che il TMAO, possa contribuire all'insorgenza di CVD, anche se i precisi meccanismi molecolari rimangono ancora da chiarire.

LIST OF ABBREVIATIONS

TMA	Trimethylamine
TMAO	Trimethylamine N-Oxide
MtDNAcn	Mitochondrial DNA copy number
NCDs	Non-communicable diseases
CVD	Cardiovascular disease
CAD	Coronary artery disease
LINE-1	Long interspersed nuclear element 1
PAMPs	Pathogen-associated molecular pattern molecules
DAMPs	Damage-associated molecular patterns
DNMTs	DNA methyltransferases
SIRTs	Sirtuins

Contents

Chapter 1

General Introduction

1.1 THE NUTRITION TRANSITION AND "MODERN" DISEASES

With increasing urbanization, industrialization and economic development, global diet has been experiencing a fast transition. Dietary regimens based on the consumption of food rich in complex carbohydrates and fiber have been replaced by dietary patterns based on processed food, low in fiber but rich in refined sugars and saturated fat mainly of animal origin [1]. However, the Western diet, as this new dietary pattern has been defined, came with a cost. In fact, the "Westernization" has been associated to a concurrent epidemiologic transition from mainly infectious or nutrient deficiency diseases toward a higher prevalence of non-communicable diseases (NCDs), such as metabolic disorders, cardiovascular diseases, and cancer. NCDs account for up to 72% of worldwide deaths [2], thus they represent a serious burden for the modern society [3]. NCDs have a multifactorial aetiology, thus they result from a complex interplay between genetic predisposition, environmental factors, and dysregulated immune responses. A common feature of NCDs is a chronic low-grade inflammatory state, induced by repeated pro-inflammatory stimuli and characterized by persistent elevated systemic concentrations of pro-inflammatory cytokines, which eventually disrupts cell and tissue homeostasis, accelerating ageing and disease progression [4]. Diet fits into this context, since food, nutrients and non-nutrient food components can modulate the inflammatory process [5].

1.2 DIET AND INFLAMMATION

Inflammation is part of the body's defence mechanism against stimuli of various kind (microbes, noxious compounds, autoimmune disorders, cellular defects, or biochemical triggers) (Figure 1). Inflammation is usually initiated by the activation of pattern recognition receptors (PRRs) expressed by immune and non-immune cells [6]. PRRs can be activated not only by pathogen-associated molecular pattern molecules (PAMPs) derived from microorganisms, but also by endogenous damage-associated molecular patterns (DAMPs), including nucleic acids, small metabolites (e.g., adenosine triphosphate (ATP) upon release into the extracellular environment) and proteins (e.g., calreticulin). In physiological conditions DAMPs are unable to activate the PRR signalling, but they can be triggered by cellular stress or perturbed cellular homeostasis. Once initiated, inflammation generally resolves rapidly, thanks to the activation of anti-inflammatory programs, but if perpetuated over long periods of time, it becomes chronic and compromises health. In fact, a persistent inflammatory response can lead to the breakdown of the immune tolerance [7], causing deleterious alterations in the normal cellular physiology, that cause collateral damage to tissues and organs and increase the risk for NCDs [8]. Currently, chronic inflammation is considered responsible for most of the diseases of industrialized countries (e.g., cardiovascular disease,

metabolic disorders, Alzheimer's disease, and cancer), and the primary cause of death worldwide [9,10]. Chronic inflammation usually initiates when activated immune cells (macrophages, neutrophils, and eosinophils) release pro-inflammatory cytokines and chemokines with autocrine, paracrine, and endocrine effects. The inflammatory cascade releases reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals, that compromise epithelium which ultimately leads to inflammatory bowel diseases (IBDs) [11].

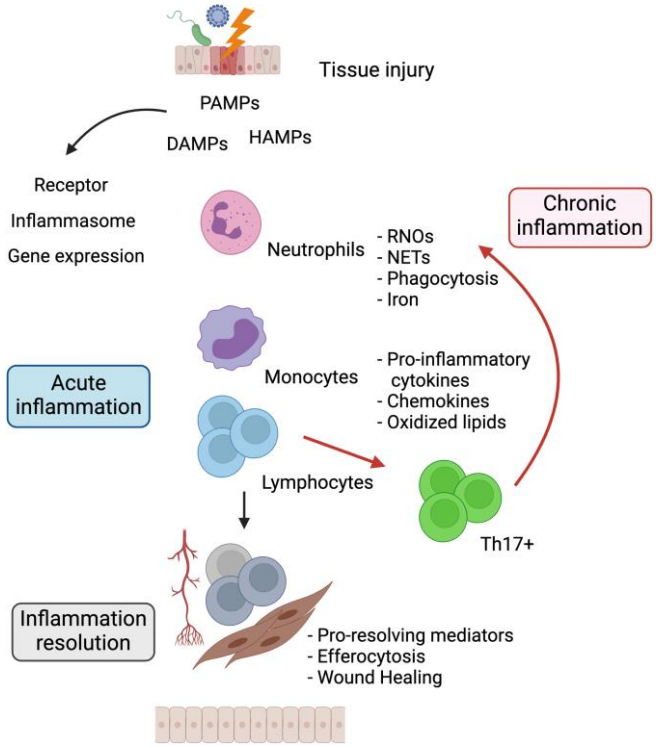


Figure 1. The relationship between acute and chronic inflammation [12].

As mentioned above, several risk factors can promote chronic inflammation, and apart from infections, and environmental or industrial toxicants, lifestyle factors (e.g., physical inactivity, psychological stress, and poor diet) can also have a prominent role (Figure 2). In particular, based on their nature, nutrients and dietary patterns can trigger or not the molecular pathways that activate the immune components. Significant associations with low-grade inflammation have been established for glycemic index (GI) and carbohydrates load, fibers, fatty acid composition, micronutrients, and bioactive compounds, like flavonoids. Carbohydrates exert different effects on health depending on their quantity and quality [13]. In general, a low-carbohydrate diet (35% of total energy or less) has been inversely associated to inflammatory markers [14–16]. The same effect on low-grade inflammation, has been observed with the consumption of low-glycemic index carbohydrates [17,18]. However, the impact of carbohydrates on systemic inflammation can be

attenuated by dietary fiber (both soluble and insoluble), which attenuates the glycemic load induced by carbohydrates. Dietary fiber has been also inversely associated with the levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), all important mediators of inflammation, as well as with the levels of C-reactive protein, a well-known biomarker of inflammation [19,20] . Dietary fats are key molecules for the organism, since they can elicit a number of essential cellular functions; however, for optimal health both the quantity and quality of dietary fats matters, since they may trigger low-grade inflammatory processes [21]. In particular, trans-fatty acids and saturated fatty acids (SFAs) have been positively associated with markers of systemic inflammation [22]. By contrast, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) have been inversely associated with inflammation [23]. In particular, ω-3 PUFAs have long been recognized to have anti-inflammatory activity. In fact, ω-3 PUFAs metabolism results in the synthesis of anti-inflammatory eicosanoids, which inhibit the synthesis of proinflammatory cytokines [24]. Instead, for ω-6 PUFAs both pro-inflammatory and anti-inflammatory effects have been described [25]. However, a very high ω-6/ω-3 ratio (typical of Western diets) has been associated with the pathogenesis of many diseases, including cardiovascular disease, cancer, inflammatory and autoimmune diseases, suggesting that the balance between the two species, rather than the single species, is important for disease prevention [26]. Potent antioxidant and anti-inflammatory properties have been attributed also to flavonoids (e.g., quercetin, kaempferol, malvidin, peonidin, daidzein, and genistein), bioactive molecules widely occurring in plant-based foods [27], even though their poor bioavailability may hinder their beneficial effects [28]. However, while it is important to consider the role of single nutrients in promoting or decreasing inflammation, it is also fundamental to consider the whole diet or “dietary pattern” (consisting of complex combinations of food and in different proportions) when monitoring the impact of food on health. Indeed, certain dietary patterns have been correlated to chronic inflammation [29]. Dietary patterns are usually assessed using a priori diet scores (pre-defined scores based on dietary guidelines, such as the Healthy Eating Index (HEI)) [30,31] or are well-established patterns of food consumption, such as the Mediterranean diet [32]. On this regard, in most observational and interventional studies, the Mediterranean dietary pattern (typically characterized by high MUFAs/SFAs and ω-3/ω-6 ratios, and rich in fruits, vegetables, legumes, and grains) has shown anti-inflammatory effects. For this reason, the Mediterranean diet could be used in clinical practice as a strategy for diminishing chronic inflammation. On the other hand, Western-type diets (WDs), which are typically energy-dense regimens, rich in processed foods, ‘‘fast food,’’ snacks, and sugary soft drinks, but poor in fiber, vitamins, phytochemicals, and minerals, have been associated with weight gain, pathological changes in lipids and energy metabolism, and activation of the immune system [33]. The inflammatory reaction can be induced either by the excess or the lack of several components of the WD. Besides the role of SFAs and the

high ω-6/ω-3 ratio that has been discussed earlier, cholesterol, which is highly abundant in the WD, is considered a well-established risk factor for atherosclerosis development. In addition, WD is rich in components and derivatives from red meat, eggs, and dairy products, such as L-carnitine and phosphatidylcholine, which have been linked to increased risk of CVDs, by means of the gut microbiota activity.

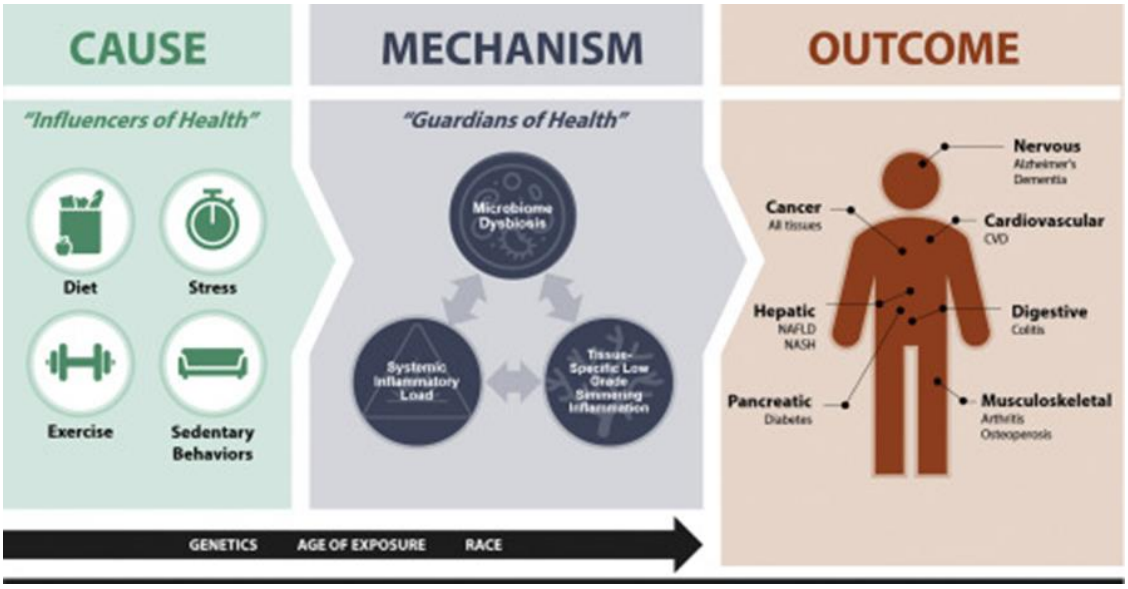


Figure 2. The impact of several risk factors on chronic inflammation and on health outcomes [34].

Indeed, the existence of the gut microbiota, which is placed at the intersection between diet and human health, further complicates this scenario. The gut microbiota has been living in a symbiotic relationship with the host for millions of years, affecting its energy balance, promoting tits intestinal immunity, and protecting against pathogens, while receiving an optimal niche to live in. Moreover, a strict relationship between the gut microbiota and diet exists. Dietary habits not only shape the gut microbiota composition and function, but also provide dietary precursors that the intestinal bacteria will transform for energetic purposes into secondary metabolites with a relevant impact (positive or not) on human health [35]. The gut microbial composition decides the nature of metabolites of bacterial origin released in the intestinal lumen, thus alterations of the gut microbiome may result in metabolic dysfunction and chronic inflammation [36] (Figure 3). Among the metabolic products of anaerobic bacteria fermentation, short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate that result from the digestion of the non-absorbable dietary fiber and resistant starches, are not only fuel for enterocytes, and modulate electrolyte and water absorption, but also known for their role in contrasting inflammation. Their anti-inflammatory effect seems to be due, among others, to the suppression of the LPS- and cytokine-stimulated release of pro-inflammatory mediators and the stimulation of anti-inflammatory cytokines release,

mainly via the inhibition of histone deacetylases (HDACs) [37]. On the other hand, trimethylamine (TMA), another by-product of bacterial metabolism of dietary precursors, together with its oxidized derivative trimethylamine-N oxide (TMAO), have been implicated in various chronic health conditions [38,39] (Figure 4).

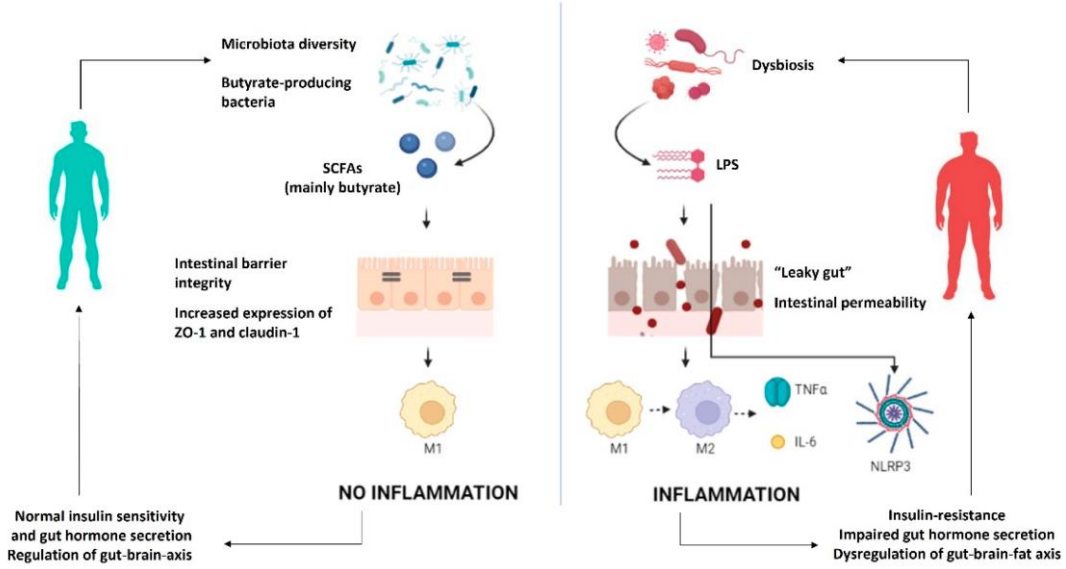


Figure 3. Mechanisms by which gut microbiota can modulate low-grade inflammation and obesity. IL-6—interleukin 6; LPS—lipopolysaccharides; M1—macrophages M1; M2—macrophages M2; NLRP3—inflammasome; SCFA—short-chain fatty acids; TNFα—tumor necrosis factor α; ZO-1—zonulin [40].

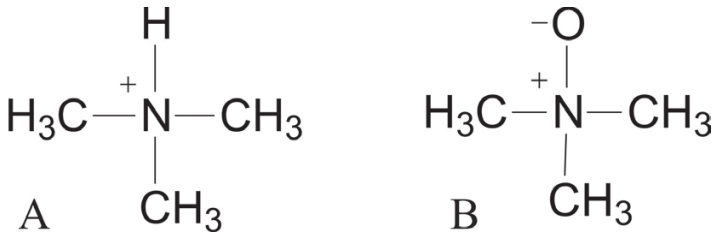


Figure 4. Structure of (A) trimethylamine (TMA) and (B) trimethylamine-N-oxide (TMAO) [41].

1.3 DIET, TMA AND TMAO

A direct relationship between diet, TMA and TMAO exists (Figure 5). In fact, TMA, a volatile tertiary aliphatic amine, derives from diet by either the consumption of foods rich in TMA, such as fish (which also contains TMAO), or the consumption of food rich in TMA precursors such as choline,

betaine, L-carnitine, dimethylglycine, and their precursors (e.g., phosphatidylcholine, crono-betaine, γ-butyrobetaine) mainly found in eggs, milk, red meat and cheese [42]. Another TMA precursor is ergothioneine, a biogenic amine (a derivative of histidine) that can be found in some animal products (mainly liver and kidney), but also in mushrooms and several types of beans as well. These precursors are used by the intestinal (caecum and colon) anaerobic bacteria (mainly belonging to *Firmicutes, Actinobacteria, and Proteobacteria*) for energetic purposes and converted into TMA according to two main metabolic pathways. The first involves the gene cluster *CutC/CutD* which codes for the choline TMA-lyase (CutC) and its activating choline trimethylamine-lyase enzyme (CutD) and needs choline as a substrate; while the second, that requires the Rieske-type oxygenase/reductase complex (CntA/B), has carnitine and gamma-butyrobetaine as substrates [43]. Moreover, betaine (choline-derived metabolite) reduction by the microbial glycine betaine reductase encoded by the gene *GrdH* is also associated with TMA production, albeit to a lesser extent[44]. Recently, the involvement of another metabolic pathway based on the enzyme complex (YeaW/X), has been also hypothesized [45]. Furthermore, TMA can be produced upon bacterial reduction of TMAO, a water-soluble osmolyte introduced in its natural form through seafood [46].

1.4 TMA AND TMAO METABOLISM

Once ingested or produced, most of TMA is rapidly absorbed by passive diffusion through the intestinal walls and is transported by the portal circulation to the liver, where is oxidized to TMAO. This oxidation is mediated by the hepatic flavin monooxygenases (FMO), mainly FMO3 [47] and generally leads to the consumption of up to 95% of TMA. TMAO's fate is to be either accumulated in extrahepatic tissues as an osmolyte or more frequently be excreted with urines and, in small percentages, also through sweat, faeces (4%), exhaled air (less and 1%) or other body secretions [48]. A small fraction of TMAO can remain into the circulation and interact with circulating proteins. It's through this mechanism that TMAO mediates the non-enzymatic oxidation of cholesterol by bonding with the zinc protoporphyrin IX dimethyl ester [ZnPPDME] to yield [TMAOZnPPDME] [49]. Since most of TMA undergoes hepatic oxidation to TMAO, the circulating levels of TMA are usually low, even though there could be some exceptions. Among the factors that can influence TMA levels in blood, diet is one of the most important. In fact, besides driving the amount of the ingested precursors, dietary habits can shape the gut microbiota composition. On this regard, it has been documented that vegetarian or vegan dietary regimens are accompanied by a reduced capacity to convert L-carnitine into TMA, not only because of dietary sources of TMA precursors are not part of the diet, but also because they seem to induce the selection of non-TMA producing bacteria. On the other hand, western-like diets rich in saturated fats negatively modify the gut microbial

composition, inducing an increase in TMA level [50]. However, also the genetic background of the host, its excretion capacity, and its liver health status could influence the levels of circulating TMA. In fact, genetic polymorphisms in the FMO3 gene impair (totally or partially) TMA hepatic oxidation and consequently the hematic TMA concentration increases, a condition known as trimethylaminuria [51]. In addition, the FMOs' activity is sensitive to certain molecules of dietary origin. For example, a decreased activity of FMOs has been reported in association with the consumption of grapefruit juice [52] and indole-containing cruciferous vegetables (e.g., Brussels sprouts, broccoli, cabbage, and cauliflower) [53]. A similar effect has been observed in case of ascorbic acid deficiency in guinea pigs [54].

So far, still little is known about the physiological roles of TMA and TMAO in humans. However, several studies have attributed potential toxic effects to both TMA and TMAO. In particular, elevated TMAO levels in blood have been linked to serious health conditions (Figure 6). However, this scenario is quite controversial and not well understood yet.

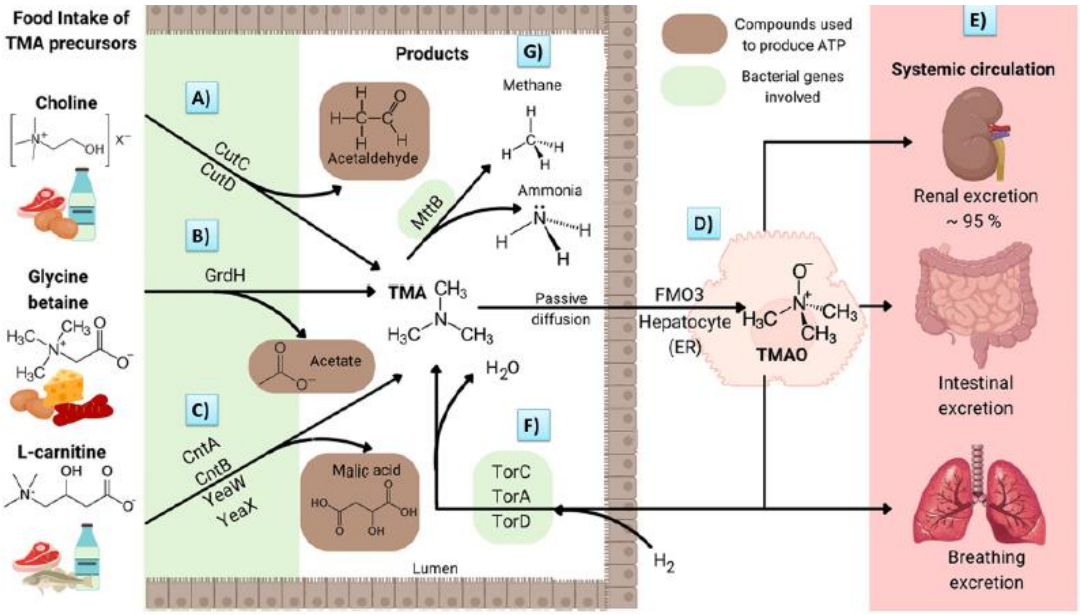


Figure 5. Biosynthesis and metabolism of trimethylamine N-oxide (TMAO). Precursors of trimethylamine (TMA), (A) choline, (B) glycine betaine, and (C) L-carnitine, are ingested with certain foods. They are then catabolized by the gut microbiota to obtain the end products (e.g., TMA) utilized in the formation of ATP. (D) Afterwards, 95% of TMA is diffused and oxidized by flavin-containing monooxygenase 3 (FMO3) in the liver to TMAO; (E) TMAO is transported to other organs via systemic circulation. (F) TMAO can serve as a final electron acceptor in bacteria that colonize the gut, thus reducing TMAO to TMA and H_2O. (G) TMA can be also used by some methanobacteria to generate methane and ammonia [55].

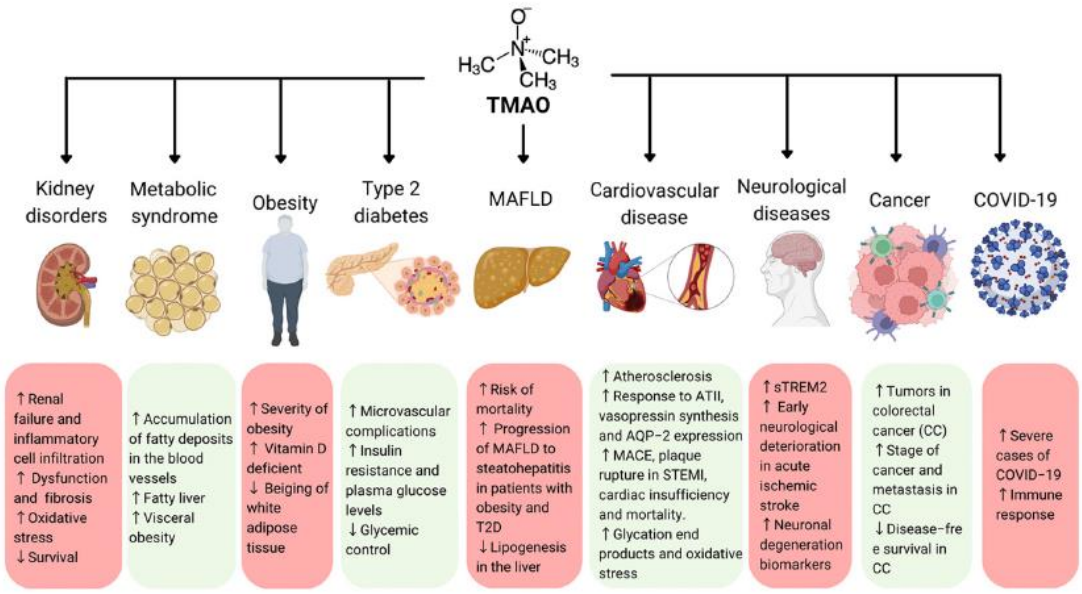


Figure 6. TMAO and human health. TMAO has been associated with an enhanced immune response, which is involved in the pathogenesis of several diseases, such as kidney and metabolic disorders, obesity, type 2 diabetes, metabolic dysfunction-associated fatty liver disease (MAFLD), cardiovascular and neurological diseases, cancer, and severe cases of COVID-19 [55].

1.5 TMA, TMAO AND HUMAN HEALTH

1.5.1 TMA and intestinal inflammation

The conversion of dietary components (choline, carnitine, and betaine) to TMA occurs mostly in the caecum and colon by anaerobic bacteria. TMA passively diffuses into blood circulation through the enterocyte membrane on its way to the liver, where is oxidized to TMAO. Approximately 95% of TMA is oxidized and then excreted in urine in a 3:95 TMA:TMAO ratio within 24h. The detected concentration of TMA in urine of healthy individuals, ranges from few µM to 50 mM [56,57] and is affected by dietary habits and by the sensitivity of instrument used for its detection. Lasty, less than 1% of TMA is eliminated with breath and only a small fraction (4%) is excreted in feces [47]. The reported amount of TMA in the fecal sample from healthy volunteers is about 0.146-0.5 µmol/g [58,59], but may vary according to the sensitivity of the detection method [46].

Considering that the intestine is the site of TMA production, that TMA may remain in the intestinal lumen until fecal excretion and considering TMA's toxic potential, several studies have investigated the potential genotoxic and cytotoxic effects of TMA on colonic cells. Jalandra and colleagues, showed that both acute and long-term exposures to TMA affect not only morphological characteristics, but also proliferation of human adenocarcinoma cell lines HCT116 and HT29, mostly via the induction of cell cycle arrest and the reduction of ATP production [60]. The inflammatory

effects of TMA on the colon and rectal epithelium come also from *in vivo* studies, where TMA was found to significantly increase the infiltration of inflammatory cells in the intestinal epithelium, and to cause extensive mucosal damage and distortion in the epithelium [60]. Moreover, meta-omic studies on colorectal cancer, reported increased levels of TMA precursors choline and choline-containing compounds in colorectal tumour biopsies compared to normal tissue from the same patients [61]. Increased levels of TMA precursors were found in stool from a high-risk group for colorectal cancer compared to a low-risk group, as well [62]. Lastly, several carnitine derivatives were reported to be differentially abundant in intestinal bowel disease compared to healthy controls [63]. Overall, these findings support the hypothesis that TMA is able to trigger inflammation not only systemically, but primarily, in its production site, with detrimental effects for the intestinal environment.

1.5.2 TMAO and kidney disease

Considering that kidneys are the main contributors to TMAO clearance, TMAO has been suggested as a biomarker for renal disease. In fact, several studies have found elevated plasma and/or urine TMAO concentration in patients with chronic kidney disease (CKD) [64,65] and end-stage renal failure [66]. Moreover, TMAO seems to exacerbate kidney dysfunction and renal fibrosis and to increase the mortality risk in CKD (1.7-fold increase when TMAO levels are elevated) [64,67].

1.5.3 TMAO and cardiovascular disease

Furthermore, elevated circulating TMAO levels have been directly correlated with atherosclerosis [50,68], heart failure [69], and hypertension [70]. Up to date, many studies (including systematic reviews and meta-analysis) further investigated and confirmed the association between TMAO and cardiovascular health. TMAO has been suggested as an independent predictor of CVD risk and mortality [71,72] with a dose-dependent effect [73]. In fact, TMAO was able to predict short- and long-term mortality and poor neurological outcome in out-of-hospital cardiac arrest patients [74]. In addition, elevated plasma TMAO independently correlated with plaque rupture in patients with myocardial infarction [75], while it was associated with increased risk of hypertension and reduced high-density lipoprotein cholesterol levels in apparently healthy individuals [76]. Thus, considering the previously mentioned role of TMAO in CKD and that a tight relationship between renal function and cardiovascular health exists, TMAO could represent a potential candidate biomarker either for early diagnosis or prognosis of cardiovascular diseases.

However, up to date the scientific community has not been able to provide a mechanistic explanation for this association between TMAO and CVD. Among the hypotheses that have been

proposed there are the promotion of platelet aggregation [77], vascular inflammation [78], cholesterol accumulation and foam cell formation [79], the increase in pro-inflammatory monocytes [80], oxidative stress [81] and the impairment of methyl metabolism [82] (Figure 7). Moreover, another issue to clarify is whether elevated levels of circulating TMAO are a cause or a consequence of the CVD.

Nevertheless, despite numerous proofs of the direct association between TMAO and CVD, contrasting evidence on the relation between TMAO and CVD emerged [83–87] and few studies suggested that TMA may have a primary role in CVD and that TMAO may be even protective [88–90], instead. On this regard, Jaworska and colleagues, suggested that TMA should be used as a marker of cardiovascular risk, based on the evidence that only TMA was responsible for the reduction of cardiomyocytes viability *in vitro,* while TMAO exerted a protective role against TMA-induced cytotoxicity [90]. Moreover, they measured increased plasma TMA levels in cardiovascular patients, which were also inversely correlated with the glomerular filtration rate (GFR) [90]. Evidence of the detrimental effects of TMA comes also from animal studies. In particular, chronic exposure to TMA increased blood pressure and the markers of kidney damage in rats [91]. Furthermore, the toxic effect of TMA is not limited to CVD. In fact, *in vivo* and *in vitro* studies suggested broad-spectrum effects of TMA, from the induction of neurological alterations [92] to carcinogenicity via the formation of N-nitrosodimethylamine [93], a well-known carcinogen. That TMA is a harmful compound, even more than TMAO, is not surprising since the liver, which detoxifies the human body from harmful molecules, promotes the almost total oxidation of TMA to TMAO in normal conditions, but if this physiological homeostasis is somehow perturbed, a pathological condition may arise.

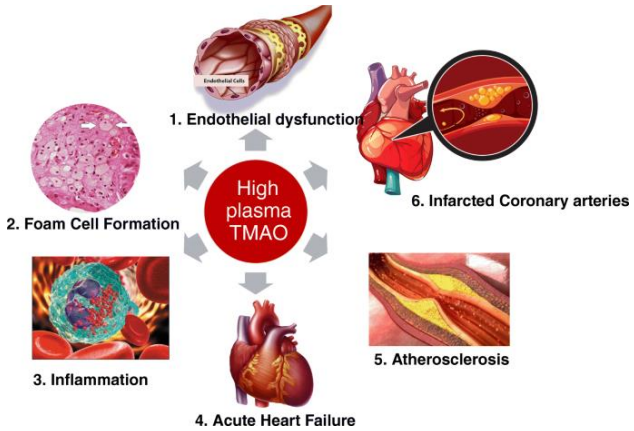


Figure 7. Elevated plasma levels of TMAO have been suggested to increase the risk for heart disease via the promotion of (1) endothelial dysfunction, (2) foam cell formation, (3) inflammation in vascular cells, (4) acute heart failure, (5) atherosclerosis, and (6) infarcted coronary arteries [94].

1.6 TMA AND TMAO AS THERAPEUTIC TARGET FOR DISEASE PREVENTION

From what abovementioned, it follows that both the diet and the gut microbiota are major actors when it comes to modulate the predisposition to NCDs, first among others metabolic and cardiovascular disorders, which represent a serious burden for the modern society. For this reason, TMA and TMAO, alone or in combination with other lifestyle factors (e.g., dietary habits) have been proposed as therapeutic targets to limit the onset of NCDs. Several therapeutic strategies are being explored to reduce TMA and TMAO levels. A strategy could be to decrease the dietary intake of TMA precursors to minimize the synthesis of TMA and TMAO. However, some of the TMA precursors are essential nutrients for humans and should be part of a normal diet. Just to mention few, L-carnitine is important for long-chain fatty acids metabolism [95], while choline is essential for neurotransmission (through the formation of acetylcholine) and for the constitution of cell membranes (through the synthesis of phosphatidylcholine and sphingomyelin); moreover, one of the end products of choline metabolism is S-adenosylmethionine (SAM), the primary methyl donor for the methylation reactions. In addition, TMAO levels not always increase after TMA precursors' ingestion [96].

For these reasons, other strategies acting on distinct levels should be also adopted. For example, increasing consumption of food that can target enzymes involved in TMAO metabolism or gut bacterial activity could help reduce the downstream formation of TMA and TMAO. As mentioned earlier, cruciferous vegetables impair FMO3 activity, thus may decrease TMA oxidation [97], while food with antimicrobial properties, such as garlic, might help prevent TMA formation in the intestine [98].

However, up to date, targeting the gut microbiome seems to be the easiest and most feasible approach to reduce TMA and TMAO formation, also because changes in the microbiome composition occur relatively fast. This goal could be achieved via pre- and pro-biotic supplementation, but also adopting strategies aimed to remodel the microbiome composition (e.g., bioremediation of TMA by methanogenic archaea) [99] for example promoting the growth of bacteria that use TMAO as substrate.

1.7 TMA AND TMAO AS BIOMARKERS FOR DISEASE PREVENTION

Given the increasing evidence supporting their positive role in the development and progress of major NCDs, such as cardiovascular events, kidney disease and neurological disorders, TMAO and TMA could represent two valuable biomarkers, for early disease prediction and population risk

stratification. Moreover, the simplicity of their detection (e.g., liquid chromatography-mass spectrometry, proton nuclear magnetic resonance spectrometry, headspace gas chromatography) in human specimens (e.g., plasma, urine, and feces) makes them extremely easy, accessible, and non-invasive biomarkers for primary and secondary disease prevention. In addition, TMA and TMAO levels not only might have a prognostic value and support ongoing therapeutic interventions, but they might be used tertiary interventions to prevent aggravation of existing illnesses.

However, some critical aspects have to be mentioned. First of all, the pathological cut-off values for plasma TMA and TMAO in humans have not been clearly defined yet. Some data are available, but they may change based on the disease or the study design and a unified scientific opinion on the topic is still missing [73,100–102]. Secondly, there are many confounding factors that could influence the association between TMAO and NCDs, such as the genetic background and the gut microbiota composition and function. Lastly, despite those numerous studies, a mechanistic explanation of the association between the abovementioned conditions and TMA or TMAO levels is still missing. On this regard, mitochondrial dynamics could help clarify this topic.

1.8 MITOCHONDRIA AND INFLAMMATION

Despite mitochondria are the evolutionary remnants of ancestral Alphaproteobacteria (the ancestors of modern Gram-negative bacteria) [103], they are extremely vital to human cells. Indeed, mitochondria are the core of multiple metabolic pathways critical for maintaining cellular homeostasis, such as oxidative phosphorylation, macromolecules biosynthesis, ion homeostasis (i.e., calcium), apoptosis and redox signaling. Based on these considerations, it seems likely that mitochondria have a key role in triggering inflammatory processes.

First of all, mitochondria are a major source of DAMPs. Indeed, given they bacterial origins, some mitochondrial components are similar to bacterial ones (e.g., differently from genomic DNA, mitochondrial DNA (mtDNA) is circular) and might function as PRR ligands. Also, the ATP they produce may function as a DAMP via P2X receptors when released by apoptotic cells [104,105]. Lastly, mitochondria are the major site of ROS production, thus mtDNA could be very vulnerable to ROS-induced damage. The oxidative stress induced by ROS overproduction leads to mitophagy inhibition, mtDNA mutations and mtDNA release, ultimately causing the decline in mitochondrial renewal and function. Mitochondria are also sensitive to other pro-inflammatory mediators, including the cytokines TNFα and IL-1β, which may induce mitochondrial damage, leading to a decrease of ATP production (through down regulation of complex I of the electron transport chain) and alteration of mitochondrial membrane potential ($\Delta\psi m$) [106].

The imbalanced mitochondrial dynamics, further contribute to cell damage, inflammation, and disease. In fact, from recent mechanistic studies it emerged that dysfunctional mitochondria are implicated in the development of chronic low-grade inflammation [107]. For example, alterations in mitochondrial function are recognized in cardiovascular disease [108–110], neurodegenerative disorders and inflammatory bowel disease. Thus, mitochondria not only have a significant role in pro-inflammatory signaling, but vice versa, one common manifestation of inflammation is a change in these organelles' physiology causing mitochondrial dysfunctions.

1.8.1 mtDNA copy number

Human mtDNA is a small circular genome, within the inner matrix of mitochondria (Figure 8). It consists of 37 genes, 13 of which code for the components of the mitochondrial electron transport chain, including seven genes that code for subunits within complex I (*MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6*), one for complex III (*MT-CYB*), three for complex IV (*MT-CO1, MT-CO2, MT-CO3*) and two for complex V (*MT-ATP6, MT-ATP8*), besides 22 transfer RNAs and 2 ribosomal RNAs (*MT-RNR1* and *MT-RNR2*). Each mitochondrion contains 2-10 copies of the mtDNA, and up to 1000 mitochondria can be found in each cell. Mitochondrial DNA content of a cell, also referred to as mtDNA copy number (mtDNAcn), is the measure of the number of mitochondrial genomes per cell and is determined mainly by the mtDNA replication rate and by the energy demand of the cell [111,112]. MtDNAcn is influenced by altered energy metabolism and ROS overproduction. For this reason, changes in mtDNAcn have been proposed as a biomarker of mitochondrial health [113,114]. Indeed, mtDNAcn is associated with mitochondrial enzyme activity and ATP production, thus changes in mtDNAcn may indicate mitochondrial damage and dysfunction, as well as the possibility of mitochondrial involvement in the onset of complex diseases. On this regard, a reduction of mtDNAcn has been reported in ageing and in various ageing-related diseases such as diabetes, obesity, and cancer [115–117], while an association between mtDNAcn and cardiovascular disease has been also reported. Lower mtDNAcn has also been found associated to frailty and all-cause mortality [118].

Up to date, the mechanisms that regulate mtDNAcn and that influence disease onset are not completely understood. One proposed mechanism could be via regulation of nuclear DNA (nDNA) methylation at specific loci that may impact human health through alteration in cell signaling [119]. The possibility to use mtDNAcn as a biomarker comes mainly from studies on peripheral blood cells. However, the recent discovery of cell-free respiratory competent mitochondria in blood [120], lead to the idea that quantifying mtDNAcn in the whole blood could be more representative of the health status of an individual.

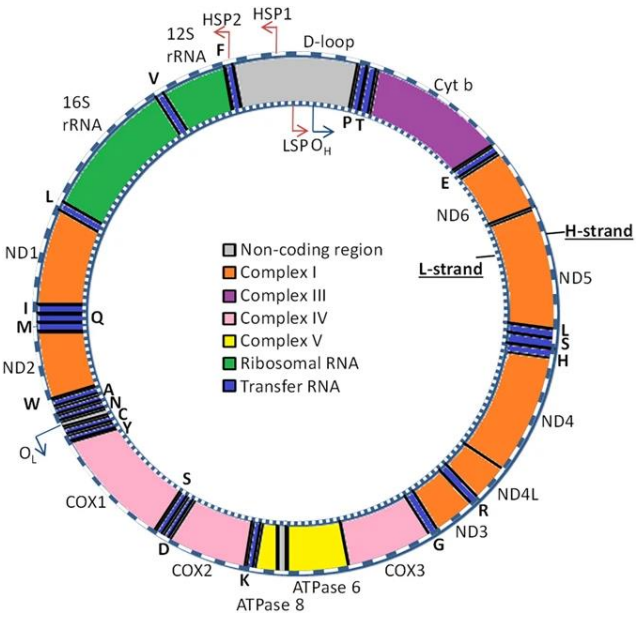


Figure 8. Mitochondrial DNA (mtDNA). The human mtDNA is a 16,569 bp circular DNA, containing a heavy (H, outer ring) and light (L, inner ring) strand. The genes encoded from the L-strand are written inside the circular DNA, whereas genes encoded from the H-strand are written on the outside. The protein-coding genes encode for the complexes required for oxidative phosphorylation (Complex I: orange, complex III: purple, complex IV: pink, complex V: yellow). The D-loop region contains the promoters for the L- and H-strand (LSP, HSP1, HSP2) and the origin of replication of the H-strand (OH) [121].

1.8.2 mtDNA epigenetics

MtDNA contains only three promoter regions, which are LSP (for genes encoded by the light (L)-strand), and HSP1 and HSP2 (for the genes encoded by the heavy (H)-strand), that produce polycistronic transcripts [122]. These three promoters are found within or in the proximity of the mitochondrial displacement loop (D-loop) region, which also host the origin of replication of the H-strand. MtDNA transcription and replication are regulated by several proteins of nuclear origin, including the mitochondrial RNA polymerase, the transcription and mtDNA maintenance factor (TFAM), the transcription specificity factors (TFB1M and TFB2M) and the transcription termination factor (mTERF). However, recent evidence suggests that mtDNA transcription and replication could also be controlled by epigenetic mechanisms. The term “mitoepigenetics” has been proposed to refer not only to the epigenetic modifications that regulate mtDNA, but also to describe the crosstalk between mitochondria and the nuclear epigenome. In fact, mitochondria provide key intermediate metabolites that are essential for epigenetic modifications on nDNA, which in turn control the expression of mitochondrial proteins.

However, contrary to nuclear epigenetics, mtDNA epigenetics has been less investigated, mainly because the existence of such mechanisms has been questioned for years, due to the different nature and structure of mtDNA compared to nDNA. Despite that, growing evidence is supporting the existence of "mitoepigenetics" and studies are focusing mainly on mtDNA methylation [123], although it is still a controversial topic. Indeed, while some authors have denied the existence of DNA methylation in the mitochondrial genome or have linked its retrieval to technical limitations [124,125], others have supported its existence in the D-loop regulatory region [126] and even suggested a functional role of DNA methylation in mtDNA replication and mtDNA gene transcription [127,128]. Moreover, the discovery of DNA methyltransferases 1, 3A and 3B (DNMT1, DNMT3A and DNMT3B) in mitochondria further corroborates the hypothesis of the existence of mtDNA methylation [129].

Changes in mtDNA methylation have been associated with the aetiology of several human diseases, such as cancer, obesity, diabetes, and cardiovascular and neurodegenerative diseases [130]. Several authors also observed a significant inverse correlation between the mtDNA methylation levels in the D-loop region and mtDNAcn [131,132], thus further suggesting the important role of DNA methylation in mitochondrial regulation.

In conclusion, although the existence of "mitoepigenetics" and its involvement in human diseases is still debated, the research in this field is still open, in the hope of finding new biomarkers for disease diagnosis and progression, as well as potential therapeutic interventions.

1.9 REFERENCES

1. Drewnowski, A.; Popkin, B.M. The Nutrition Transition: New Trends in the Global Diet. *Nutr Rev* **1997**, *55*, 31–43, doi:10.1111/j.1753-4887.1997.tb01593.x.
2. 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. *Lancet* **2017**, *390*, 1151–1210, doi:10.1016/S0140-6736(17)32152-9.
3. Beaglehole, R.; Bonita, R.; Horton, R.; Adams, C.; Alleyne, G.; Asaria, P.; Baugh, V.; Bekedam, H.; Billo, N.; Casswell, S.; et al. Priority Actions for the Non-Communicable Disease Crisis. *Lancet* **2011**, *377*, 1438–1447, doi:10.1016/S0140-6736(11)60393-0.
4. Hotamisligil, G.S. Inflammation, Metaflammation and Immunometabolic Disorders. *Nature* **2017**, *542*, 177–185, doi:10.1038/nature21363.
5. Calder, P.C.; Ahluwalia, N.; Brouns, F.; Buetler, T.; Clement, K.; Cunningham, K.; Esposito, K.; Jönsson, L.S.; Kolb, H.; Lansink, M.; et al. Dietary Factors and Low-Grade Inflammation in Relation to Overweight and Obesity. *Br J Nutr* **2011**, *106 Suppl*, S5-78, doi:10.1017/S0007114511005460.
6. Kroemer, G.; Galassi, C.; Zitvogel, L.; Galluzzi, L. Immunogenic Cell Stress and Death. *Nat Immunol* **2022**, *23*, 487–500, doi:10.1038/S41590-022-01132-2.
7. Kotas, M.E.; Medzhitov, R. Homeostasis, Inflammation, and Disease Susceptibility. *Cell* **2015**, *160*, 816–827, doi:10.1016/J.CELL.2015.02.010.
8. Bennett, J.M.; Reeves, G.; Billman, G.E.; Sturmberg, J.P. Inflammation-Nature's Way to Efficiently Respond to All Types of Challenges: Implications for Understanding and Managing "the Epidemic" of Chronic Diseases. *Front Med (Lausanne)* **2018**, *5*, doi:10.3389/FMED.2018.00316.
9. Roth, G.A.; Abate, D.; Abate, K.H.; Abay, S.M.; Abbafati, C.; Abbasi, N.; Abbastabar, H.; Abd-Allah, F.; Abdela, J.; Abdelalim, A.; et al. Global, Regional, and National Age-Sex-Specific Mortality for 282 Causes of Death in 195 Countries and Territories, 1980-2017: A Systematic Analysis for the Global Burden of Disease Study 2017. *Lancet* **2018**, *392*, 1736–1788, doi:10.1016/S0140-6736(18)32203-7.
10. Kyu, H.H.; Abate, D.; Abate, K.H.; Abay, S.M.; Abbafati, C.; Abbasi, N.; Abbastabar, H.; Abd-Allah, F.; Abdela, J.; Abdelalim, A.; et al. Global, Regional, and National Disability-Adjusted Life-Years (DALYs) for 359 Diseases and Injuries and Healthy Life Expectancy (HALE) for 195 Countries and Territories, 1990-2017: A Systematic Analysis for the Global Burden of Disease Study 2017. *The Lancet* **2018**, *392*, 1859–1922, doi:10.1016/S0140-6736(18)32335-3.
11. Tian, T.; Wang, Z.; Zhang, J. Pathomechanisms of Oxidative Stress in Inflammatory Bowel Disease and Potential Antioxidant Therapies. *Oxid Med Cell Longev* **2017**, *2017*, doi:10.1155/2017/4535194.
12. Chavez-Dominguez, R.; Perez-Medina, M.; Aguilar-Cazares, D.; Galicia-Velasco, M.; Meneses-Flores, M.; Islas-Vazquez, L.; Camarena, A.; Lopez-Gonzalez, J.S. Old and New Players of Inflammation and Their Relationship With Cancer Development. *Front Oncol* **2021**, *11*, 722999, doi:10.3389/FONC.2021.722999/BIBTEX.
13. Slavin, J.; Carlson, J. Carbohydrates. *Adv Nutr* **2014**, *5*, 760–761, doi:10.3945/AN.114.006163.
14. Jonasson, L.; Guldbrand, H.; Lundberg, A.K.; Nystrom, F.H. Advice to Follow a Low-Carbohydrate Diet Has a Favourable Impact on Low-Grade Inflammation in Type 2 Diabetes Compared with Advice to Follow a Low-Fat Diet. *Ann Med* **2014**, *46*, 182–187, doi:10.3109/07853890.2014.894286.
15. Tavakoli, A.; Mirzababaei, A.; Sajadi, F.; Mirzaei, K. Circulating Inflammatory Markers May Mediate the Relationship between Low Carbohydrate Diet and Circadian Rhythm in Overweight and Obese Women. *BMC Womens Health* **2021**, *21*, doi:10.1186/S12905-021-01240-5.
16. Seshadri, P.; Iqbal, N.; Stern, L.; Williams, M.; Chicano, K.L.; Daily, D.A.; McGrory, J.; Gracely, E.J.; Rader, D.J.; Samaha, F.F. A Randomized Study Comparing the Effects of a Low-Carbohydrate Diet and a Conventional Diet on Lipoprotein Subfractions and C-Reactive Protein Levels in Patients with Severe Obesity. *Am J Med* **2004**, *117*, 398–405, doi:10.1016/J.AMJMED.2004.04.009.
17. Gomes, J.M.G.; Fabrini, S.P.; Alfenas, R. de C.G. Low Glycemic Index Diet Reduces Body Fat and Attenuates Inflammatory and Metabolic Responses in Patients with Type 2 Diabetes. *Arch Endocrinol Metab* **2017**, *61*, 137–144, doi:10.1590/2359-3997000000206.
18. Gögebakan, Ö.; Kohl, A.; Osterhoff, M.A.; Van Baak, M.A.; Jebb, S.A.; Papadaki, A.; Martinez, J.A.; Handjieva-Darlenska, T.; Hlavaty, P.; Weickert, M.O.; et al. Effects of Weight Loss and Long-Term Weight Maintenance with Diets Varying in Protein and Glycemic Index on Cardiovascular Risk Factors: The Diet, Obesity, and Genes (DiOGenes) Study: A Randomized, Controlled Trial. *Circulation* **2011**, *124*, 2829–2838, doi:10.1161/CIRCULATIONAHA.111.033274.
19. North, C.J.; Venter, C.S.; Jerling, J.C. The Effects of Dietary Fibre on C-Reactive Protein, an Inflammation Marker Predicting Cardiovascular Disease. *Eur J Clin Nutr* **2009**, *63*, 921–933, doi:10.1038/EJCN.2009.8.
20. Ma, Y.; Hébert, J.R.; Li, W.; Bertone-Johnson, E.R.; Olendzki, B.; Pagoto, S.L.; Tinker, L.; Rosal, M.C.; Ockene, I.S.; Ockene, J.K.; et al. Association between Dietary Fiber and Markers of Systemic Inflammation in the Women's Health Initiative Observational Study. *Nutrition* **2008**, *24*, 941–949, doi:10.1016/J.NUT.2008.04.005.
21. Lichtenstein, A.H.; Kennedy, E.; Barrier, P.; Danford, D.; Ernst, N.D.; Grundy, S.M.; Leveille, G.A.; Van Horn, L.; Williams, C.L.; Booth, S.L. Dietary Fat Consumption and Health. *Nutr Rev* **1998**, *56*, 3–19, doi:10.1111/J.1753-4887.1998.TB01728.X.
22. Mozaffarian, D.; Pischon, T.; Hankinson, S.E.; Rifai, N.; Joshipura, K.; Willett, W.C.; Rimm, E.B. Dietary Intake of Trans Fatty Acids and Systemic Inflammation in Women. *Am J Clin Nutr* **2004**, *79*, 606–612, doi:10.1093/AJCN/79.4.606.
23. Rocha, D.M.; Bressan, J.; Hermsdorff, H.H. The Role of Dietary Fatty Acid Intake in Inflammatory Gene Expression: A Critical Review. *Sao Paulo Med J* **2017**, *135*, 157–168, doi:10.1590/1516-3180.2016.008607072016.
24. Riediger, N.D.; Othman, R.A.; Suh, M.; Moghadasian, M.H. A Systemic Review of the Roles of N-3 Fatty Acids in Health and Disease. *J Am Diet Assoc* **2009**, *109*, 668–679, doi:10.1016/J.JADA.2008.12.022.
25. de Lorgeril, M. Essential Polyunsaturated Fatty Acids, Inflammation, Atherosclerosis and Cardiovascular Diseases. *Subcell Biochem* **2007**, *42*, 283–297, doi:10.1007/1-4020-5688-5_13.
26. Simopoulos, A.P. The Importance of the Ratio of Omega-6/Omega-3 Essential Fatty Acids. *Biomed Pharmacother* **2002**, *56*, 365–379, doi:10.1016/S0753-3322(02)00253-6.
27. González, R.; Ballester, I.; López-Posadas, R.; Suárez, M.D.; Zarzuelo, A.; Martínez-Augustin, O.; Sánchez de Medina, F. Effects of Flavonoids and Other Polyphenols on Inflammation. *Crit Rev Food Sci Nutr* **2011**, *51*, 331–362, doi:10.1080/10408390903584094.

28. Martin, K.R.; Appel, C.L. Polyphenols as Dietary Supplements: A Double-Edged Sword. *Nutr Diet Suppl* **2009**, *2*, 1–12, doi:10.2147/NDS.S6422.
29. Hu, F.B. Dietary Pattern Analysis: A New Direction in Nutritional Epidemiology. *Curr Opin Lipidol* **2002**, *13*, 3–9, doi:10.1097/00041433-200202000-00002.
30. Guenther, P.M.; Reedy, J.; Krebs-Smith, S.M. Development of the Healthy Eating Index-2005. *J Am Diet Assoc* **2008**, *108*, 1896–1901, doi:10.1016/J.JADA.2008.08.016.
31. Guenther, P.M.; Casavale, K.O.; Reedy, J.; Kirkpatrick, S.I.; Hiza, H.A.B.; Kuczynski, K.J.; Kahle, L.L.; Krebs-Smith, S.M. Update of the Healthy Eating Index: HEI-2010. *J Acad Nutr Diet* **2013**, *113*, 569–580, doi:10.1016/J.JAND.2012.12.016.
32. Panagiotakos, D.B.; Pitsavos, C.; Stefanadis, C. Dietary Patterns: A Mediterranean Diet Score and Its Relation to Clinical and Biological Markers of Cardiovascular Disease Risk. *Nutr Metab Cardiovasc Dis* **2006**, *16*, 559–568, doi:10.1016/J.NUMECD.2005.08.006.
33. Christ, A.; Lauterbach, M.; Latz, E. Western Diet and the Immune System: An Inflammatory Connection. *Immunity* **2019**, *51*, 794–811, doi:10.1016/J.IMMUNI.2019.09.020.
34. Hofseth, L.J.; Hébert, J.R. Diet and Acute and Chronic, Systemic, Low-Grade Inflammation. *Diet, Inflammation, and Health* **2022**, 85–111, doi:10.1016/B978-0-12-822130-3.00011-9.
35. Gentile, C.L.; Weir, T.L. The Gut Microbiota at the Intersection of Diet and Human Health. *Science* **2018**, *362*, 776–780, doi:10.1126/science.aau5812.
36. Sommer, F.; Bäckhed, F. The Gut Microbiota — Masters of Host Development and Physiology. *Nature Reviews Microbiology 2013 11:4* **2013**, *11*, 227–238, doi:10.1038/nrmicro2974.
37. Vinolo, M.A.R.; Rodrigues, H.G.; Nachbar, R.T.; Curi, R. Regulation of Inflammation by Short Chain Fatty Acids. *Nutrients* **2011**, *3*, 858–876, doi:10.3390/nu3100858.
38. Jalandra, R.; Makharia, G.K.; Sharma, M.; Kumar, A. Inflammatory and Deleterious Role of Gut Microbiota-Derived Trimethylamine on Colon Cells. *Front Immunol* **2023**, *13*, 8161, doi:10.3389/FIMMU.2022.1101429/BIBTEX.
39. Yang, S.; Li, X.; Yang, F.; Zhao, R.; Pan, X.; Liang, J.; Tian, L.; Li, X.; Liu, L.; Xing, Y.; et al. Gut Microbiota-Dependent Marker TMAO in Promoting Cardiovascular Disease: Inflammation Mechanism, Clinical Prognostic, and Potential as a Therapeutic Target. *Front Pharmacol* **2019**, *10*, doi:10.3389/FPHAR.2019.01360.
40. Vetrani, C.; Di Nisio, A.; Paschou, S.A.; Barrea, L.; Muscogiuri, G.; Graziadio, C.; Savastano, S.; Colao, A. From Gut Microbiota through Low-Grade Inflammation to Obesity: Key Players and Potential Targets. *Nutrients 2022, Vol. 14, Page 2103* **2022**, *14*, 2103, doi:10.3390/NU14102103.
41. Mackay, R.J.; McEntyre, C.J.; Henderson, C.; Lever, M.; George, P.M. Trimethylaminuria: Causes and Diagnosis of a Socially Distressing Condition. *Clin Biochem Rev* **2011**, *32*, 33.
42. Zhang, A.Q.; Mitchell, S.C.; Smith, R.L. Dietary Precursors of Trimethylamine in Man: A Pilot Study. *Food Chem Toxicol* **1999**, *37*, 515–520, doi:10.1016/s0278-6915(99)00028-9.
43. Rath, S.; Heidrich, B.; Pieper, D.H.; Vital, M. Uncovering the Trimethylamine-Producing Bacteria of the Human Gut Microbiota. *Microbiome* **2017**, *5*, 54, doi:10.1186/s40168-017-0271-9.
44. Rath, S.; Heidrich, B.; Pieper, D.H.; Vital, M. Uncovering the Trimethylamine-Producing Bacteria of the Human Gut Microbiota. *Microbiome* **2017**, *5*, doi:10.1186/S40168-017-0271-9.
45. Koeth, R.A.; Levison, B.S.; Culley, M.K.; Buffa, J.A.; Wang, Z.; Gregory, J.C.; Org, E.; Wu, Y.; Li, L.; Smith, J.D.; et al. γ-Butyrobetaine Is a Proatherogenic Intermediate in Gut Microbial Metabolism of L-Carnitine to TMAO. *Cell Metab* **2014**, *20*, 799–812, doi:10.1016/j.cmet.2014.10.006.
46. Hoyles, L.; Jiménez-Pranteda, M.L.; Chilloux, J.; Brial, F.; Myridakis, A.; Aranias, T.; Magnan, C.; Gibson, G.R.; Sanderson, J.D.; Nicholson, J.K.; et al. Metabolic Retroconversion of Trimethylamine N-Oxide and the Gut Microbiota. *Microbiome* **2018**, *6*, 73, doi:10.1186/s40168-018-0461-0.
47. Zeisel, S.H.; Warrier, M. Trimethylamine N-Oxide, the Microbiome, and Heart and Kidney Disease. *Annu Rev Nutr* **2017**, *37*, 157–181, doi:10.1146/annurev-nutr-071816-064732.
48. Gatarek, P.; Kaluzna-Czaplinska, J. Trimethylamine N-Oxide (TMAO) in Human Health. *EXCLI J* **2021**, *20*, 301–319, doi:10.17179/excli2020-3239.
49. Paul, N.; Sarkar, R.; Sarkar, S. Zinc Protoporphyrin-Trimethylamine-N-Oxide Complex Involves Cholesterol Oxidation Causing Atherosclerosis. *J Biol Inorg Chem* **2021**, *26*, 367–374, doi:10.1007/s00775-021-01861-z.
50. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis. *Nat Med* **2013**, *19*, 576–585, doi:10.1038/nm.3145.
51. Dolphin, C.T.; Janmohamed, A.; Smith, R.L.; Shephard, E.A.; Phillips, I.R. Missense Mutation in Flavin-Containing Mono-Oxygenase 3 Gene, FMO3, Underlies Fish-Odour Syndrome. *Nat Genet* **1997**, *17*, 491–494, doi:10.1038/ng1297-491.
52. Chung, W.G.; Kang, J.H.; Lee, K.H.; Roh, H.K.; Cha, Y.N. Determination of Flavin-Containing Monooxygenase Activity Using Human Caffeine Metabolism and Differential Inhibition of FMO Activity with Grapefruit Juice. *Clin Pharmacol Ther* **1997**, *61*, 225.
53. Cashman, J.R.; Xiong, Y.; Lin, J.; Verhagen, H.; van Poppel, G.; van Bladeren, P.J.; Larsen-Su, S.; Williams, D.E. In Vitro and in Vivo Inhibition of Human Flavin-Containing Monooxygenase Form 3 (FMO3) in the Presence of Dietary Indoles. *Biochem Pharmacol* **1999**, *58*, 1047–1055, doi:10.1016/s0006-2952(99)00166-5.
54. Brodfuehrer, J.I.; Zannoni, V.G. Flavin-Containing Monooxygenase and Ascorbic Acid Deficiency: Qualitative and Quantitative Differences. *Biochem Pharmacol* **1987**, *36*, 3161–3167, doi:https://doi.org/10.1016/0006-2952(87)90627-7.
55. Constantino-Jonapa, L.A.; Espinoza-Palacios, Y.; Escalona-Montaño, A.R.; Hernández-Ruiz, P.; Amezcua-Guerra, L.M.; Amedei, A.; Aguirre-García, M.M. Contribution of Trimethylamine N-Oxide (TMAO) to Chronic Inflammatory and Degenerative Diseases. *Biomedicines 2023, Vol. 11, Page 431* **2023**, *11*, 431, doi:10.3390/BIOMEDICINES11020431.
56. Lee, S.-K.; Kim, D.-H.; Jin, C.-B.; Yoo, hye hyun Determination of Urinary Trimethylamine and Trimethylamine N-Oxide by Liquid Chromatography-Tandem Mass Spectrometry Using Mixed-Mode Stationary Phases. *Bull Korean Chem Soc* **2010**, *31*, 483–486, doi:10.5012/bkcs.2010.31.02.483.
57. D'Angelo, R.; Scimone, C.; Esposito, T.; Bruschetta, D.; Rinaldi, C.; Ruggeri, A.; Sidoti, A. Fish Odor Syndrome (Trimethylaminuria) Supporting the Possible FMO3 down Expression in Childhood: A Case Report. *J Med Case Rep* **2014**, *8*, 328, doi:10.1186/1752-1947-8-328.
58. Borrel, G.; McCann, A.; Deane, J.; Neto, M.C.; Lynch, D.B.; Brugère, J.-F.; O'Toole, P.W. Genomics and Metagenomics of Trimethylamine-Utilizing Archaea in the Human Gut Microbiome. *ISME J* **2017**, *11*, 2059–2074, doi:10.1038/ismej.2017.72.

59. Fiori, J.; Turroni, S.; Candela, M.; Brigidi, P.; Gotti, R. Simultaneous HS-SPME GC-MS Determination of Short Chain Fatty Acids, Trimethylamine and Trimethylamine N-Oxide for Gut Microbiota Metabolic Profile. *Talanta* **2018**, *189*, 573–578, doi:10.1016/j.talanta.2018.07.051.
60. Jalandra, R.; Makharia, G.K.; Sharma, M.; Kumar, A. Inflammatory and Deleterious Role of Gut Microbiota-Derived Trimethylamine on Colon Cells. *Front Immunol* **2022**, *13*, 1101429, doi:10.3389/fimmu.2022.1101429.
61. Chan, E.C.Y.; Koh, P.K.; Mal, M.; Cheah, P.Y.; Eu, K.W.; Backshall, A.; Cavill, R.; Nicholson, J.K.; Keun, H.C. Metabolic Profiling of Human Colorectal Cancer Using High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HR-MAS NMR) Spectroscopy and Gas Chromatography Mass Spectrometry (GC/MS). *J Proteome Res* **2009**, *8*, 352–361, doi:10.1021/pr8006232.
62. Ocvirk, S.; Wilson, A.S.; Posma, J.M.; Li, J. V; Koller, K.R.; Day, G.M.; Flanagan, C.A.; Otto, J.E.; Sacco, P.E.; Sacco, F.D.; et al. A Prospective Cohort Analysis of Gut Microbial Co-Metabolism in Alaska Native and Rural African People at High and Low Risk of Colorectal Cancer. *Am J Clin Nutr* **2020**, *111*, 406–419, doi:10.1093/ajcn/nqz301.
63. Lloyd-Price, J.; Arze, C.; Ananthakrishnan, A.N.; Schirmer, M.; Avila-Pacheco, J.; Poon, T.W.; Andrews, E.; Ajami, N.J.; Bonham, K.S.; Brislawn, C.J.; et al. Multi-Omics of the Gut Microbial Ecosystem in Inflammatory Bowel Diseases. *Nature* **2019**, *569*, 655–662, doi:10.1038/s41586-019-1237-9.
64. Tang, W.H.W.; Wang, Z.; Kennedy, D.J.; Wu, Y.; Buffa, J.A.; Agatisa-Boyle, B.; Li, X.S.; Levison, B.S.; Hazen, S.L. Gut Microbiota-Dependent Trimethylamine N-Oxide (TMAO) Pathway Contributes to Both Development of Renal Insufficiency and Mortality Risk in Chronic Kidney Disease. *Circ Res* **2015**, *116*, 448–455, doi:10.1161/CIRCRESAHA.116.305360.
65. Bell, J.D.; Lee, J.A.; Lee, H.A.; Sadler, P.J.; Wilkie, D.R.; Woodham, R.H. Nuclear Magnetic Resonance Studies of Blood Plasma and Urine from Subjects with Chronic Renal Failure: Identification of Trimethylamine-N-Oxide. *Biochim Biophys Acta* **1991**, *1096*, 101–107, doi:10.1016/0925-4439(91)90046-c.
66. Bain, M.A.; Faull, R.; Fornasini, G.; Milne, R.W.; Evans, A.M. Accumulation of Trimethylamine and Trimethylamine-N-Oxide in End-Stage Renal Disease Patients Undergoing Haemodialysis. *Nephrol Dial Transplant* **2006**, *21*, 1300–1304, doi:10.1093/ndt/gfk056.
67. Fogelman, A.M. TMAO Is Both a Biomarker and a Renal Toxin. *Circ Res* 2015, *116*, 396–397.
68. Bordoni, L.; Samulak, J.J.; Sawicka, A.K.; Pelikant-Malecka, I.; Radulska, A.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Trimethylamine N-Oxide and the Reverse Cholesterol Transport in Cardiovascular Disease: A Cross-Sectional Study. *Sci Rep* **2020**, *10*, 18675, doi:10.1038/s41598-020-75633-1.
69. Mamic, P.; Chaikijurajai, T.; Tang, W.H.W. Gut Microbiome - A Potential Mediator of Pathogenesis in Heart Failure and Its Comorbidities: State-of-the-Art Review. *J Mol Cell Cardiol* **2021**, *152*, 105–117, doi:10.1016/j.yjmcc.2020.12.001.
70. Ge, X.; Zheng, L.; Zhuang, R.; Yu, P.; Xu, Z.; Liu, G.; Xi, X.; Zhou, X.; Fan, H. The Gut Microbial Metabolite Trimethylamine N-Oxide and Hypertension Risk: A Systematic Review and Dose-Response Meta-Analysis. *Adv Nutr* **2020**, *11*, 66–76, doi:10.1093/advances/nmz064.
71. Heianza, Y.; Ma, W.; Manson, J.E.; Rexrode, K.M.; Qi, L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. *J Am Heart Assoc* **2017**, *6*, doi:10.1161/JAHA.116.004947.
72. Senthong, V.; Wang, Z.; Li, X.S.; Fan, Y.; Wu, Y.; Tang, W.H.W.; Hazen, S.L. Intestinal Microbiota-Generated Metabolite Trimethylamine-N-Oxide and 5-Year Mortality Risk in Stable Coronary Artery Disease: The Contributory Role of Intestinal Microbiota in a COURAGE-Like Patient Cohort. *J Am Heart Assoc* **2016**, *5*, doi:10.1161/JAHA.115.002816.
73. Schiattarella, G.G.; Sannino, A.; Toscano, E.; Giugliano, G.; Gargiulo, G.; Franzone, A.; Trimarco, B.; Esposito, G.; Perrino, C. Gut Microbe-Generated Metabolite Trimethylamine-N-Oxide as Cardiovascular Risk Biomarker: A Systematic Review and Dose-Response Meta-Analysis. *Eur Heart J* **2017**, *38*, 2948–2956, doi:10.1093/eurheartj/ehx342.
74. Hochstrasser, S.R.; Metzger, K.; Vincent, A.M.; Becker, C.; Keller, A.K.J.; Beck, K.; Perrig, S.; Tisljar, K.; Sutter, R.; Schuetz, P.; et al. Trimethylamine-N-Oxide (TMAO) Predicts Short- and Long-Term Mortality and Poor Neurological Outcome in out-of-Hospital Cardiac Arrest Patients. *Clin Chem Lab Med* **2020**, *59*, 393–402, doi:10.1515/cclm-2020-0159.
75. Tan, Y.; Sheng, Z.; Zhou, P.; Liu, C.; Zhao, H.; Song, L.; Li, J.; Zhou, J.; Chen, Y.; Wang, L.; et al. Plasma Trimethylamine N-Oxide as a Novel Biomarker for Plaque Rupture in Patients With ST-Segment-Elevation Myocardial Infarction. *Circ Cardiovasc Interv* **2019**, *12*, e007281, doi:10.1161/CIRCINTERVENTIONS.118.007281.
76. Abbasalizad Farhangi, M.; Vajdi, M. Gut Microbiota-Associated Trimethylamine N-Oxide and Increased Cardiometabolic Risk in Adults: A Systematic Review and Dose-Response Meta-Analysis. *Nutr Rev* **2021**, *79*, 1022–1042, doi:10.1093/nutrit/nuaa111.
77. Zhu, W.; Gregory, J.C.; Org, E.; Buffa, J.A.; Gupta, N.; Wang, Z.; Li, L.; Fu, X.; Wu, Y.; Mehrabian, M.; et al. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. *Cell* **2016**, *165*, 111–124, doi:10.1016/j.cell.2016.02.011.
78. Sun, X.; Jiao, X.; Ma, Y.; Liu, Y.; Zhang, L.; He, Y.; Chen, Y. Trimethylamine N-Oxide Induces Inflammation and Endothelial Dysfunction in Human Umbilical Vein Endothelial Cells via Activating ROS-TXNIP-NLRP3 Inflammasome. *Biochem Biophys Res Commun* **2016**, *481*, 63–70, doi:10.1016/j.bbrc.2016.11.017.
79. Canyelles, M.; Tondo, M.; Cedó, L.; Farràs, M.; Escolà-Gil, J.C.; Blanco-Vaca, F. Trimethylamine N-Oxide: A Link among Diet, Gut Microbiota, Gene Regulation of Liver and Intestine Cholesterol Homeostasis and HDL Function. *Int J Mol Sci* **2018**, *19*, doi:10.3390/ijms19103228.
80. Haghikia, A.; Li, X.S.; Liman, T.G.; Bledau, N.; Schmidt, D.; Zimmermann, F.; Kränkel, N.; Widera, C.; Sonnenschein, K.; Haghikia, A.; et al. Gut Microbiota-Dependent Trimethylamine N-Oxide Predicts Risk of Cardiovascular Events in Patients With Stroke and Is Related to Proinflammatory Monocytes. *Arterioscler Thromb Vasc Biol* **2018**, *38*, 2225–2235, doi:10.1161/ATVBAHA.118.311023.
81. Zhou, S.; Xue, J.; Shan, J.; Hong, Y.; Zhu, W.; Nie, Z.; Zhang, Y.; Ji, N.; Luo, X.; Zhang, T.; et al. Gut-Flora-Dependent Metabolite Trimethylamine-N-Oxide Promotes Atherosclerosis-Associated Inflammation Responses by Indirect ROS Stimulation and Signaling Involving AMPK and SIRT1. *Nutrients* 2022, *14*.
82. Obeid, R.; Awwad, H.M.; Rabagny, Y.; Graeber, S.; Herrmann, W.; Geisel, J. Plasma Trimethylamine N-Oxide Concentration Is Associated with Choline, Phospholipids, and Methyl Metabolism. *Am J Clin Nutr* **2016**, *103*, 703–711, doi:10.3945/ajcn.115.121269.
83. Olek, R.A.; Samulak, J.J.; Sawicka, A.K.; Hartmane, D.; Grinberga, S.; Pugovics, O.; Lysiak-Szydlowska, W. Increased Trimethylamine N-Oxide Is Not Associated with Oxidative Stress Markers in Healthy Aged Women. *Oxid Med Cell Longev* **2019**, *2019*, 6247169, doi:10.1155/2019/6247169.

84. Samulak, J.J.; Sawicka, A.K.; Hartmane, D.; Grinberga, S.; Pugovics, O.; Lysiak-Szydlowska, W.; Olek, R.A. L-Carnitine Supplementation Increases Trimethylamine-N-Oxide but Not Markers of Atherosclerosis in Healthy Aged Women. *Ann Nutr Metab* **2019**, *74*, 11–17, doi:10.1159/000495037.
85. Bordoni, L.; Sawicka, A.K.; Szarmach, A.; Winklewski, P.J.; Olek, R.A.; Gabbianelli, R. A Pilot Study on the Effects of L -Carnitine and Trimethylamine-N-Oxide on Platelet Mitochondrial DNA Methylation and CVD Biomarkers in Aged Women. *Internationa Journal of Molecular Sciences* **2020**, 1–17.
86. Yin, J.; Liao, S.-X.; He, Y.; Wang, S.; Xia, G.-H.; Liu, F.-T.; Zhu, J.-J.; You, C.; Chen, Q.; Zhou, L.; et al. Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide Level in Patients With Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack. *J Am Heart Assoc* **2015**, *4*, doi:10.1161/JAHA.115.002699.
87. Collins, H.L.; Drazul-Schrader, D.; Sulpizio, A.C.; Koster, P.D.; Williamson, Y.; Adelman, S.J.; Owen, K.; Sanli, T.; Bellamine, A. L-Carnitine Intake and High Trimethylamine N-Oxide Plasma Levels Correlate with Low Aortic Lesions in ApoE(-/-) Transgenic Mice Expressing CETP. *Atherosclerosis* **2016**, *244*, 29–37, doi:10.1016/j.atherosclerosis.2015.10.108.
88. Jaworska, K.; Bielinska, K.; Gawrys-Kopczynska, M.; Ufnal, M. TMA (Trimethylamine), but Not Its Oxide TMAO (Trimethylamine-Oxide), Exerts Haemodynamic Effects: Implications for Interpretation of Cardiovascular Actions of Gut Microbiome. *Cardiovasc Res* **2019**, *115*, 1948–1949, doi:10.1093/cvr/cvz231.
89. Restini, C.B.A.; Fink, G.D.; Watts, S.W. Vascular Reactivity Stimulated by TMA and TMAO: Are Perivascular Adipose Tissue and Endothelium Involved? *Pharmacol Res* **2021**, *163*, 105273, doi:10.1016/j.phrs.2020.105273.
90. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kapłon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins (Basel)* **2019**, *11*, doi:10.3390/toxins11090490.
91. Maksymiuk, K.M.; Szudzik, M.; Gawryś-Kopczyńska, M.; Onyszkiewicz, M.; Samborowska, E.; Mogilnicka, I.; Ufnal, M. Trimethylamine, a Gut Bacteria Metabolite and Air Pollutant, Increases Blood Pressure and Markers of Kidney Damage Including Proteinuria and KIM-1 in Rats. *J Transl Med* **2022**, *20*, 470, doi:10.1186/s12967-022-03687-y.
92. Vanholder, R.; Pletinck, A.; Schepers, E.; Glorieux, G. Biochemical and Clinical Impact of Organic Uremic Retention Solutes: A Comprehensive Update. *Toxins (Basel)* **2018**, *10*, doi:10.3390/toxins10010033.
93. Koppang, N. Dimethylnitrosamine--Formation in Fish Meal and Toxic Effects in Pigs. *Am J Pathol* **1974**, *74*, 95–108.
94. Thomas, M.S.; Fernandez, M.L. Trimethylamine N-Oxide (TMAO), Diet and Cardiovascular Disease. *Curr Atheroscler Rep* **2021**, *23*, 1–7, doi:10.1007/S11883-021-00910-X/TABLES/1.
95. Longo, N.; Frigeni, M.; Pasquali, M. Carnitine Transport and Fatty Acid Oxidation. *Biochim Biophys Acta* **2016**, *1863*, 2422–2435, doi:10.1016/j.bbamcr.2016.01.023.
96. Zhu, C.; Sawrey-Kubicek, L.; Bardagjy, A.S.; Houts, H.; Tang, X.; Sacchi, R.; Randolph, J.M.; Steinberg, F.M.; Zivkovic, A.M. Whole Egg Consumption Increases Plasma Choline and Betaine without Affecting TMAO Levels or Gut Microbiome in Overweight Postmenopausal Women. *Nutr Res* **2020**, *78*, 36–41, doi:10.1016/J.NUTRES.2020.04.002.
97. Janeiro, M.H.; Ramírez, M.J.; Milagro, F.I.; Martínez, J.A.; Solas, M. Implication of Trimethylamine N-Oxide (TMAO) in Disease: Potential Biomarker or New Therapeutic Target. *Nutrients* **2018**, *10*, doi:10.3390/nu10101398.
98. Wu, W.-K.; Panyod, S.; Ho, C.-T.; Kuo, C.-H.; Wu, M.-S.; Sheen, L.-Y. Dietary Allicin Reduces Transformation of L-Carnitine to TMAO through Impact on Gut Microbiota. *J Funct Foods* **2015**, *15*, 408–417, doi:10.1016/j.jff.2015.04.001.
99. Fadhlaoui, K.; Arnal, M.-E.; Martineau, M.; Camponova, P.; Ollivier, B.; O'Toole, P.W.; Brugère, J.-F. Archaea, Specific Genetic Traits, and Development of Improved Bacterial Live Biotherapeutic Products: Another Face of next-Generation Probiotics. *Appl Microbiol Biotechnol* **2020**, *104*, 4705–4716, doi:10.1007/s00253-020-10599-8.
100. Duranton, F.; Cohen, G.; De Smet, R.; Rodriguez, M.; Jankowski, J.; Vanholder, R.; Argiles, A. Normal and Pathologic Concentrations of Uremic Toxins. *Journal of the American Society of Nephrology* **2012**, *23*, 1258–1270, doi:10.1681/ASN.2011121175/-/DCSUPPLEMENTAL.
101. Tang, W.H.W.; Wang, Z.; Levison, B.S.; Koeth, R.A.; Britt, E.B.; Fu, X.; Wu, Y.; Hazen, S.L. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. *N Engl J Med* **2013**, *368*, 1575–1584, doi:10.1056/NEJMOA1109400.
102. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis. *Nature Medicine 2013 19:5* **2013**, *19*, 576–585, doi:10.1038/nm.3145.
103. Roger, A.J.; Muñoz-Gómez, S.A.; Kamikawa, R. The Origin and Diversification of Mitochondria. *Curr Biol* **2017**, *27*, R1177–R1192, doi:10.1016/J.CUB.2017.09.015.
104. Denning, N.L.; Aziz, M.; Gurien, S.D.; Wang, P. DAMPs and NETs in Sepsis. *Front Immunol* **2019**, *10*, doi:10.3389/FIMMU.2019.02536.
105. Dosch, M.; Gerber, J.; Jebbawi, F.; Beldi, G. Mechanisms of ATP Release by Inflammatory Cells. *Int J Mol Sci* **2018**, *19*, doi:10.3390/IJMS19041222.
106. Zell, R.; Geck, P.; Werdan, K.; Boekstegers, P. TNF-Alpha and IL-1 Alpha Inhibit Both Pyruvate Dehydrogenase Activity and Mitochondrial Function in Cardiomyocytes: Evidence for Primary Impairment of Mitochondrial Function. *Mol Cell Biochem* **1997**, *177*, 61–67, doi:10.1023/A:1006896832582.
107. Yu, E.; Calvert, P.A.; Mercer, J.R.; Harrison, J.; Baker, L.; Figg, N.L.; Kumar, S.; Wang, J.C.; Hurst, L.A.; Obaid, D.R.; et al. Mitochondrial DNA Damage Can Promote Atherosclerosis Independently of Reactive Oxygen Species through Effects on Smooth Muscle Cells and Monocytes and Correlates with Higher-Risk Plaques in Humans. *Circulation* **2013**, *128*, 702–712, doi:10.1161/CIRCULATIONAHA.113.002271/-/DC1.
108. Murphy, E.; Ardehali, H.; Balaban, R.S.; DiLisa, F.; Dorn, G.W.; Kitsis, R.N.; Otsu, K.; Ping, P.; Rizzuto, R.; Sack, M.N.; et al. Mitochondrial Function, Biology, and Role in Disease: A Scientific Statement From the American Heart Association. *Circ Res* **2016**, *118*, 1960–1991, doi:10.1161/RES.0000000000000104.
109. Johri, A.; Beal, M.F. Mitochondrial Dysfunction in Neurodegenerative Diseases. *J Pharmacol Exp Ther* **2012**, *342*, 619–630, doi:10.1124/JPET.112.192138.
110. Novak, E.A.; Mollen, K.P. Mitochondrial Dysfunction in Inflammatory Bowel Disease. *Front Cell Dev Biol* **2015**, *3*, 62, doi:10.3389/FCELL.2015.00062.
111. Ekstrand, M.I.; Falkenberg, M.; Rantanen, A.; Park, C.B.; Gaspari, M.; Hultenby, K.; Rustin, P.; Gustafsson, C.M.; Larsson, N.G. Mitochondrial Transcription Factor A Regulates MtDNA Copy Number in Mammals. *Hum Mol Genet* **2004**, *13*, 935–944, doi:10.1093/HMG/DDH109.
112. Melser, S.; Lavie, J.; Bénard, G. Mitochondrial Degradation and Energy Metabolism. *Biochim Biophys Acta* **2015**, *1853*, 2812–2821, doi:10.1016/J.BBAMCR.2015.05.010.

113. Malik, A.N.; Czajka, A. Is Mitochondrial DNA Content a Potential Biomarker of Mitochondrial Dysfunction? *Mitochondrion* **2013**, *13*, 481–492, doi:10.1016/J.MITO.2012.10.011.
114. Clay Montier, L.L.; Deng, J.J.; Bai, Y. Number Matters: Control of Mammalian Mitochondrial DNA Copy Number. *J Genet Genomics* **2009**, *36*, 125–131, doi:10.1016/S1673-8527(08)60099-5.
115. Castellani, C.A.; Longchamps, R.J.; Sun, J.; Guallar, E.; Arking, D.E. Thinking Outside the Nucleus: Mitochondrial DNA Copy Number in Health and Disease. *Mitochondrion* **2020**, *53*, 214–223, doi:10.1016/J.MITO.2020.06.004.
116. Guha, M.; Avadhani, N.G. Mitochondrial Retrograde Signaling at the Crossroads of Tumor Bioenergetics, Genetics and Epigenetics. *Mitochondrion* **2013**, *13*, 577–591, doi:10.1016/J.MITO.2013.08.007.
117. Dechsupa, S.; Yingsakmongkol, W.; Limthongkul, W.; Singhatanadgige, W.; Honsawek, S. Relative Telomere Length and Oxidative DNA Damage in Hypertrophic Ligamentum Flavum of Lumbar Spinal Stenosis. *PeerJ* **2018**, *2018*, doi:10.7717/PEERJ.5381/SUPP-4.
118. Ashar, F.N.; Moes, A.; Moore, A.Z.; Grove, M.L.; Chaves, P.H.M.; Coresh, J.; Newman, A.B.; Matteini, A.M.; Bandeen-Roche, K.; Boerwinkle, E.; et al. Association of Mitochondrial DNA Levels with Frailty and All-Cause Mortality. *J Mol Med (Berl)* **2015**, *93*, 177–186, doi:10.1007/S00109-014-1233-3.
119. Castellani, C.A.; Longchamps, R.J.; Sumpter, J.A.; Newcomb, C.E.; Lane, J.A.; Grove, M.L.; Bressler, J.; Brody, J.A.; Floyd, J.S.; Bartz, T.M.; et al. Mitochondrial DNA Copy Number Can Influence Mortality and Cardiovascular Disease via Methylation of Nuclear DNA CpGs. *Genome Med* **2020**, *12*, doi:10.1186/S13073-020-00778-7.
120. Al Amir Dache, Z.; Otandault, A.; Tanos, R.; Pastor, B.; Meddeb, R.; Sanchez, C.; Arena, G.; Lasorsa, L.; Bennett, A.; Grange, T.; et al. Blood Contains Circulating Cell-Free Respiratory Competent Mitochondria. *FASEB J* **2020**, *34*, 3616–3630, doi:10.1096/FJ.201901917RR.
121. Van Der Wijst, M.G.P.; Van Tilburg, A.Y.; Ruiters, M.H.J.; Rots, M.G. Experimental Mitochondria-Targeted DNA Methylation Identifies GpC Methylation, Not CpG Methylation, as Potential Regulator of Mitochondrial Gene Expression. *Scientific Reports 2017 7:1* **2017**, *7*, 1–15, doi:10.1038/s41598-017-00263-z.
122. Mposhi, A.; Van Der Wijst, M.G.P.; Faber, K.N.; Rots, M.G. Regulation of Mitochondrial Gene Expression, the Epigenetic Enigma. *Front Biosci (Landmark Ed)* **2017**, *22*, 1099–1113, doi:10.2741/4535.
123. Manev, H.; Dzitoyeva, S. Progress in Mitochondrial Epigenetics. *Biomol Concepts* **2013**, *4*, 381–389, doi:10.1515/BMC-2013-0005.
124. Hong, E.E.; Okitsu, C.Y.; Smith, A.D.; Hsieh, C.-L. Regionally Specific and Genome-Wide Analyses Conclusively Demonstrate the Absence of CpG Methylation in Human Mitochondrial DNA. *Mol Cell Biol* **2013**, *33*, 2683–2690, doi:10.1128/MCB.00220-13.
125. Mechta, M.; Ingerslev, L.R.; Fabre, O.; Picard, M.; Barrès, R. Evidence Suggesting Absence of Mitochondrial DNA Methylation. *Front Genet* **2017**, *8*, doi:10.3389/FGENE.2017.00166.
126. Shmookler Reis, R.J.; Goldstein, S. Mitochondrial DNA in Mortal and Immortal Human Cells. Genome Number, Integrity, and Methylation. *Journal of Biological Chemistry* **1983**, *258*, 9078–9085, doi:10.1016/s0021-9258(17)44633-3.
127. Rebelo, A.P.; Williams, S.L.; Moraes, C.T. In Vivo Methylation of MtDNA Reveals the Dynamics of Protein-MtDNA Interactions. *Nucleic Acids Res* **2009**, *37*, 6701–6715, doi:10.1093/NAR/GKP727.
128. Dostal, V.; Churchill, M.E.A. Cytosine Methylation of Mitochondrial DNA at CpG Sequences Impacts Transcription Factor A DNA Binding and Transcription. *Biochim Biophys Acta Gene Regul Mech* **2019**, *1862*, 598–607, doi:10.1016/j.bbagrm.2019.01.006.
129. Saini, S.K.; Mangalhara, K.C.; Prakasam, G.; Bamezai, R.N.K. DNA Methyltransferase1 (DNMT1) Isoform3 Methylates Mitochondrial Genome and Modulates Its Biology. *Scientific Reports 2017 7:1* **2017**, *7*, 1–10, doi:10.1038/s41598-017-01743-y.
130. Stoccoro, A.; Coppedè, F. Mitochondrial DNA Methylation and Human Diseases. *Int J Mol Sci* **2021**, *22*, doi:10.3390/IJMS22094594.
131. Gao, J.; Wen, S.; Zhou, H.; Feng, S. De-Methylation of Displacement Loop of Mitochondrial DNA Is Associated with Increased Mitochondrial Copy Number and Nicotinamide Adenine Dinucleotide Subunit 2 Expression in Colorectal Cancer. *Mol Med Rep* **2015**, *12*, 7033–7038, doi:10.3892/MMR.2015.4256.
132. Tong, H.; Zhang, L.; Gao, J.; Wen, S.; Zhou, H.; Feng, S. Methylation of Mitochondrial DNA Displacement Loop Region Regulates Mitochondrial Copy Number in Colorectal Cancer. *Mol Med Rep* **2017**, *16*, 5347–5353, doi:10.3892/MMR.2017.7264.

Chapter 2

Diet, trimethylamine metabolism, and mitochondrial DNA: an observational study

2.1 STATE OF ART

Mitochondria are cellular organelles responsible for energy production, as well as the synthesis of phospholipids, calcium homeostasis, apoptotic activation, and cell death [1]. Remarkably, the mitochondrion is the only mammalian cellular organelle containing an independent genome, which is the circular double-stranded mitochondrial DNA (mtDNA) of ≈16.6 kb, encoding 13 structural subunits of complex I, III, IV, and V, 22 tRNA and two ribosomal RNA genes used for RNA translation [2].

MtDNA can be found in a different copy number (mtDNAcn) depending on the cell and tissue type, as well as the health status of cells [3,4]. Tissues that have greater energy demands naturally have a higher mtDNAcn (i.e., more mtDNA copies per cell). For example, in the heart there are ≈2000–5000 copies of mtDNAcn per nucleus, ≈1000–3000 in skeletal muscles, ≈500–1000 copies in liver, and ≈150–600 copies in blood leukocytes [5]. Despite some authors described a direct correlation between mtDNAcn, mitochondrial number, and functions [6], others reported inverse or no correlations [7], evoking a picture that is more dynamic and complex than initially speculated. Interestingly, beyond its role as a template for the respiratory chain, mtDNA is also actively engaged in triggering the innate immune signaling [8,9]. In spite of the complexity of this context, a robust body of literature associated circulating levels of mtDNAcn to metabolic and cardiovascular health [10–13]. Nevertheless, conflicting results concerning the direction of this association emerged [14], and the reason mtDNAcn is associated to health outcomes is still to be completely elucidated. Remarkably, it has been shown that mtDNA might also be methylated [15–17], and the identification of the mitochondrial isoform of the DNA methyltransferase 1 (DNMT1) supported this hypothesis [18]. Methylation has been measured throughout the mitochondrial genome, in CpG sites but also in CpH (non-CG) dinucleotides [19,20],and differ among tissues and cell types [21,22]. Strand-specific methylation levels in the D-loop area of mtDNA have been found as well and reported to vary in response to environmental stimuli [23–25], including diet [26–28], even though a clear mechanistic role of this epigenetic modification has not been defined yet.

The diet's impact on mitochondrial function has been widely studied [29] due to its role as the powerhouse of the cell and involvement in the metabolism of nutrients [1]. In particular, glucose is metabolized by glycolysis to pyruvate and enters mitochondria to undergo the Krebs cycle. Also, fatty acids enter the mitochondria through carnitine palmitoyltransferase (CPT)-1 and go through β-oxidation to make acetyl coenzyme A, which is further metabolized in the Krebs cycle. The Krebs cycle and β-oxidation produce reducing equivalents, such as NADH and FADH2, which donate electrons to the electron transport chain boosting the ATP synthesis [30]. Thus, the ability to

process these macronutrients is strictly associated to mitochondrial functions. Also, when calorie intake is in excess or the capacity of oxidative phosphorylation is limited, the electron transport is compromised and there is an increased risk to generate reactive oxygen species (ROS). Additionally, food provides substrates and cofactors for mitochondrial enzymes that are essential for mitochondrial functions [1,31]. Among them, single nutrients (including vitamins, fatty acids, minerals, and amino acids) and bioactive compounds present in a balanced diet should be regarded as promoting optimal functioning of the cellular-mitochondrial-metabolic axis [32]. On the other hand, an unhealthy diet (e.g., high in sugar and/or fat) can also affect mitochondrial functions [1]. Trimethylamine-N-oxide (TMAO) is listed among diet-associated metabolites that have been studied for a potentially harmful effect on human health [33]. TMAO is a low-molecular-weight compound formed in the process of oxidation of trimethylamine (TMA), which is primarily produced from nutritional substrates from the metabolism of phosphatidylcholine/choline, carnitine, betaine, dimethylglycine, and ergothioneine by intestinal microbiota in the colon [34]. TMA is absorbed in the gut and then oxidized in the liver by hepatic flavin monooxygenases (FMO1 and FMO3) into TMAO [35]. Circulating levels of TMAO are determined by many factors, such as age, gender, diet, intestinal microbiota composition, kidney function, and liver flavin monooxygenase activity [36]. The evidence suggests that high blood levels of TMAO may constitute potential risk factors for cardiovascular diseases, independent of other, traditional ones [34,37]. Interestingly, several studies indicated that the impact of TMAO on human health might result from its effect on mitochondrial dynamics [38–43].

2.2 AIM OF THE STUDY

The available evidence on the effect of diet, TMA, and TMAO on mtDNA is limited. Only a few studies reporting on the impact of diet on mtDNAcn and methylation have been published to this date [26–28]. Therefore, this research aims to assess whether diet, TMA, and TMAO are associated with levels of mitochondrial DNA copy number and methylation in a group of healthy subjects having extremely healthy or unhealthy dietary patterns.

2.3 MATERIALS AND METHODS

2.3.1 Studied Population and Dietary Assessments

A detailed description of the course of research, recruitment procedure, and inclusion/exclusion criteria were published previously [44,45].In particular, inclusion criteria was age between 31 and 50 years, and either western or healthy dietary pattern evaluated by Easy Diet Screener. All participants were informed about the study and have given their written consent. The study was

approved by the Local Ethic Committee at Poznan´ University of Medical Sciences, number 486/2016. Exclusion criteria were diseases such as diabetes, cancer, gastrointestinal diseases (e.g., inflammatory bowel disease, irritable bowel syndrome, and celiac disease); the use of lipid-profile regulating medications; altered dietary habits in the last 6 months; using probiotics or antibiotics during last 6 months; and being pregnant or lactating. Subsequently, 200 subjects with extreme (healthy or unhealthy) dietary patterns were recruited. Such enrolment ensured high variance in the dietary intake within the group of participants and enabled analysing data in a broad range of intakes. Dietary records from 3 consecutive days were collected. The dietary intakes including relative percentages of energy obtained from proteins (%ENP), carbohydrates (%ENC), simple sugars (%EsimpC), fat (%ENF), saturated (%ESFA), and polyunsaturated (%EPUFA) fatty acids were calculated using the Dieta 6.0 software (National Food and Nutrition Institute, Warsaw, Poland). Additionally, intakes of choline and betaine were calculated using the USDA database for the choline content of common foods [46]. The quality of diet was estimated by establishing the value of the HEI [47,48]. Body composition was assessed by calculating the BMI and measuring the %FM using the whole-body densitometry method with the predicted thoracic gas volume (BodPod, Cosmed, USA). PA) level (1-low, 2-medium, 3-high) was estimated by means of a short form of the international physical activity questionnaire (IPAQ) [49].

2.3.2 Circulating Levels of TMA Metabolites and Precursors

Plasma levels of TMA and TMAO and circulating levels of TMA precursors (choline, l-carnitine, O-acetyl-l-carnitine, betaine) were measured by means of an ultra-high-performance liquid chromatography electrospray ionization mass spectrometry (RP-UHPLC-ESI-MS) analysis performed using Dionex UltiMate 3000 UHPLC, Sunnyvale, CA, USA) coupled to a Bruker maXis impact ultrahigh resolution orthogonal quadrupole-time-of-flight accelerator (qTOF) equipped with an ESI source and operated in a positive ion mode (Bruker Daltonik, Bremen, Germany). The TMA was derivatized with ethyl bromoacetate to form ethyl betaine bromide prior to testing [50]. Then, the isotope dilution analysis was performed. This approach had been already described and validated by Koc et al. [51]. Choline chloride-(trimethyl-D9), l-Carnitine-(methyl-D3) hydrochloride, acetyl-D3- l-carnitine hydrochloride, betaine-1,2–13C2, trimethylamine-D9N-oxide (D9-TMAO), and trimethyl-D9-amine hydrochloride (D9-TMA) were used as internal standards for choline, l-carnitine, O-acetyl-l-carnitine, betaine, TMAO, and TMA, respectively.

2.3.3 DNA Extraction, Quantification of mtDNA Copy Number and DNA Methylation

DNA was extracted from whole blood using the NucleoSpin Blood kit (Macherey-Nagel, Germany). Relative mtDNAcn quantification was performed by means of real-time PCR (Biorad CFX96, USA) considering nDNA as a normalizer, according to the previously published data [10,52]. Since the amount of mtDNA amplified in each sample was normalized to the amount of nDNA, relative mtDNAcn was determined by the ΔCt method ($2^{-\Delta Ct}$). All DNA samples were processed under the same conditions and using an identical DNA extraction method, since its variations might affect the evaluation of mtDNAcn [52,53]. The relative quantification was a commonly used method for mtDNAcn assessment [10,53–59] which was useful to compare the mtDNAcn between different samples in the same study but did not provide an absolute quantification of this parameter. The mitochondrial primers were validated by Fazzini et al. [52] for their specificity (unique amplification of mtDNA) and the absence of coamplified NUMTs. An inter-run calibrator sample was applied to adjust the results obtained from different amplification plates.

Bisulfite pyrosequencing was performed to assess DNA methylation levels in both nuclear (LINE-1) and mitochondrial sequences (LDLR2). DNA methylation in the LINE-1, a repetitive DNA retrotransposon, was regarded to be a surrogate marker of global DNA methylation. Moreover, since the mitochondrial genome lacks histone complexes and retrotransposons (such as LINE-1), this estimation of global DNA methylation referred exclusively to the nuclear and not to the mitochondrial DNA. LINE-1 methylation was determined interrogating the L1Hs sequences as described by Tabish et al. [60]. On the contrary, the selected mitochondrial area was located in proximity to the light-strand transcription promoter, and it was selected because it had been reported that DNA methylation in mtDNA might differ between two strands [24] and levels measured in the light strand may be higher compared to those in the heavy strand [23,61]. Furthermore, D-loop was one of the few regions on mtDNA that was not included in the nuclear DNA as NUMTs [62]. For this reason, NUMTs did not represent significant biases in the analysis of DNA methylation through bisulfite pyrosequencing in this area without previous isolation of the mtDNA from nuclear DNA. This allows simultaneous quantification of nuclear (LINE-1) and mitochondrial DNA methylation in the same sample. To avoid inaccurate bisulfite conversion resulting from the 3D structure of mtDNA, 600ng of DNA were digested with BamHI (New England Biolabs, USA) in accordance with the manufacturer's instruction. Digestion with BamHI was performed in order to linearize mtDNA, thus reducing the risk of falsely positive methylation results [63]. Subsequently, DNA was converted with bisulfite using the EZ-96 DNA Methylation Gold Kit (Zymo Research, Orange, USA) following the manufacturer's instructions. The selected areas were amplified by means of the Pyromark PCR Kit (Qiagen, Germany) and sequenced using the Pyromark

Q24 instrument (Qiagen, Germany) (LDLR2: Fw-5'- TTTTTAGTGTATTGTTTTGAGGAGGTAAGT-3'; Rv-5'-CACTCCCATACTACTAATCTCATCA-3'; Seq-5'-TTTTTGGGGTTTGGT-3'; LINE-1: Fw-5'-TTTTGAGTTAGGTGTGGGATATA-3'; Rv-5'- AAAATCAAAAAATTCCCTTTC-3'; Seq-5'-AGTTAGGTGTGGATATAGT-3'). The degree of methylation was defined as a percentage of methylated cytosines over the sum of methylated and unmethylated cytosines.

2.3.4 Statistical Analysis

Data were presented as means ± standard deviations unless otherwise indicated. The normality of data distribution was evaluated using the Kolmogorov–Smirnov test. Parameters with skewed distributions were appropriately log-transformed before the analyses. Differences between groups were assessed through t-test or ANOVA tests. Association tests were calculated after adjusting for sex, age, FM%, PA (as indicated in the text) through GLM. Pearson's correlation and linear regression were used to test correlations and associations (adjusting for covariates) between continuous variables, respectively. A two-sided p-value under 0.05 was considered statistically significant. Since this study can be described as explorative, the study adjusted for multiple testing and reported nominal correlations as indicated in the text. SPSS [64] and R software (ggplot2 package [65]) were used for statistical analysis and visualization.

2.4 RESULTS

2.4.1 Descriptive Statistics

Detailed characteristics of the study group, including age, smoking status, physical activity (PA), body composition, and dietary intake, were published previously [45]. The group of participants consisted of 100 subjects having a healthy dietary pattern (H group) and 100 individuals with a western (W group) dietary pattern according to the Easy Diet Screener [44]. The mean age of persons under study was 38 ± 5 y/o in the H group and 37 ± 5 y/o in the W group (p = 0.215). 50% of participants were males and 50% were females, equally distributed in the two groups (p = 0.556). With regard to PA, lower levels were recorded in the W group compared to those measured in the H group (p = 0.028). Individuals assigned to the W group were characterized by higher %FM (fat mass), BMI (body mass index), %ESFA (percentage energy from saturated fatty acids), and %EsimpC (percentage energy from simple sugars), as well as lower HEI (healthy eating index), %ENP (percentage energy from proteins), and %EPUFA (percentage energy from polyunsaturated fatty acids) [45].

2.4.2 TMA and TMA Dietary Precursors

Participants with the western dietary pattern had lower TMA levels than participants maintaining a healthy diet (H: 1.87±2.51; W: 1.01±1.13; $p = 5.02 \times 10^{-4}$) (Figure 1A). No significant differences were measured in terms of the TMAO levels between the two groups (H: 5.81 ± 3.13; W: 5.90 ± 5.60; $p = 0.401$) (Figure 1B). The TMAO/TMA ratio was higher in the W group than in the H group (H: 7.59 ± 8.6; W: 8.92 ± 6.76; $p = 0.006$) (Figure 1C).

In terms of the circulating levels of TMA dietary precursors (Table 1), significantly lower levels of plasma l-carnitine were recorded in the W group compared to the H group ($p = 0.001$). l-carnitine circulating levels were correlated with %ENP in both groups (Pearson's correlation = 0.158; $p = 0.025$). No other TMA dietary precursors were significantly associated with %ENP. O-acetyl-l-carnitine ($p = 0.03$) and betaine ($p = 0.05$) plasmatic levels were nominally lower in the W group than in the H group.

Dietary intakes of choline were correlated with circulating choline levels in the overall population (Pearson's correlation = 0.175; $p = 0.013$) but did not differ between the two groups under study (H: 452.83±278.54 mg d^{-1}; 404.21±202.23 mg d^{-1}; $p = 0.331$). On the contrary, betaine intake was not associated with circulating betaine levels (p = 0.972), but it was significantly higher in the W group than in the group maintaining a healthy diet (H: 82.33 ± 75.12 mg d^{-1}; W: 135.78 ± 97.00 mg d^{-1}; $p = 2.1 \times 10^{-5}$). In fact, betaine intake was strongly linked to HEI (Pearson's correlation = −0.388; $p = 1.75 \times 10^{-8}$). Betaine intake, but not choline intake ($p = 0.917$), was also nominally correlated with TMA circulating levels (Pearson's correlation = −0.159; $p = 0.025$) and negatively correlated with %ENP (Pearson's correlation = −0.176; $p = 0.013$), while choline intake showed a strong positive correlation with %ENP (Pearson's correlation = 0.409; $p = 1.7 \times 10^{-9}$). Table S1 in supporting information shows the correlations between circulating levels of TMA precursors and TMA or TMAO circulating levels in the entire cohort A) and the two groups B).

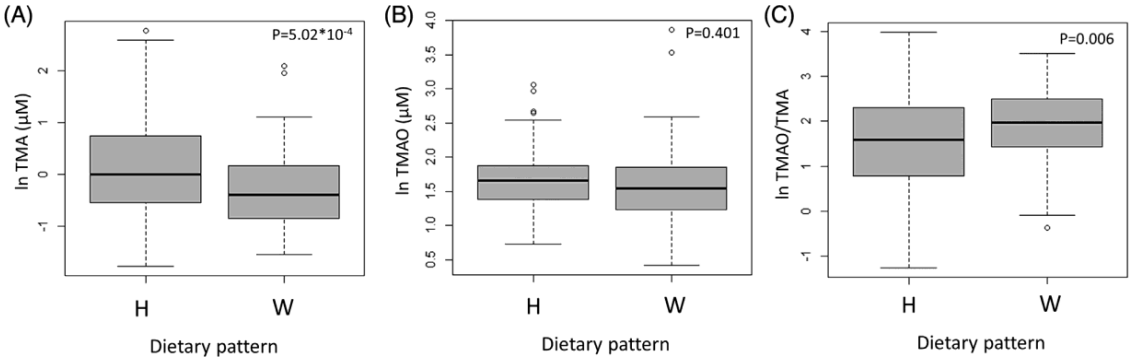


Figure 1. Levels of plasma trimethylamine (TMA) A) trimethylamine-N-oxide (TMAO) B), and the TMAO/TMA ratio C) in groups with healthy (H) and western (W) dietary patterns.

Table 1. Descriptive statistics of levels of the trimethylamine (TMA) dietary precursors in a study group stratified by the dietary patterns.

	Healthy diet group (*N* = 100) Min	Healthy diet group (*N* = 100) Max	Healthy diet group (*N* = 100) Mean	Healthy diet group (*N* = 100) SD	Western diet group (*N* = 100) Min	Western diet group (*N* = 100) Max	Western diet group (*N* = 100) Mean	Western diet group (*N* = 100) SD	*p*
Plasma choline [μM L^{-1}]	5.99	18.31	9.49	2.23	4.29	19.16	9.41	2.28	0.81
Plasma O-acetyl-L-carnitine [μM L^{-1}]	3.75	14.76	7.23	2.11	2.85	13.40	6.62	1.80	0.03
Plasma betaine [μM L^{-1}]	4.59	32.60	15.83	5.57	4.80	34.71	14.40	5.20	0.05
Plasma L-carnitine [μM L^{-1}]	10.92	53.40	26.85	9.75	9.91	48.32	22.49	8.97	**0.001**

Statistically significant results after Bonferroni's multiple correction ($p < 0.05/4 = 0.01$) are highlighted in bold. SD, standard deviation.

2.4.3 PA, but not Dietary Patterns, Associated with mtDNAcn

No difference in mtDNAcn levels was observed between the H and the W diet groups ($p = 0.660$). More in detail, general linear model (GLM) analysis adjusted for age, sex, smoking, and FM% showed that no significant associations occurred between mtDNAcn and the H or W diet group ($p = 0.416$) (Figure 2A). To test whether PA is associated with mtDNAcn (Figure 2B), the GLM analysis was performed on the whole group after adjustment for age, sex, smoking, FM%, and HEI (to exclude confounding effects of diet). In general, mtDNAcn was associated with PA levels ($p = 0.026$). After Bonferroni's correction, mtDNAcn was significantly lower in PA 1 than PA 3 (+18.03%; $p = 0.020$), but not than PA 2 (+12.78%; $p = 0.169$), even though no significant differences were measured between PA 2 and PA 3 (+4.71%; $p = 0.815$). According to our findings, only intense PA appears to be associated with mtDNAcn, which is independent of the overall diet quality ($p = 0.883$). In fact, after adjusting the analysis for factors such as age, sex, smoking, FM%, and PA, mtDNAcn was not associated with HEI ($p = 0.912$), nor with the dietary intake of macronutrients (%ENC (percentage energy from carbohydrates), %ENP, %ENF (percentage energy from fat), %ENA (percentage energy from alcohol); data not shown).

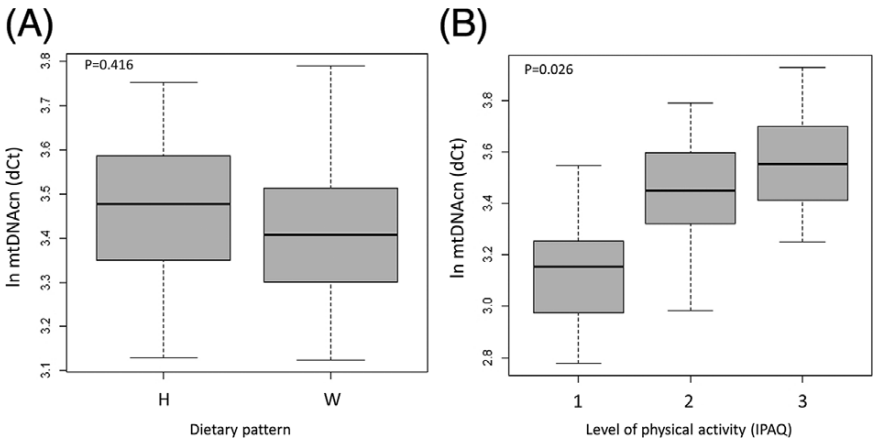


Figure 2. MtDNAcn levels in the population according to healthy (H) and western (W) dietary pattern groups A) or physical activity levels B). Boxplots adjusted for age, sex, smoking, and (fat mass percentage) FM%) or age, sex, smoking, FM%, and healthy eating index (HEI) Overall p-values from the general linear model analysis are shown.

2.4.4 MtDNAcn Association with TMAO/TMA Ratio only in the H Group

After adjusting the analysis for age, sex, smoking, FM%, and PA, mtDNAcn was not associated with TMAO (p = 0.317), TMA (p = 0.138) nor the TMAO/TMA ratio (p = 0.063). To minimize confounding effects resulting from diet, the analysis was repeated after its further adjustment for the circulating levels of TMA precursors (l-carnitine levels, betaine and choline levels, and intakes), a nominal association was detected between mtDNAcn and the TMAO/TMA ratio in the whole group (β = 0.162; p = 0.035). Testing this association in the two dietary pattern subgroups, we found that mtDNAcn was strongly correlated with the TMAO/TMA ratio in the H group (β = 0.298; p = 0.007) but not in the W group (p = 0.493).

2.4.5 D-Loop Methylation and Dietary Factors

The analysis of light-strand D-loop region (LDLR2) showed that the first CpG in this area had higher methylation levels (methylation % = 10.55 ± 3.94) than the second (methylation % = 4.84 ± 3.81) and the third one (methylation % = 5.59 ± 4.75). Mean methylation levels measured in the H group were not significantly different from those in the W group (p = 0.866) (Figure 3A) and were not directly associated with HEI (p = 0.855) after adjusting the analysis for age, sex, smoking, PA, and FM%. Methylation in this mitochondrial area was not directly correlated with mtDNAcn (p = 0.442). D-Loop methylation was neither associated with PA (p = 0.493) (Figure 3B) nor body FM% (p = 0.970). No significant associations with dietary factors that might impact methylation levels (i.e.,

intake of B12, B2, B6 vitamins, or folate) were detected (data not shown). After adjusting the analysis for covariates (age, sex, smoking, FM%, PA, HEI), LDLR2 methylation was nominally associated with %ENC (β = 0.160; p = 0.032) and %ENP (β = −0.150; p = 0.049). Furthermore, the ENP/ENC ratio showed a negative association with LDLR2 methylation (β = −0.168; p = 0.024). However, after adjusting the analysis for circulating levels of TMA precursors (p = 0.066), this relation was no longer significant, which suggests that the association between %ENP/%ENC and LDLR2 methylation might be mediated by the intake of TMA precursors and their metabolism (see the following section).

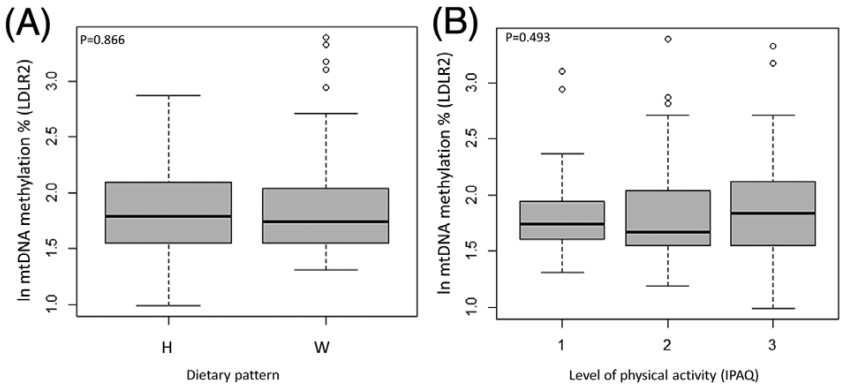


Figure 3. MtDNA methylation levels in groups divided according to healthy (H) or western (W) dietary pattern groups A) or three levels of PA B). Boxplots adjusted for age, sex, smoking, and fat mass percentage (FM%) or age, sex, smoking, FM%, and healthy eating index (HEI) Overall p-values from the general linear model analysis are shown.

2.4.6 D-Loop Methylation Association with the TMAO/TMA Ratio

Pearson's correlation analysis showed that LDLR2 methylation was not correlated with TMA (Pearson's Rho = 0.102; p = 0.154), but significantly correlated with TMAO (Pearson's Rho = −0.212; p = 0.003) and TMAO/TMA (Pearson's Rho = −0.203; p = 0.004). The adjustment of the analysis for age, sex, smoking, FM%, PA, and HEI revealed that LDLR2 methylation was not linked to TMA levels (p = 0.151) and simultaneously negatively associated with TMAO (β = −0.234; p = 0.002) and TMAO/TMA ratio (β = −0.210; p = 0.004) (Figure 4). The association with TMAO/TMA was significant also after adjusting the analysis for the plasma concentrations of TMA precursors and their dietary intakes (β = −0.275; p = 5.1 × 10–4). l-carnitine significantly contributed to the model (β = −0.224; p = 0.004). The association between LDLR2 methylation and the TMAO/TMA ratio was further confirmed after dividing the population according to dietary patterns (in H: β = −0.210; p = 0.049;

in W: β = −0.294; p = 0.006), suggesting an independent effect between the two variables in the cohort.

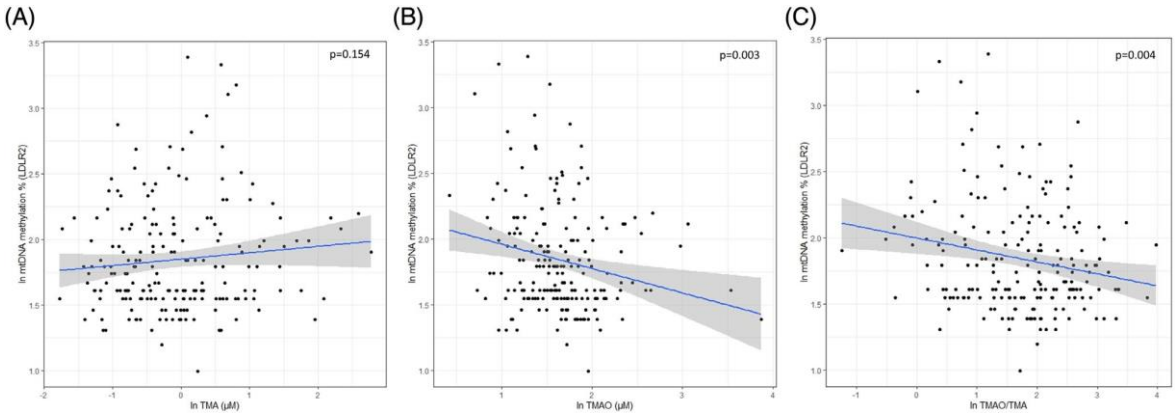


Figure 4. MtDNA methylation and trimethylamine (TMA) metabolites. Scatter plots presenting Pearson's correlations between mtDNA methylation and TMA A), trimethylamine-N-oxide (TMAO) B), or TMAO/TMA C).

2.4.7 Nuclear DNA Methylation (LINE-1), TMAO/TMA, and Dietary TMA Precursors

Long interspersed nuclear element 1 (LINE-1) methylation did not differ between participants with healthy (mean % = 74.64 ± 1.46) and western (mean % = 74.93 ± 1.69) dietary patterns (p = 0.244). After adjusting the analysis for age, sex, FM%, and PA, LINE-1 methylation was not associated with TMA (p = 0.129), TMAO (p = 0.564) nor TMAO/TMA (p = 0.098). Moreover, LINE-1 methylation proved not to be associated with mitochondrial D- loop methylation (β = −0.023; p = 0.742), nor mtDNAcn (p = 0.202).

2.5 DISCUSSION AND CONCLUSIONS

Numerous studies in the last decades have proven the existence of a causal link between Westernized dietary patterns and the increased prevalence of diet-related chronic diseases [66] (e.g., obesity [67], diabetes [68], cardiovascular diseases [69], hypertension [70] and cancer [71,72]). Diet-derived metabolites, which have been indexed as potentially harmful for human health, include TMA [73,74] and TMAO [75–80] that may impact a person's condition also through their impact on mitochondria. Previous studies advocated that TMAO can cause NLRP3 inflammasome activation through inhibition of the SIRT3-SOD2- mitochondrial ROS signaling pathway in human umbilical vein endothelial cells and aortas of ApoE-/- mice [43]. Moreover, it has been suggested that TMAO impairs pyruvate and fatty acid oxidation in cardiac mitochondria [40].

At the same time, mitochondria contribute to the detoxification of trimethylamine N-oxide by the mitochondrial Amidoxime Reducing Component (mARC) [42]. Nevertheless, other findings have shown different effects of TMAO, which protected mitochondrial energy metabolism and cardiac functionality in a rat model of right ventricle heart failure [39]. In fact, the mechanistic role of TMA and TMAO has not been elucidated, and several contrasting hypotheses on its impact on human health have been raised [73,74,81].

In this observational study, a western dietary pattern was associated with lower circulating levels of l-carnitine and TMA, while the TMAO/TMA ratio was higher in the W group compared to the H group. These findings are in line with evidence obtained to this date, which suggests that low TMA levels and high TMAO/TMA ratio may constitute a risk factor for complex multifactorial pathologies, such as cardiovascular diseases [10,82]. Since most of the previous research focused on circulating TMAO levels without measuring TMA, the results of this study support the hypothesis that the measurement of the TMAO/TMA ratio may be more informative than of TMAO alone.

Due to the potential link between TMAO and mitochondria and because of the tight metabolic relationship between diet and these organelles [1], we measured mtDNAcn and methylation in the circulating blood of the recruited individuals. In this study, the dietary pattern was not directly associated with mtDNAcn, despite previous investigations suggesting the possibility that nutrition may modulate mtDNAcn [83–86]. On the other hand, we found a significant association between mtDNAcn and PA levels, with higher mtDNAcn in subjects practicing intense and regular PA. Considering the pivotal role on mitochondria in PA, this finding is consistent with evidence suggesting that low mtDNAcn is a biomarker of mitochondrial dysfunction (correlated with energy reserves, oxidative stress, and changes in mitochondrial membrane potential) [3]. Regardless of the fact that observational studies revealed conflicting results on the direction of the association between mtDNAcn and disease risk (which has been extensively reviewed here [14]), low mtDNAcn has been recently described as a risk factor for type 2 diabetes in a prospective cohort [87]. Further investigations in interventional and longitudinal studies may also facilitate establishing the role of PA in the modulation of this parameter.

Furthermore, this study revealed a mild association between mtDNAcn and TMA. After adjusting the analysis for numerous confounding factors, such as age, sex, %FM, TMA dietary pre- cursors, and overall diet quality (HEI), mtDNAcn was directly associated with the TMAO/TMA ratio, especially in the H group. Since it has been previously suggested that high TMAO may impair energy metabolism and increase the number of mitochondria in vitro [38], it is possible to assume that increased mtDNAcn correlated with high TMAO/TMA levels may be a compensatory reaction to an increased number of mitochondria. In this study, an inverse correlation between the TMAO/TMA ratio and mitochondrial D-Loop methylation was measured for the first time. Changes in mitochondrial D-loop methylation have been evaluated in association with environmental

exposures [88], such as pollutants [23,89,90], and a few pieces of evidence suggested a potential impact of dietary factors on this parameter [28]. However, the influence of the TMAO/TMA ratio on mtDNA methylation had been never investigated before. Despite mtDNA methylation still being a controversial topic [91], we measured significant levels of this phenomenon in the analyzed area in the LDLR2, similar to levels previously measured by Vos et al. [23] in the same area. Since mtDNA methylation is a topic discussed particularly due to methodological issues, we conducted the analyses 1) using bisulphite-pyrosequencing, which is the gold standard technology for DNA methylation analysis at specific DNA loci [92]; 2) by linearizing mtDNA before bisulphite conversion in order to avoid falsely positive outcome resulting from mtDNA supercoiling [63]; 3) by amplifying the area of mtDNA that is not present in the nuclear DNA as a nuclear mitochondrial DNA sequence (NUMT) [23]; and 4) taking into account strand-specific differences that have been recorded for this parameter [23]. This approach increased the reliability of mtDNA methylation data, despite several intrinsic issues associated with this analysis.

Dietary patterns can modulate DNA methylation, as has been previously demonstrated [93–97]. However, there is not much information on the impact of diet on methylation in mitochondrial DNA [28]. In our study, the overall diet quality was not associated with mtDNA methylation levels. Nevertheless, we found that a lower ENP/ENC ratio was nominally correlated with higher mtDNA methylation. After adjusting the analysis for circulating levels of L-carnitine, choline and betaine (dietary TMA precursors), this association was no longer significant. In fact, the strongest association detected in terms of mtDNA methylation involved TMAO and the TMAO/TMA ratio, which were negatively correlated with this parameter. This relation remained significant even after adjusting the analysis for several covariates, including FM%, PA, HEI, and circulating levels of TMA precursors, suggesting an independent association between these metabolisms. Despite the fact that in this study, D-loop methylation levels were not directly associated with mtDNAcn, both mtDNAcn and D-loop methylation were correlated with the TMAO/TMA ratio, which is increased by a western dietary pattern. Interestingly, recent reports have highlighted that a short open reading frame into mtDNA also encodes for three mitochondrial-derived peptides (MDPs; i.e., Humanin, MOTS-c, and SHLP1-6), with a potential role in cell metabolism, inflammation, and response to stressors [98,99]. A tight correlation between glucose metabolism and the activity of MDPs has been shown [100,101]. Also, a cytoprotective effect of MDPs in age-related diseases, including cardiovascular diseases, has been suggested [102]. In senescent primary human fibroblasts, MDPs were involved in the regulation of glycolipids metabolism, with a potential protective effect from atherosclerosis, and a role of mtDNA methylation in senescence has been hypothesized [103]. Due to the correlation between a TMAO/TMA ratio and mtDNA methylation emerged from this study, further mechanistic investigations aimed to assess if diet can affect MDPs synthesis, also through a modulation of mtDNA methylation, might enlighten a new focus for age-

related diseases, such as cardiovascular diseases. Additionally, since methylation might affect the binding of mtDNA with mitochondrial transcription factor A (TFAM) [104], which has a histone-like function and can be post-translationally modified as well [105], further prospectives might also include the evaluation of the impact of diet on mtDNA-TFAM binding and the consequential transcriptional activity of the mitochondrial genome. Finally, since mtDNA is also released and has potentially immunomodulatory functions, it has been suggested that differential methylation signatures and/or hypomethylation may cause mtDNA to appear more "foreign" than "self" [9] thus enlightening an additional role of mtDNA methylation (and environmental factors associated with it) on human health.

This study has several limitations. First, since we analyzed whole blood samples, we could not retrieve data from single-cell types and the complex blood composition might contribute to noise, especially for mtDNAcn measures. Therefore, we were not able to establish whether the measured effects were limited to specific cell types or not. This also reduces the number of mechanistic speculations, especially for epigenetic data that is cell-specific. Further to this point, bisulfite pyrosequencing has some limitations, e.g., assessment of methylation limited to CpGs, preferential amplification of low-methylated sequences, impossibility to distinguish between methylation and hydroxymethylation, potential failure of bisulfite conversion due to three-dimensional structures of the DNA. Despite these limitations, this technology remains the gold standard for targeted analyses of DNA methylation, especially in molecular epidemiology, also because of its high scalability, specificity, and robustness of the results given as a percentage of the overall amplicons. Concerning the samples recruitment, the two dietary pattern groups (H and W) differed not only in dietary intakes but also in PA levels and body composition. To overcome this obstacle, all analyses were adjusted for these confounding factors. Additionally, we cannot exclude other differences between the two groups regarding parameters that we did not measure. On the other hand, one of the advantages of this research is that the variability in terms of dietary patterns was high (including people with extreme dietary patterns), which enabled studying associations of dietary intakes on biological parameters. Moreover, all recruited participants were healthy and of similar age and ethnicity, which reduced the risk of unexpected variation in the population with regard to other parameters.

In conclusion, the emerging connection between diet, TMAO and mitochondrial DNA indicates the need for further replication studies and supports additional investigations aimed to elucidate the mechanistic role of mtDNA methylation in affecting human health.

2.6 REFERENCES

1. García-García, F.J.; Monistrol-Mula, A.; Cardellach, F.; Garrabou, G. Nutrition, Bioenergetics, and Metabolic Syndrome. *Nutrients* **2020**, *12*, doi:10.3390/nu12092785.
2. Anderson, S.; Bankier, A.T.; Barrell, B.G.; de Bruijn, M.H.; Coulson, A.R.; Drouin, J.; Eperon, I.C.; Nierlich, D.P.; Roe, B.A.; Sanger, F.; et al. Sequence and Organization of the Human Mitochondrial Genome. *Nature* **1981**, *290*, 457–465, doi:10.1038/290457a0.
3. Castellani, C.A.; Longchamps, R.J.; Sun, J.; Guallar, E.; Arking, D.E. Thinking Outside the Nucleus: Mitochondrial DNA Copy Number in Health and Disease. *Mitochondrion* **2020**, *53*, 214–223, doi:10.1016/J.MITO.2020.06.004.
4. Mengel-From, J.; Thinggaard, M.; Dalgård, C.; Kyvik, K.O.; Christensen, K.; Christiansen, L. Mitochondrial DNA Copy Number in Peripheral Blood Cells Declines with Age and Is Associated with General Health among Elderly. *Hum Genet* **2014**, *133*, 1149–1159, doi:10.1007/S00439-014-1458-9.
5. Rausser, S.; Trumpff, C.; McGill, M.A.; Junker, A.; Wang, W.; Ho, S.H.; Mitchell, A.; Karan, K.R.; Monk, C.; Segerstrom, S.C.; et al. Mitochondrial Phenotypes in Purified Human Immune Cell Subtypes and Cell Mixtures. *Elife* **2021**, *10*, doi:10.7554/ELIFE.70899.
6. Larsen, S.; Nielsen, J.; Hansen, C.N.; Nielsen, L.B.; Wibrand, F.; Stride, N.; Schroder, H.D.; Boushel, R.; Helge, J.W.; Dela, F.; et al. Biomarkers of Mitochondrial Content in Skeletal Muscle of Healthy Young Human Subjects. *J Physiol* **2012**, *590*, 3349–3360, doi:10.1113/JPHYSIOL.2012.230185.
7. Brinckmann, A.; Weiss, C.; Wilbert, F.; Von Moers, A.; Zwirner, A.; Stoltenburg-Didinger, G.; Wilichowski, E.; Schuelke, M. Regionalized Pathology Correlates with Augmentation of MtDNA Copy Numbers in a Patient with Myoclonic Epilepsy with Ragged-Red Fibers (MERRF-Syndrome). *PLoS One* **2010**, *5*, e13513, doi:10.1371/JOURNAL.PONE.0013513.
8. Riley, J.S.; Tait, S.W. Mitochondrial DNA in Inflammation and Immunity. *EMBO Rep* **2020**, *21*, doi:10.15252/EMBR.201949799.
9. West, A.P.; Shadel, G.S. Mitochondrial DNA in Innate Immune Responses and Inflammatory Pathology. *Nat Rev Immunol* **2017**, *17*, 363–375, doi:10.1038/NRI.2017.21.
10. Bordoni, L.; Petracci, I.; Pelikant-Malecka, I.; Radulska, A.; Piangerelli, M.; Samulak, J.J.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Mitochondrial DNA Copy Number and Trimethylamine Levels in the Blood: New Insights on Cardiovascular Disease Biomarkers. *FASEB J* **2021**, *35*, e21694, doi:10.1096/fj.202100056R.
11. Castellani, C.A.; Longchamps, R.J.; Sumpter, J.A.; Newcomb, C.E.; Lane, J.A.; Grove, M.L.; Bressler, J.; Brody, J.A.; Floyd, J.S.; Bartz, T.M.; et al. Mitochondrial DNA Copy Number Can Influence Mortality and Cardiovascular Disease via Methylation of Nuclear DNA CpGs. *Genome Med* **2020**, *12*, 84–84, doi:10.1186/S13073-020-00778-7.
12. Kaaman, M.; Sparks, L.M.; Van Harmelen, V.; Smith, S.R.; Sjölin, E.; Dahlman, I.; Arner, P. Strong Association between Mitochondrial DNA Copy Number and Lipogenesis in Human White Adipose Tissue. *Diabetologia* **2007**, *50*, 2526–2533, doi:10.1007/S00125-007-0818-6.
13. Tin, A.; Grams, M.E.; Ashar, F.N.; Lane, J.A.; Rosenberg, A.Z.; Grove, M.L.; Boerwinkle, E.; Selvin, E.; Coresh, J.; Pankratz, N.; et al. Association between Mitochondrial DNA Copy Number in Peripheral Blood and Incident CKD in the Atherosclerosis Risk in Communities Study. *J Am Soc Nephrol* **2016**, *27*, 2467–2473, doi:10.1681/ASN.2015060661.
14. Filograna, R.; Mennuni, M.; Alsina, D.; Larsson, N.G. Mitochondrial DNA Copy Number in Human Disease: The More the Better? *FEBS Lett* **2021**, *595*, 976–1002, doi:10.1002/1873-3468.14021.
15. Castegna, A.; Iacobazzi, V.; Infantino, V. The Mitochondrial Side of Epigenetics. *Physiol Genomics* **2015**, *47*, 299–307, doi:10.1152/PHYSIOLGENOMICS.00096.2014.
16. Coppedè, F.; Stoccoro, A. Mitoepigenetics and Neurodegenerative Diseases. *Front Endocrinol (Lausanne)* **2019**, *10*, 86, doi:10.3389/fendo.2019.00086.
17. Iacobazzi, V.; Castegna, A.; Infantino, V.; Andria, G. Mitochondrial DNA Methylation as a Next-Generation Biomarker and Diagnostic Tool. *Mol Genet Metab* **2013**, *110*, 25–34, doi:10.1016/j.ymgme.2013.07.012.
18. Shock, L.S.; Thakkar, P. V; Peterson, E.J.; Moran, R.G.; Taylor, S.M. DNA Methyltransferase 1, Cytosine Methylation, and Cytosine Hydroxymethylation in Mammalian Mitochondria. *Proc Natl Acad Sci U S A* **2011**, *108*, 3630–3635, doi:10.1073/pnas.1012311108.
19. Bellizzi, D.; D'aquila, P.; Scafone, T.; Giordano, M.; Riso, V.; Riccio, A.; Passarino, G. The Control Region of Mitochondrial DNA Shows an Unusual CpG and Non-CpG Methylation Pattern. *DNA Res* **2013**, *20*, 537–547, doi:10.1093/DNARES/DST029.
20. Sun, Z.; Terragni, J.; Borgaro, J.G.; Liu, Y.; Yu, L.; Guan, S.; Wang, H.; Sun, D.; Cheng, X.; Zhu, Z.; et al. High-Resolution Enzymatic Mapping of Genomic 5-Hydroxymethylcytosine in Mouse Embryonic Stem Cells. *Cell Rep* **2013**, *3*, 567–576, doi:10.1016/j.celrep.2013.01.001.
21. Sirard, M.-A. Distribution and Dynamics of Mitochondrial DNA Methylation in Oocytes, Embryos and Granulosa Cells. *Sci Rep* **2019**, *9*, 11937, doi:10.1038/s41598-019-48422-8.
22. Stoccoro, A.; Coppedè, F. Mitochondrial DNA Methylation and Human Diseases. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22094594.
23. Vos, S.; Nawrot, T.S.; Martens, D.S.; Byun, H.M.; Janssen, B.G. Mitochondrial DNA Methylation in Placental Tissue: A Proof of Concept Study by Means of Prenatal Environmental Stressors. *Epigenetics* **2021**, *16*, 121, doi:10.1080/15592294.2020.1790923.
24. Dou, X.; Boyd-Kirkup, J.D.; McDermott, J.; Zhang, X.; Li, F.; Rong, B.; Zhang, R.; Miao, B.; Chen, P.; Cheng, H.; et al. The Strand-Biased Mitochondrial DNA Methylome and Its Regulation by DNMT3A. *Genome Res* **2019**, *29*, 1622–1634, doi:10.1101/gr.234021.117.
25. Shukla, A.; Bunkar, N.; Kumar, R.; Bhargava, A.; Tiwari, R.; Chaudhury, K.; Goryacheva, I.Y.; Mishra, P.K. Air Pollution Associated Epigenetic Modifications: Transgenerational Inheritance and Underlying Molecular Mechanisms. *Sci Total Environ* **2019**, *656*, 760–777, doi:10.1016/j.scitotenv.2018.11.381.
26. Bordoni, L.; Sawicka, A.K.; Szarmach, A.; Winklewski, P.J.; Olek, R.A.; Gabbianelli, R. A Pilot Study on the Effects of L-Carnitine and Trimethylamine-N-Oxide on Platelet Mitochondrial DNA Methylation and CVD Biomarkers in Aged Women. *Int J Mol Sci* **2020**, *21*, doi:10.3390/ijms21031047.
27. Bordoni, L.; Smerilli, V.; Nasuti, C.; Gabbianelli, R. Mitochondrial DNA Methylation and Copy Number Predict Body Composition in a Young Female Population. *J Transl Med* **2019**, *17*, 1–11, doi:10.1186/S12967-019-02150-9/FIGURES/6.

28. Corsi, S.; Iodice, S.; Shannon, O.; Siervo, M.; Mathers, J.; Bollati, V.; Byun, H.-M. Mitochondrial DNA Methylation Is Associated with Mediterranean Diet Adherence in a Population of Older Adults with Overweight and Obesity. *Proceedings of the Nutrition Society* **2020**, *79*, E95, doi:10.1017/S0029665120000439.
29. Wesselink, E.; Koekkoek, W.A.C.; Grefte, S.; Witkamp, R.F.; van Zanten, A.R.H. Feeding Mitochondria: Potential Role of Nutritional Components to Improve Critical Illness Convalescence. *Clin Nutr* **2019**, *38*, 982–995, doi:10.1016/J.CLNU.2018.08.032.
30. Saraste, M. Oxidative Phosphorylation at the Fin de Siècle. *Science* **1999**, *283*, 1488–1493, doi:10.1126/SCIENCE.283.5407.1488.
31. Bordoni, L.; Gabbianelli, R. Mitochondrial DNA and Neurodegeneration: Any Role for Dietary Antioxidants? *Antioxidants (Basel)* **2020**, *9*, doi:10.3390/antiox9080764.
32. F C Lopes, A. Mitochondrial Metabolism and DNA Methylation: A Review of the Interaction between Two Genomes. *Clin Epigenetics* **2020**, *12*, 182, doi:10.1186/s13148-020-00976-5.
33. Thomas, M.S.; Fernandez, M.L. Trimethylamine N-Oxide (TMAO), Diet and Cardiovascular Disease. *Curr Atheroscler Rep* **2021**, *23*, 12, doi:10.1007/s11883-021-00910-x.
34. Cho, C.E.; Caudill, M.A. Trimethylamine-N-Oxide: Friend, Foe, or Simply Caught in the Cross-Fire? *Trends Endocrinol Metab* **2017**, *28*, 121–130, doi:10.1016/j.tem.2016.10.005.
35. Bennett, B.J.; de Aguiar Vallim, T.Q.; Wang, Z.; Shih, D.M.; Meng, Y.; Gregory, J.; Allayee, H.; Lee, R.; Graham, M.; Crooke, R.; et al. Trimethylamine-N-Oxide, a Metabolite Associated with Atherosclerosis, Exhibits Complex Genetic and Dietary Regulation. *Cell Metab* **2013**, *17*, 49–60, doi:10.1016/j.cmet.2012.12.011.
36. Gatarek, P.; Kaluzna-Czaplinska, J. Trimethylamine N-Oxide (TMAO) in Human Health. *EXCLI J* **2021**, *20*, 301–319, doi:10.17179/excli2020-3239.
37. Zhou, W.; Cheng, Y.; Zhu, P.; Nasser, M.I.; Zhang, X.; Zhao, M. Implication of Gut Microbiota in Cardiovascular Diseases. *Oxid Med Cell Longev* **2020**, *2020*, 5394096, doi:10.1155/2020/5394096.
38. Savi, M.; Bocchi, L.; Bresciani, L.; Falco, A.; Quaini, F.; Mena, P.; Brighenti, F.; Crozier, A.; Stilli, D.; Del Rio, D. Trimethylamine-N-Oxide (TMAO)-Induced Impairment of Cardiomyocyte Function and the Protective Role of Urolithin B-Glucuronide. *Molecules* **2018**, *23*, doi:10.3390/molecules23030549.
39. Videja, M.; Vilskersts, R.; Korzh, S.; Cirule, H.; Sevostjanovs, E.; Dambrova, M.; Makrecka-Kuka, M. Microbiota-Derived Metabolite Trimethylamine N-Oxide Protects Mitochondrial Energy Metabolism and Cardiac Functionality in a Rat Model of Right Ventricle Heart Failure. *Front Cell Dev Biol* **2020**, *8*, 622741, doi:10.3389/fcell.2020.622741.
40. Makrecka-Kuka, M.; Volska, K.; Antone, U.; Vilskersts, R.; Grinberga, S.; Bandere, D.; Liepinsh, E.; Dambrova, M. Trimethylamine N-Oxide Impairs Pyruvate and Fatty Acid Oxidation in Cardiac Mitochondria. *Toxicol Lett* **2017**, *267*, 32–38, doi:10.1016/j.toxlet.2016.12.017.
41. Vallance, H.D.; Koochin, A.; Branov, J.; Rosen-Heath, A.; Bosdet, T.; Wang, Z.; Hazen, S.L.; Horvath, G. Marked Elevation in Plasma Trimethylamine-N-Oxide (TMAO) in Patients with Mitochondrial Disorders Treated with Oral l-Carnitine. *Mol Genet Metab Rep* **2018**, *15*, 130–133, doi:10.1016/j.ymgmr.2018.04.005.
42. Schneider, J.; Girreser, U.; Havemeyer, A.; Bittner, F.; Clement, B. Detoxification of Trimethylamine N-Oxide by the Mitochondrial Amidoxime Reducing Component MARC. *Chem Res Toxicol* **2018**, *31*, 447–453, doi:10.1021/acs.chemrestox.7b00329.
43. Chen, M.-L.; Zhu, X.-H.; Ran, L.; Lang, H.-D.; Yi, L.; Mi, M.-T. Trimethylamine-N-Oxide Induces Vascular Inflammation by Activating the NLRP3 Inflammasome Through the SIRT3-SOD2-MtROS Signaling Pathway. *J Am Heart Assoc* **2017**, *6*, doi:10.1161/JAHA.117.006347.
44. Malinowska, A.M. Easy Diet Screener: A Quick and Easy Tool for Determining Dietary Patterns Associated with Lipid Profile and Body Adiposity. *Journal of Human Nutrition and Dietetics* **2022**, *35*, 590–604, doi:10.1111/JHN.12973.
45. Malinowska, A.M.; Schmidt, M.; Kok, D.E.; Chmurzynska, A. Ex Vivo Folate Production by Fecal Bacteria Does Not Predict Human Blood Folate Status: Associations between Dietary Patterns, Gut Microbiota, and Folate Metabolism. *Food Res Int* **2022**, *156*, doi:10.1016/J.FOODRES.2022.111290.
46. USDA Database for the Choline Content of Common Foods, Release 2 (2008) | Ag Data Commons Available online: https://data.nal.usda.gov/dataset/usda-database-choline-content-common-foods-release-2-2008 (accessed on 26 June 2023).
47. Reedy, J.; Lerman, J.L.; Krebs-Smith, S.M.; Kirkpatrick, S.I.; Pannucci, T.R.E.; Wilson, M.M.; Subar, A.F.; Kahle, L.L.; Tooze, J.A. Evaluation of the Healthy Eating Index-2015. *J Acad Nutr Diet* **2018**, *118*, 1622, doi:10.1016/J.JAND.2018.05.019.
48. Krebs-Smith, S.M.; Pannucci, T.R.E.; Subar, A.F.; Kirkpatrick, S.I.; Lerman, J.L.; Tooze, J.A.; Wilson, M.M.; Reedy, J. Update of the Healthy Eating Index: HEI-2015. *J Acad Nutr Diet* **2018**, *118*, 1591–1602, doi:10.1016/J.JAND.2018.05.021.
49. Krebs-Smith, C.L.; Marshall, A.L.; Sjöström, M.; Bauman, A.E.; Booth, M.L.; Ainsworth, B.E.; Pratt, M.; Ekelund, U.; Yngve, A.; Sallis, J.F.; et al. International Physical Activity Questionnaire: 12-Country Reliability and Validity. *Med Sci Sports Exerc* **2003**, *35*, 1381–1395, doi:10.1249/01.MSS.0000078924.61453.FB.
50. Johnson, D.W. A Flow Injection Electrospray Ionization Tandem Mass Spectrometric Method for the Simultaneous Measurement of Trimethylamine and Trimethylamine N-Oxide in Urine. *J Mass Spectrom* **2008**, *43*, 495–499, doi:10.1002/JMS.1339.
51. Koc, H.; Mar, M.H.; Ranasinghe, A.; Swenberg, J.A.; Zeisel, S.H. Quantitation of Choline and Its Metabolites in Tissues and Foods by Liquid Chromatography/Electrospray Ionization-Isotope Dilution Mass Spectrometry. *Anal Chem* **2002**, *74*, 4734–4740, doi:10.1021/AC025624X.
52. Fazzini, F.; Schöpf, B.; Blatzer, M.; Coassin, S.; Hicks, A.A.; Kronenberg, F.; Fendt, L. Plasmid-Normalized Quantification of Relative Mitochondrial DNA Copy Number. *Sci Rep* **2018**, *8*, 15347, doi:10.1038/s41598-018-33684-5.
53. Longchamps, R.J.; Castellani, C.A.; Yang, S.Y.; Newcomb, C.E.; Sumpter, J.A.; Lane, J.; Grove, M.L.; Guallar, E.; Pankratz, N.; Taylor, K.D.; et al. Evaluation of Mitochondrial DNA Copy Number Estimation Techniques. *PLoS One* **2020**, *15*, doi:10.1371/JOURNAL.PONE.0228166.
54. Koller, A.; Fazzini, F.; Lamina, C.; Rantner, B.; Kollerits, B.; Stadler, M.; Klein-Weigel, P.; Fraedrich, G.; Kronenberg, F. Mitochondrial DNA Copy Number Is Associated with All-Cause Mortality and Cardiovascular Events in Patients with Peripheral Arterial Disease. *J Intern Med* **2020**, *287*, 569–579, doi:10.1111/JOIM.13027.
55. Fazzini, F.; Lamina, C.; Raftopoulou, A.; Koller, A.; Fuchsberger, C.; Pattaro, C.; Del Greco, F.M.; Döttelmayer, P.; Fendt, L.; Fritz, J.; et al. Association of Mitochondrial DNA Copy Number with Metabolic Syndrome and Type 2 Diabetes in 14 176 Individuals. *J Intern Med* **2021**, *290*, 190–202, doi:10.1111/JOIM.13242.

56. Alejandro Alegría Torres, J. The Mitochondrial DNA Copy Number Used as Biomarker. *International Journal of Molecular Biology: Open Access* **2018**, *Volume 3*, doi:10.15406/IJMBOA.2018.03.00063.
57. Al-Kafaji, G.; Aljadaan, A.; Kamal, A.; Bakhiet, M. Peripheral Blood Mitochondrial DNA Copy Number as a Novel Potential Biomarker for Diabetic Nephropathy in Type 2 Diabetes Patients. *Exp Ther Med* **2018**, *16*, 1483, doi:10.3892/ETM.2018.6319.
58. Palmeira, C.M.; Rolo, A.P. Mitochondrial Regulation: Methods and Protocols. *Mitochondrial Regulation: Methods and Protocols* **2014**, 1–194, doi:10.1007/978-1-4939-1875-1/COVER.
59. Malik, A.N.; Czajka, A. Is Mitochondrial DNA Content a Potential Biomarker of Mitochondrial Dysfunction? *Mitochondrion* **2013**, *13*, 481–492, doi:10.1016/J.MITO.2012.10.011.
60. Tabish, A.M.; Baccarelli, A.A.; Godderis, L.; Barrow, T.M.; Hoet, P.; Byun, H.M. Assessment of Changes in Global DNA Methylation Levels by Pyrosequencing® of Repetitive Elements. *Methods Mol Biol* **2015**, *1315*, 201–207, doi:10.1007/978-1-4939-2715-9_15.
61. Bordoni, L.; Petracci, I.; Mlodzik-Czyzewska, M.; Malinowska, A.M.; Szwengiel, A.; Sadowski, M.; Gabbianelli, R.; Chmurzynska, A. Mitochondrial DNA and Epigenetics: Investigating Interactions with the One-Carbon Metabolism in Obesity. *Oxid Med Cell Longev* **2022**, *2022*, 9171684, doi:10.1155/2022/9171684.
62. Tsuji, J.; Frith, M.C.; Tomii, K.; Horton, P. Mammalian NUMT Insertion Is Non-Random. *Nucleic Acids Res* **2012**, *40*, 9073–9088, doi:10.1093/NAR/GKS424.
63. Liu, B.; Du, Q.; Chen, L.; Fu, G.; Li, S.; Fu, L.; Zhang, X.; Ma, C.; Bin, C. CpG Methylation Patterns of Human Mitochondrial DNA. *Sci Rep* **2016**, *6*, doi:10.1038/SREP23421.
64. IBM Corp. (2013) IBM SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY. - References - Scientific Research Publishing Available online: https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=2010524 (accessed on 26 June 2023).
65. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis.* ; 2nd ed.; Springer New York, 2009;
66. Cena, H.; Calder, P.C. Defining a Healthy Diet: Evidence for The Role of Contemporary Dietary Patterns in Health and Disease. *Nutrients* **2020**, *12*, doi:10.3390/NU12020334.
67. Seifu, C.N.; Fahey, P.P.; Hailemariam, T.G.; Frost, S.A.; Atlantis, E. Dietary Patterns Associated with Obesity Outcomes in Adults: An Umbrella Review of Systematic Reviews. *Public Health Nutr* **2021**, *24*, 6390–6414, doi:10.1017/S1368980021000823.
68. Guo, Y.; Huang, Z.; Sang, D.; Gao, Q.; Li, Q. The Role of Nutrition in the Prevention and Intervention of Type 2 Diabetes. *Front Bioeng Biotechnol* **2020**, *8*, 575442, doi:10.3389/fbioe.2020.575442.
69. Casas, R.; Castro-Barquero, S.; Estruch, R.; Sacanella, E. Nutrition and Cardiovascular Health. *Int J Mol Sci* **2018**, *19*, doi:10.3390/ijms19123988.
70. Bazzano, L.A.; Green, T.; Harrison, T.N.; Reynolds, K. Dietary Approaches to Prevent Hypertension. *Curr Hypertens Rep* **2013**, *15*, 694–702, doi:10.1007/s11906-013-0390-z.
71. Nasir, A.; Bullo, M.M.H.; Ahmed, Z.; Imtiaz, A.; Yaqoob, E.; Jadoon, M.; Ahmed, H.; Afreen, A.; Yaqoob, S. Nutrigenomics: Epigenetics and Cancer Prevention: A Comprehensive Review. *Crit Rev Food Sci Nutr* **2020**, *60*, 1375–1387, doi:10.1080/10408398.2019.1571480.
72. Mayne, S.T.; Playdon, M.C.; Rock, C.L. Diet, Nutrition, and Cancer: Past, Present and Future. *Nat Rev Clin Oncol* **2016**, *13*, 504–515, doi:10.1038/nrclinonc.2016.24.
73. Jaworska, K.; Bielinska, K.; Gawrys-Kopczynska, M.; Ufnal, M. TMA (Trimethylamine), but Not Its Oxide TMAO (Trimethylamine-Oxide), Exerts Haemodynamic Effects: Implications for Interpretation of Cardiovascular Actions of Gut Microbiome. *Cardiovasc Res* **2019**, *115*, 1948–1949, doi:10.1093/cvr/cvz231.
74. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kapłon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins (Basel)* **2019**, *11*, doi:10.3390/toxins11090490.
75. He, M.; Tan, C.P.; Xu, Y.J.; Liu, Y. Gut Microbiota-Derived Trimethylamine-N-Oxide: A Bridge between Dietary Fatty Acid and Cardiovascular Disease? *Food Res Int* **2020**, *138*, doi:10.1016/J.FOODRES.2020.109812.
76. Farhangi, M.A.; Vajdi, M. Novel Findings of the Association between Gut Microbiota-Derived Metabolite Trimethylamine N-Oxide and Inflammation: Results from a Systematic Review and Dose-Response Meta-Analysis. *Crit Rev Food Sci Nutr* **2020**, *60*, 2801–2823, doi:10.1080/10408398.2020.1770199.
77. Papandreou, C.; Moré, M.; Bellamine, A. Trimethylamine N-Oxide in Relation to Cardiometabolic Health—Cause or Effect? *Nutrients 2020, Vol. 12, Page 1330* **2020**, *12*, 1330, doi:10.3390/NU12051330.
78. Vogt, N.M.; Romano, K.A.; Darst, B.F.; Engelman, C.D.; Johnson, S.C.; Carlsson, C.M.; Asthana, S.; Blennow, K.; Zetterberg, H.; Bendlin, B.B.; et al. The Gut Microbiota-Derived Metabolite Trimethylamine N-Oxide Is Elevated in Alzheimer's Disease. *Alzheimers Res Ther* **2018**, *10*, doi:10.1186/S13195-018-0451-2.
79. Heianza, Y.; Ma, W.; Manson, J.A.E.; Rexrode, K.M.; Qi, L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. *J Am Heart Assoc* **2017**, *6*, doi:10.1161/JAHA.116.004947.
80. Qi, J.; You, T.; Li, J.; Pan, T.; Xiang, L.; Han, Y.; Zhu, L. Circulating Trimethylamine N-Oxide and the Risk of Cardiovascular Diseases: A Systematic Review and Meta-Analysis of 11 Prospective Cohort Studies. *J Cell Mol Med* **2018**, *22*, 185–194, doi:10.1111/JCMM.13307.
81. Landfald, B.; Valeur, J.; Berstad, A.; Raa, J. Microbial Trimethylamine- N-Oxide as a Disease Marker: Something Fishy? *Microb Ecol Health Dis* **2017**, *28*, 1327309, doi:10.1080/16512235.2017.1327309.
82. Roncal, C.; Martínez-Aguilar, E.; Orbe, J.; Ravassa, S.; Fernandez-Montero, A.; De Pipaon, G.S.; Ugarte, A.; Estella-Hermoso de Mendoza, A.; Rodriguez, J.A.; Fernández-Alonso, S.; et al. Trimethylamine (Tma) And Trimethylamine-N-Oxide (Tmao) As Predictors Of Cardiovascular Mortality In Peripheral Artery Disease. *Atherosclerosis* **2019**, *287*, e233, doi:10.1016/j.atherosclerosis.2019.06.716.
83. Hernández-Ríos, R.; Hernández-Estrada, S.; Cruz-Robles, D.; Hernández-Lobato, S.; Villalobos-Martín, M.; Johnson, R.J.; Rodríguez-Castellanos, F.; Salazar, J.; García-Arroyo, F.; Sánchez-Lozada, L.G.; et al. Low Fructose and Low Salt Diets Increase Mitochondrial DNA in White Blood Cells of Overweight Subjects. *Exp Clin Endocrinol Diabetes* **2013**, *121*, 535–538, doi:10.1055/S-0033-1349144.
84. Liao, K.; Yan, J.; Mai, K.; Ai, Q. Dietary Lipid Concentration Affects Liver Mitochondrial DNA Copy Number, Gene Expression and DNA Methylation in Large Yellow Croaker (Larimichthys Crocea). *Comp Biochem Physiol B Biochem Mol Biol* **2016**, *193*, 25–32, doi:10.1016/J.CBPB.2015.11.012.

85. Cioffi, F.; Senese, R.; Lasala, P.; Ziello, A.; Mazzoli, A.; Crescenzo, R.; Liverini, G.; Lanni, A.; Goglia, F.; Iossa, S. Fructose-Rich Diet Affects Mitochondrial DNA Damage and Repair in Rats. *Nutrients* **2017**, *9*, doi:10.3390/NU9040323.
86. Bai, Y.; Carrillo, J.A.; Li, Y.; He, Y.; Song, J. Diet Induced the Change of MtDNA Copy Number and Metabolism in Angus Cattle. *J Anim Sci Biotechnol* **2020**, *11*, 1–13, doi:10.1186/S40104-020-00482-X/FIGURES/6.
87. Memon, A.A.; Sundquist, J.; Hedelius, A.; Palmér, K.; Wang, X.; Sundquist, K. Association of Mitochondrial DNA Copy Number with Prevalent and Incident Type 2 Diabetes in Women: A Population-Based Follow-up Study. *Sci Rep* **2021**, *11*, 4608, doi:10.1038/S41598-021-84132-W.
88. Sharma, N.; Pasala, M.S.; Prakash, A. Mitochondrial DNA: Epigenetics and Environment. *Environ Mol Mutagen* **2019**, *60*, 668–682, doi:10.1002/EM.22319.
89. Janssen, B.G.; Byun, H.M.; Gyselaers, W.; Lefebvre, W.; Baccarelli, A.A.; Nawrot, T.S. Placental Mitochondrial Methylation and Exposure to Airborne Particulate Matter in the Early Life Environment: An ENVIRONAGE Birth Cohort Study. *Epigenetics* **2015**, *10*, 536–544, doi:10.1080/15592294.2015.1048412.
90. Byun, H.M.; Colicino, E.; Trevisi, L.; Fan, T.; Christiani, D.C.; Baccarelli, A.A. Effects of Air Pollution and Blood Mitochondrial DNA Methylation on Markers of Heart Rate Variability. *J Am Heart Assoc* **2016**, *5*, doi:10.1161/JAHA.116.003218.
91. van der Wijst, M.G.P.; Rots, M.G. Mitochondrial Epigenetics: An Overlooked Layer of Regulation? *Trends Genet* **2015**, *31*, 353–356, doi:10.1016/J.TIG.2015.03.009.
92. Poulin, M.; Zhou, J.Y.; Yan, L.; Shioda, T. Pyrosequencing Methylation Analysis. *Methods Mol Biol* **2018**, *1856*, 283–296, doi:10.1007/978-1-4939-8751-1_17.
93. Barchitta, M.; Maugeri, A.; Lio, R.M.S.; Favara, G.; La Rosa, M.C.; La Mastra, C.; Quattrocchi, A.; Agodi, A. Dietary Patterns Are Associated with Leukocyte LINE-1 Methylation in Women: A Cross-Sectional Study in Southern Italy. *Nutrients* **2019**, *11*, doi:10.3390/NU11081843.
94. 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.; et al. Whole Blood DNA Methylation Signatures of Diet Are Associated With Cardiovascular Disease Risk Factors and All-Cause Mortality. *Circ Genom Precis Med* **2020**, *13*, E002766, doi:10.1161/CIRCGEN.119.002766.
95. Nicoletti, C.F.; Cortes-Oliveira, C.; Noronha, N.Y.; Pinhel, M.A.S.; Dantas, W.S.; Jácome, A.; Marchini, J.S.; Gualano, B.; Crujeiras, A.B.; Nonino, C.B. DNA Methylation Pattern Changes Following a Short-Term Hypocaloric Diet in Women with Obesity. *Eur J Clin Nutr* **2020**, *74*, 1345–1353, doi:10.1038/S41430-020-0660-1.
96. Hibler, E.; Huang, L.; Andrade, J.; Spring, B. Impact of a Diet and Activity Health Promotion Intervention on Regional Patterns of DNA Methylation. *Clin Epigenetics* **2019**, *11*, doi:10.1186/S13148-019-0707-0.
97. Maugeri, A.; Barchitta, M. How Dietary Factors Affect DNA Methylation: Lesson from Epidemiological Studies. *Medicina (B Aires)* **2020**, *56*, 1–26, doi:10.3390/MEDICINA56080374.
98. Conte, M.; Sabbatinelli, J.; Chiariello, A.; Martucci, M.; Santoro, A.; Monti, D.; Arcaro, M.; Galimberti, D.; Scarpini, E.; Bonfigli, A.R.; et al. Disease-Specific Plasma Levels of Mitokines FGF21, GDF15, and Humanin in Type II Diabetes and Alzheimer's Disease in Comparison with Healthy Aging. *Geroscience* **2021**, *43*, 985, doi:10.1007/S11357-020-00287-W.
99. Yang, Y.; Gao, H.; Zhou, H.; Liu, Q.; Qi, Z.; Zhang, Y.; Zhang, J. The Role of Mitochondria-Derived Peptides in Cardiovascular Disease: Recent Updates. *Biomed Pharmacother* **2019**, *117*, doi:10.1016/J.BIOPHA.2019.109075.
100. Wang, X.; Wu, Z.; He, Y.; Zhang, H.; Tian, L.; Zheng, C.; Shang, T.; Zhu, Q.; Li, D.; He, Y. Humanin Prevents High Glucose-Induced Monocyte Adhesion to Endothelial Cells by Targeting KLF2. *Mol Immunol* **2018**, *101*, 245–250, doi:10.1016/J.MOLIMM.2018.07.008.
101. Bhullar, K.S.; Shang, N.; Kerek, E.; Wu, K.; Wu, J. Mitofusion Is Required for MOTS-c Induced GLUT4 Translocation. *Scientific Reports 2021 11:1* **2021**, *11*, 1–12, doi:10.1038/s41598-021-93735-2.
102. Gong, Z.; Tas, E.; Muzumdar, R. Humanin and Age-Related Diseases: A New Link? *Front Endocrinol (Lausanne)* **2014**, *5*, doi:10.3389/FENDO.2014.00210.
103. Kim, S.J.; Mehta, H.H.; Wan, J.; Kuehnemann, C.; Chen, J.; Hu, J.F.; Hoffman, A.R.; Cohen, P. Mitochondrial Peptides Modulate Mitochondrial Function during Cellular Senescence. *Aging* **2018**, *10*, 1239–1256, doi:10.18632/AGING.101463.
104. Dostal, V.; Churchill, M.E.A. Cytosine Methylation of Mitochondrial DNA at CpG Sequences Impacts Transcription Factor A DNA Binding and Transcription. *Biochim Biophys Acta Gene Regul Mech* **2019**, *1862*, 598–607, doi:10.1016/J.BBAGRM.2019.01.006.
105. King, G.A.; Hashemi Shabestari, M.; Taris, K.K.H.; Pandey, A.K.; Venkatesh, S.; Thilagavathi, J.; Singh, K.; Krishna Koppisetti, R.; Temiakov, D.; Roos, W.H.; et al. Acetylation and Phosphorylation of Human TFAM Regulate TFAM-DNA Interactions via Contrasting Mechanisms. *Nucleic Acids Res* **2018**, *46*, 3633–3642, doi:10.1093/NAR/GKY204.

2.7 SUPPORTING INFORMATION

Supplementary Table 1. Correlations between circulating levels of dietary TMA precursors and TMA or TMAO in the entire group (A) and in the healthy and western diet groups (B)

A

		Circulating choline levels	Circulating acetyl-carnitine levels	Circulating betaine levels	Circulating carnitine levels
TMA	Pearson's correlation	-0.135	0.103	-0.002	0.366
	P	0.057	0.147	0.982	**0.000**
TMAO	Pearson's correlation	0.106	-0.002	0.236	0.061
	P	0.137	0.973	**0.001**	0.390

B

			Circulating choline levels	Circulating acetyl-carnitine levels	Circulating betaine levels	Circulating carnitine levels
HEALTHY DIET GROUP	TMA	Pearson's correlation	-0.059	-0.026	-0.051	0.310
		P	0.559	0.800	0.615	**0.002**
	TMAO	Pearson's correlation	-0.001	-0.097	0.260	-0.155
		P	0.993	0.336	**0.009**	0.123
WESTERN DIET GROUP	TMA	Pearson's correlation	-0.255*	0.201	-0.020	0.357
		P	0.010	0.045	0.844	0.**000**
	TMAO	Pearson's correlation	0.192	0.065	0.208	0.212
		P	0.055	0.519	0.**038**	0.034

Chapter 3

Biological age and diet: measuring the impact of lifestyle on a 6CpG-epigenetic clock

3.1 STATE OF ART

Epigenetic marks on DNA and histones are not entirely maintained in somatic cells over aging [1]. This altered epigenomic state, referred to as 'epigenetic drift', contributes to impaired cellular and molecular functions in aged cells [2]. With regards to DNA methylation in mammals, aging is characterized by a global DNA hypomethylation and local changes in the methylation levels of specific DNA loci [3,4]. Global hypomethylation in particular affects highly methylated repeated sequences, such as transposable elements [5,6]. Since CpG sites are particularly enriched at transposable elements (accounting for > 65% of those found throughout the human genome), the analysis of the methylation levels in these areas of the genome has been considered as a surrogate marker of global levels of DNA methylation. Among transposable elements, the long interspersed nuclear elements (LINEs) are those whose methylation can be altered in response to stress, infection, diseases [7] and have been associated to genomic instability [8,9]. Moreover, LINE-1 hypomethylation is permissive for LINE-1 transcription [10] and it is a common condition in aging and age-related diseases [5]. Nevertheless, despite these prevailing tendencies to hypomethylation in aging, DNA could be either hypomethylated or hypermethylated at specific loci [11].

Due to the association between aging and specific DNA methylation patterns [3], it has been suggested that, collectively, changes in DNA methylation at specific sites can be used as an "epigenetic clock" to predict the biological age and the lifespan of an individual [11]. Epigenetic clocks use algorithms to calculate biological age on the basis of a read- out of the extent to which dozens or even hundreds of CpGs across an individual's genome are methylated. Classically, these models are trained to estimate chronological age. While chronological age refers to the time elapsed since the birth of the individual biological age is linked to the decline of biological functions. The deviation measured between the epi- genetic and the chronological age, often referred to as either DeltaAge or age acceleration (AA), tends to be predictive of age-related health outcomes, such as mortality and disease burden. Thus, epigenetic age is thought to be a proxy for biological age, and epigenetic clocks are considered tools able to trace biological aging [12].

Although the biological and chronological ages of an individual with a healthy lifestyle should match, exposition to detrimental environmental factors can increase biological age (i.e., promoting AA). Thus, it has been suggested that the epigenetic clock might help to quantify the impact of risk factors associated to the aging process (i.e., exposure to environmental factors such as unhealthy diet, alcohol use, tobacco, and stress) on the health status, by measuring acceleration or deceleration of the epigenetic age (EA) [13].

Among environmental exposures, diet is one of the most well-described factors able to modulate the epigenome [14]. In particular, the influence of diet on DNA methylation pathways (especially through the regulation of the one-carbon metabolism [15,16]) as well as its impact on the aging process [17–20]have been widely described. Both the overall diet quality and the availability of nutrients involved in the one-carbon cycle (1CC) (i.e., B vitamins, betaine, and choline) might impact the DNA methylation pathways [16] and, consequentially, the epigenetic age. In particular choline, commonly found in dietary sources such as egg yolk, meat, fish, dairy, nuts, and soybean, is not only essential to normal cellular functions (choline is a component of phosphatidylcholine the major cell membrane phospholipid and it is also a precursor of the neurotransmitter acetylcholine) but following oxidation in mitochondria, choline is converted to betaine, an important methyl group donor in the one carbon cycle, which can influence DNA and histone methylation. The transformation of choline to trimethylamine (TMA) by intestinal bacteria can significantly impact health not only through the accumulation of trimethylamine N-oxide (TMAO) (TMAO derives from the hepatic oxidation of TMA and has been linked to increased risk of cardiovascular disease (CVD) and major adverse cardiovascular events [21–23]), but also because it decreases the bioavailability of choline and downstream metabolites involved in one-carbon metabolism, contributing to DNA hypomethylation across multiple tissues [24]. Thus, the microbial choline metabolism disturbs the host epigenome and gene expression which may favor the onset of metabolic diseases and accelerate the aging process [25–27].

Several studies on the impact of diet and lifestyle on the epigenetic clock are available [28–30]. However, contrasting evidence has been collected [31] and different approaches to measure the epigenetic clock have been used. Indeed, several epigenetic clocks have been developed that predict biological age based on the changes in methylation from a tissue or blood at different selected CpGs. One of the first and most popular epigenetic clock was developed by Horvath [12]. The Horvath clock used 353 CpG sites (obtained from publicly available datasets for various human tissues and cell types) to predict biological age. Other clocks have been developed, trying to reduce the number of CpGs used maintaining a good accuracy [13]. Indeed, despite most of the epigenetic clocks rely on the Illumina BeadChip technology, a targeted analysis limited to a few CpGs is more cost-effective, faster, and less dependent on availability of this technology and its specific BeadChip versions. For this reason, a targeted approach to epigenetic age measurement might facilitate the translation of this interesting biomarker towards an easily accessible and more standardized test, as requested by the European in vitro diagnostics regulations (IVDR). Several pyrosequencing-based epigenetic clocks have been proposed for animal models [32] and humans [33,34]. Among them, Han et al. recently elegantly described the possibility to use methylation levels at 6 CpGs to successfully estimate the biological age from human blood DNA samples [35]. To select age associated CpGs, they used a training set of 973 DNAm profiles of healthy human blood samples (1

to 101 years old), derived from 7 different studies and based on the 450 k Illumina BeadChip platform. They selected candidate CpGs either for linear correlation (Pearson's correlation), continuous non-linear association (Spearman's rank-order), or by linear correlation with the logarithm of age. After a careful evaluation and elimination of CpGs that might act as confounding factors, they identified and validated in an independent set, several epigenetic clocks based on a different number of CpGs [35]. Among these, the simpler, but still informative, was the model based on 6 CpGs, which appeared as an easily accessible tool to evaluate the epigenetic age (6CpG-EA).

3.2 AIM OF THE STUDY

Thus, the aim of this study was: 1) to validate the measurement of epigenetic age through a 6- CpG model in an independent cohort of 200 healthy subjects, 2) to test the correlation between the 6-CpG- epigenetic clock and other parameters associated with biological aging (LINE-1 methylation and telomere length (TL)), 3) to test if lifestyle factors are associated with the 6CpG-EA, with a particular focus on diet quality, intake of vitamins involved in the 1CC, and specific food-derived compounds (i.e., TMA and TMAO).

3.3 MATERIALS AND METHODS

3.3.1 Enrolment of study participants, evaluation of body composition and physical activity levels

Participants gave their written informed consent (procedures approved by the Local Ethics Committee at Poznan University of Medical Sciences (number 486/2016)). A detailed description of the course of research, recruitment procedure and inclusion/exclusion criteria has been previously published [36–38]. The mean age of the recruited cohort is 38.2 ± 4.9. Body Mass Index (BMI) was calculated as weight in kilograms (kg) divided by height in meters squared (m^2). Fat mass percentage (%FM) was measured using the whole-body densitometry method with the predicted thoracic gas volume (BodPod, Cosmed, USA). Information about smoking habits were collected. No quantitative data about the number of cigarettes smoked per day were avail- able. Physical activity (PA) level (1-low, 2-medium, 3-high) was estimated by means of a short form of the International Physical Activity Questionnaire (IPAQ) [39].

3.3.2 Dietary records, diet quality and vitamin intake

Dietary records from three consecutive days have been collected. 200 subjects with extreme (healthy or western) dietary patterns were recruited. Such enrolment ensured high variance in the

dietary intake within the group of participants and enabled analysing data in a broad range of intakes. The quality of diet was estimated by establishing the value of the Healthy Eating Index (HEI) [40,41]. The dietary intakes (vitamins B1, B2, B6, B9, B12) were calculated using the Diet 6.0 software (National Food and Nutrition Institute, Warsaw). Intakes of choline and betaine were calculated using the USDA database for the choline content of common foods [42]. Due to its connection with the 1CC, plasma homocysteine was assessed with using high-performance liquid chromatography with DAD detector (Merck Hitachi D-7000) [43,44].

3.3.3 Circulating Levels of TMA Metabolites

Plasma levels of TMA and TMAO were measured by means of an ultra-high-performance liquid chromatography electrospray ionization mass spectrometry (RP-UHPLC-ESI-MS) analysis performed using Dionex UltiMate 3000 UHPLC, Sunnyvale, CA, USA) coupled to Bruker maXis impact ultrahigh resolution orthogonal quadrupole-time-of-flight accelerator (qTOF) equipped with an ESI source and operated in a positive ion mode (Bruker Daltonik, Bremen, Germany). The TMA was derivatized with ethyl bromoacetate to form ethyl betaine bromide prior to testing [45]. Then, the isotope dilution analysis was performed. This approach had been already described and validated by Koc et al. [46].Trimethylamine-D9N-oxide (D9-TMAO), and trimethyl-D9-amine hydrochloride (D9-TMA) were used as internal standards for TMAO and TMA, respectively.

3.3.4 DNA extraction, DNA methylation and TL assessments

DNA was extracted from whole blood using the NucleoSpin Blood Kit (Macherey-Nagel, Germany). Relative TL was determined with a qPCR method normalizing the amount of telomere repeat amplification product to a single copy gene (IFNB1) product, as previously described [47]. 2 ng of gDNA were amplified and the SsoAdvanced Universal SYBR Green Supermix (Biorad) was used to optimize sensitivity and specificity of the assay. An inter-run calibrator was present in all PCR plates. Primers used are shown in Table 1 of supplementary materials (Table 1 S). Bisulfite pyrosequencing was performed to assess DNA methylation levels in long interspersed nuclear element-1 (LINE-1), and the selected CpGs for the epigenetic clock estimation, as previously described [35,48]. DNA was converted with bisulfite using the EZ-96 DNA Methylation Gold Kit (Zymo Research, Orange, USA) following the manufacturer's instructions. The selected areas were amplified by means of the Pyromark PCR Kit (Qiagen, Germany) and sequenced using the Pyromark Q24 instrument (Qiagen, Germany). Primers used are listed in Table 1S of supplementary materials. The CpGs selected for the epigenetic clock were associated with the genes of Elongation Of Very Long Chain Fatty Acids Protein 2 (ELOVL2), Immunoglobulin Superfamily Member 11 (IGSF11), Coiled-Coil Domain-

Containing Protein 102B (CCDC102B), Collagen Type I Alpha 1 Chain (COL1A1), Four And A Half LIM Domains Protein 2 (FHL2), and MEIS1 Antisense RNA 3 (MEIS1-AS3). The methylation percentages of the 6 selected CpGs were used in a multivariable model to calculate the epigenetic age (6CpG-EA), as described by Han et al. [35]. The CpGs identified by Han et al. that have been used to calculate EA in this manuscript are detailed in supplementary materials Table 2S. No transformation of the chronological age was applied since only adult (>30 y/o) were included in this study. AA was defined as the residuals from regressing predicted age on chronological age. Individuals with negative AA are considered to be biologically younger than their chronological age, and vice versa. LINE-1 methylation was determined interrogating the L1Hs sequences as described by Tabish et al. [48].

3.3.5 Statistical analysis

Data are presented as means standard deviations unless otherwise indicated. Correlations between continuous variables were tested with Pearson's or Spearman's correlations for parametric and non- parametric data, respectively. The normality of data distribution was evaluated using the Kolmogorov- Smirnov test. Parameters with skewed distributions were appropriately log-transformed before the analyses. Student's t-test or ANOVA test with Bonferroni's correction were used to test differences between means. General linear model was used to test association between categorical and continuous variables adjusting for covariates. The varimax-rotated Principal Component Analysis (PCA) was used as a technique for dimension reduction in multivariate analysis. A p-value < 0.05 was considered significant throughout the study. The Bonferroni correction was applied to correct p values in multiple comparisons. Since this study can be described as explorative, we reported also nominal correlations, as indicated in the text. IBM SPSS Statistics 18 [49] and R software version 4.0.5 (ggplot2 package [50]) was used for statistical analysis and visualization.

3.4 RESULTS

3.4.1 Descriptive statistics

No significant differences in distribution of age (healthy diet group: 38.6 ± 5.0; western diet group: 37.8 ± 4.8; p = 0.215), genders (50% of males and 50% of females in both groups; p = 0.556) or smokers (14% in the healthy, 18% in the western diet group; p = 0.281) were measured between the healthy and the western diet group. Higher prevalence of sedentary people was in the western diet group than in the healthy diet group (p = 0.028). The overall dietary quality measured with the Healthy Eating Index (HEI) was highly different between the two groups (healthy diet group: 76.90

± 9.21; western diet group: 58.71 ± 10.47; $p = 1.8*10^{-28}$). Descriptive statistics for micronutrient intakes and blood homocysteine and have been previously reported [36,51] and are summarized in Table 3S. Significantly lower intakes of B2, B6, B9 and higher intakes of betaine were detected in the western than in the healthy diet group (Table 3S). A nominally higher homocysteine level was detected in the western diet group than in the healthy diet group (Table 3S). Circulating TMA and TMAO levels have been previously reported [38] Lower TMA levels were measured in subjects following the western dietary pattern compared to those having a healthy diet (H: 1.87±2.51; W: 1.01±1.13; $p = 5.02 \times 10^{-4}$). No significant differences were measured in terms of the TMAO levels between the two groups (H: 5.81 ± 3.13; W: 5.90 ± 5.60; $p = 0.401$). The TMAO/TMA ratio was higher in the W group than in the H group (H: 7.59 ± 8.6; W: 8.92 ± 6.76; $p = 0.006$) (Figure 1S).

3.4.2 The 6CpG-epigenetic clock correlates with chronological age and other molecular hallmarks of aging (TL, LINE-1 methylation)

Mean 6CpG-EA in the whole group was 40.19 ± 7.79. 6CpG-EA was highly correlated with chronological age in the whole study group (r = 0.591; $p = 7.2*10^{-20}$, Mean Absolute Error, MAE = 4.85) (Figure 1A). Results were similar when correlation was tested with the logarithm of age (r = 0.588; $p = 1.3*10^{-19}$), which might be more suitable in young populations [52]. The 6CpG-EA was significantly lower in males than in females (mean diff: –3.05 ± 1.09; $p = 0.001$), despite no significant differences were measured for chronological age between the two groups ($p = 0.204$) (Figure 2S). The 6CpG-EA was inversely correlated with TL (r = –0.150; $p = 0.040$) (Figure 1B) and LINE-1 methylation (r = –0.240; $p = 0.001$) (Figure 1C). Mean AA was 2.04 ± 6.29 in the whole group. It was correlated with LINE-1 methylation (r = –0.217; $p = 0.002$), but not with telomere length (r = –0.051; $p = 0.483$).

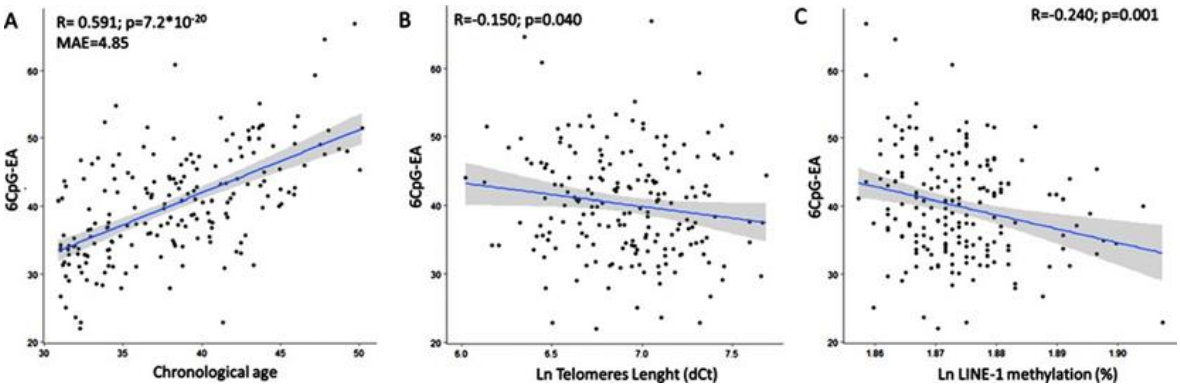


Figure 1. The 6CpG-EA is highly correlated with the chronological age (A). The 6CpG-EA is negatively correlated with telomeres length (B) and LINE-1 methylation levels (C). MAE: Mean Absolute Error.

3.4.3 The 6CpG-EA is not associated with smoking, physical activity, and body composition

Neither the 6CpG-EA (p = 0.720) nor the AA (p = 0.737) were different between smokers and non-smokers. Neither the 6CpG-EA (p = 0.561) (Figure 2A) nor the AA (p = 0.745) were different in subjects with low, medium, and high levels of physical activity. The 6CpG-EA was positively correlated with FM% (r = 0.149; p = 0.037) (Figure 2B), despite FM% was not correlated to the chronological age (r = 0.109; p = 0.124) (Figure 2C). However, there was no correlation between FM% and AA (p = 0.151).

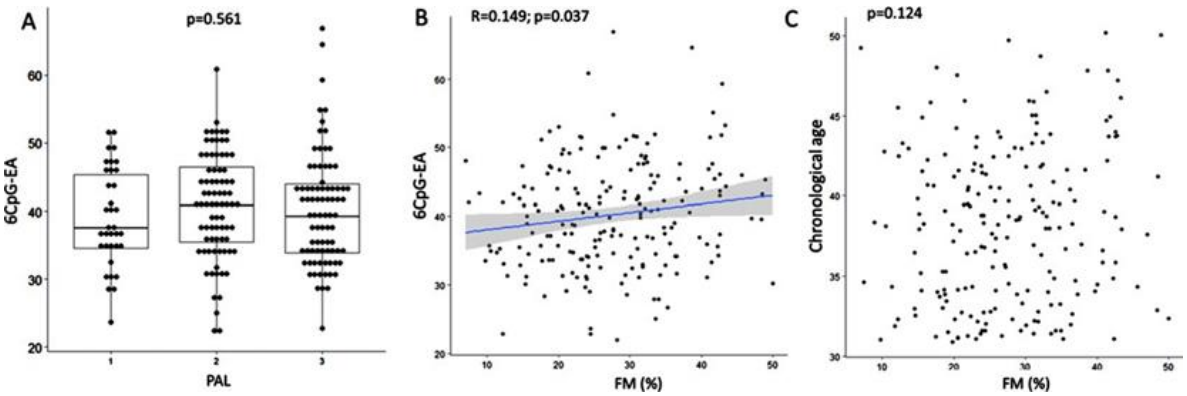


Figure 2. Boxplots show 6CpG-EA differences among individuals with different physical activity levels (A). Correlation between FM% and 6CpG-EA (B) and chronological age (C) are shown. FM: fat mass; PAL: physical activity level (1 = low; 2 = medium; 3 = high) measured by the International Physical Activity Questionnaire (IPAQ).

3.4.4 The 6CpG-EA is correlated with vitamins' intake

Biological age measured with the 6CpG-epigenetic clock (6CpG-EA) was 40.22 ± 7.6 in the healthy and 40.17 ± 8.03 (p = 0.966) in the western group (Figure 3A). No significant differences in AA were measured between the two groups (healthy diet group: 1.58 ± 6.52; western diet group: 2.51 ± 6.04; p = 0.304). No correlation was detected between the overall diet quality (HEI) and 6CpG-EA (r = 0.015; p = 0.836) (Figure 3B) or AA (r = –0.010; p = 0.873). Homocysteine blood level was not correlated to the 6CpG-EA in the whole sample (r = –0.136; p = 0.057). Remarkably, the 6CpG-EA and AA were nominally correlated with the intake of several nutrients involved in the 1CC, which were not correlated to the chronological age (Table 1). Of note, the intakes of these nutrients were significantly different between genders (Table 4S). Evaluating the association between the 6CpG-EA and genders adjusting the analysis for the intake of these nutrients, the difference in the epigenetic age previously detected between males and females was no more significant (p = 0.131). This suggests that intakes of nutrients involved in 1CC rather than sex is a determinant of the 6CpG-

EA in this sample. Due to the different distribution of these nutrients' intake also between healthy and western diet groups (Table 3S), we evaluated the correlations with the 6CpG-EA separately. Data showed that the correlation between intakes of nutrients and the 6CpG-EA was significant in the western diet group but not in the healthy diet group (Table 2). Comparable results were obtained considering the correlation between nutrients' intake and AA (Table 2). This suggests that lower intakes of B1, B2, B6, B9 and choline, especially in a western dietary pattern, is reflected into an acceleration of the epigenetic age.

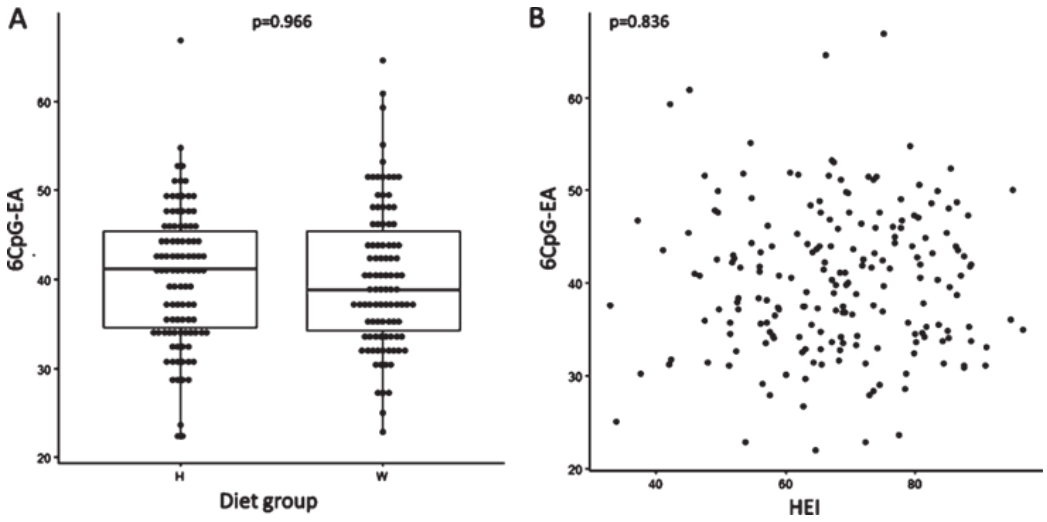


Figure 3. No differences in the 6CpG-EA between healthy and western diet group have been identified (A). No correlation between 6CpG-EA and HEI have been found (B). H: healthy diet group; W: western diet group.

3.4.5 The 6CpG-EA is correlated with TMA but not with TMAO

No correlations were detected in the entire population between the circulating TMAO levels and the AA (p = 0.477) or 6CpG-EA (p = 0.863) (Figure 4B). Similarly, no significant correlation was found between the circulating TMAO levels and the AA or 6CpG-EA neither in the western diet group (AA, p = 0.156; EA, p = 0.296) nor in the healthy diet group (AA, p = 0.614; EA, p = 0.365). Circulating TMA levels were not correlated to AA neither in the entire population (p = 0.32) nor in the diet-based groups (western diet group: p = 0.447; healthy diet group: p = 0.303). However, a significant positive correlation was found between the circulating TMA levels and the 6CpG-EA either in the whole population (r = 0.144; p = 0.044) (Figure 4A) or in the healthy diet group (r = 0.292; p = 0.045), but not in the western diet group (p =0.420).

This suggests that higher TMA blood levels, rather than TMAO, especially in a healthy dietary pattern, is reflected into an increased epigenetic age.

No significant correlation was found between TL and circulating TMA levels (p = 0.904) (Figure 4C) or TMAO levels (p = 0.877) (Figure 4D) in the entire population.

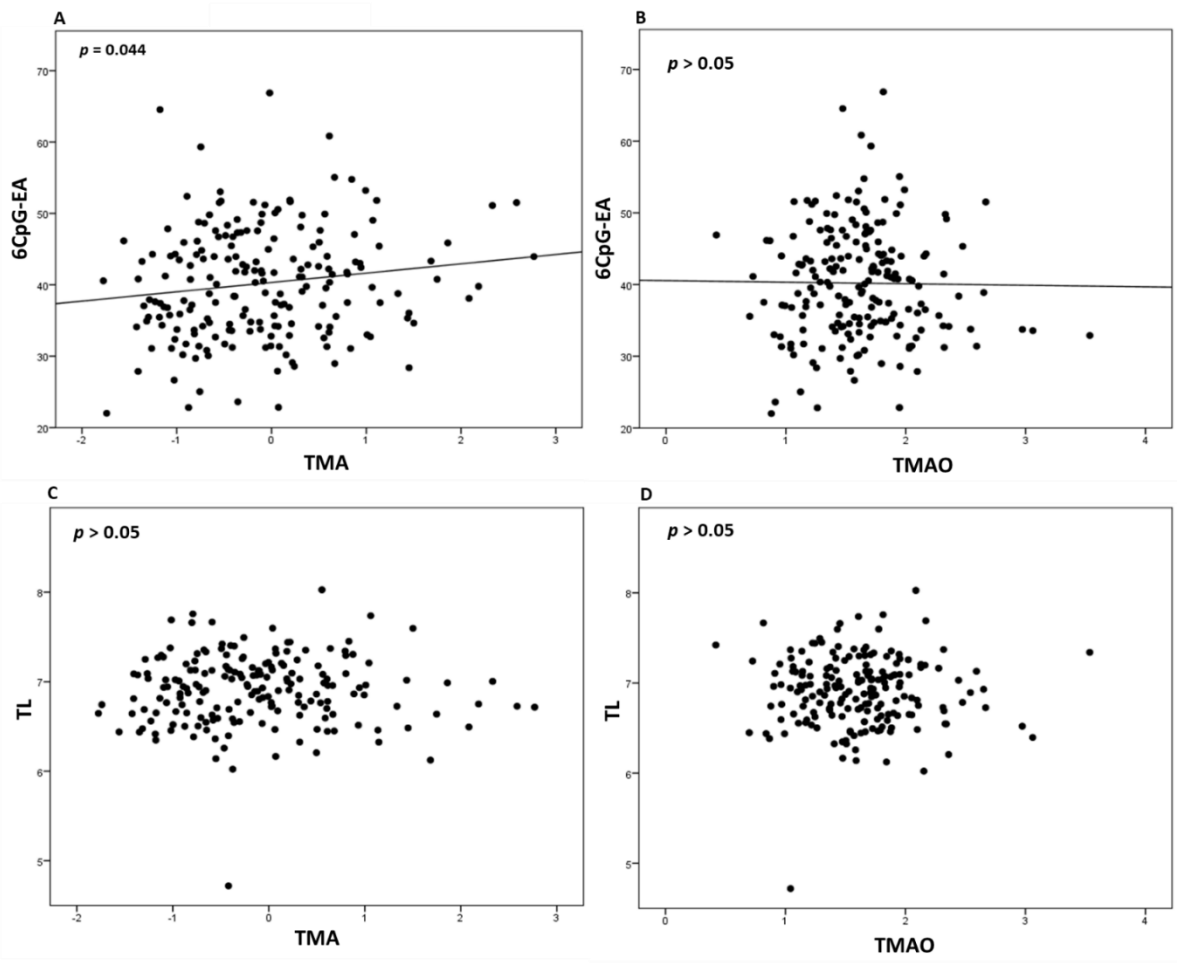


Figure 4. Scatter plots showing the correlation between the circulating levels of TMA (A) and TMAO (B) and the 6CpG-EA, or telomere length (C and D, respectively) in the whole study group.

3.4.6 Lifestyle associated factors and other molecular marks of aging (TL, LINE-1 methylation)

TL was negatively correlated with the chronological age (r = –0.163; p = 0.025). It did not differ between smokers and non-smokers (p = 0.720) or between genders (p = 0.194) and did not correlate with body composition (p = 0.489). Physical activity level was not associated to TL (p = 0.198).

TL was not different between the healthy diet and the western diet group (p = 0.194) and did not correlate with HEI (r = –0.026; p = 0.720). However, consistently to what observed for the 6CpG-EA, it showed a positive correlation with the intake of the nutrients involved in the 1CC (Table 2). This

supports the hypothesis that the intake of these nutrients can have a role against the progressive deregulation of the genome functions occurring during aging.

LINE-1 methylation level was nominally correlated with chronological age (r = –0.139; *p* = 0.053). It did not differ between smokers and non-smokers (*p* = 0.735). A slightly but significantly lower methylation in the LINE-1 was observed in the female than in males in this group (M: 75.1 1.7% vs F: 74.4 1.3%; *p* = 0.004). LINE-1 methylation did not correlate with body composition (*p* = 0.122). Physical activity level was not correlated to LINE-1 methylation (*p* = 0.155).

LINE-1 methylation was not different between the healthy diet and the western diet group (*p* = 0.206) and did not correlate with HEI (r = –0.067; *p* = 0.352). It was not correlated with micronutrients intake in the entire sample (Table 1), except for a nominal correlation with betaine intake, which was significant only in the western diet group (r = 0.284; *p* = 0.005) (Table 2).

Table 1

Spearman's correlations between 6CpG-EA, AA, TL, LINE-1 methylation, and chronological age

		6CpG-EA	AA	TL	LINE-1	Age
B1 intake (mg/d)	r	-0.199	-0.151	0.167	0.100	-0.127
	p	**0.005**	**0.034**	**0.022**	0.162	0.074
B2 intake (mg/d)	r	-0.114	-0.120	0.142	0.120	-0.045
	p	0.111	0.094	0.052	0.094	0.531
B6 intake (mg/d)	r	-0.169	-0.166	0.117	0.060	-0.080
	p	**0.018**	**0.020**	0.109	0.405	0.261
B9 intake (µg/d)	r	-0.160	-0.127	0.136	0.029	-0.102
	p	**0.025**	0.076	0.063	0.683	0.152
B12 intake (µg/d)	r	-0.117	-0.097	0.063	0.075	-0.062
	p	0.103	0.178	0.387	0.297	0.385
Choline intake (mg/d)	r	-0.184	-0.179	0.177	0.110	-0.065
	p	**0.010**	**0.012**	**0.015**	0.125	0.360
Betaine intake (mg/d)	r	-0.066	-0.004	0.077	0.170	-0.129
	p	0.361	0.960	0.296	**0.018**	0.071

Bonferroni Adj. *p* = 0.007

Table 2

Spearman's correlations between the intakes of nutrients involved in the 1CC and 6CpG-EA, TL, LINE-1 methylation, and chronological age.

		Healthy diet group					Western diet group				
		6CpG-EA	AA	TL	LINE-1	Age	6CpG-EA	AA	TL	LINE-1	Age
B1 intake (mg/d)	r	–0.175	–0.049	–0.078	0.056	–0.170	–0.223	–0.251	0.379	0.158	–0.080
	p	0.083	0.628	0.461	0.584	0.092	**0.028**	**0.013**	**$1.3*10^{-4}$**	0.122	0.431
B2 intake (mg/d)	r	–0.039	–0.010	–0.055	0.111	–0.036	–0.216	–0.231	0.335	0.164	–0.086
	p	0.700	0.921	0.603	0.273	0.719	**0.034**	**0.023**	**0.001**	0.108	0.395
B6 intake (mg/d)	r	–0.026	–0.058	–0.143	0.053	0.043	–0.314	–0.287	0.352	0.114	–0.214
	p	0.799	0.571	0.173	0.605	0.673	**0.002**	**0.004**	**$3.9*10^{-4}$**	0.268	0.033
B9 intake (µg/d)	r	–0.113	–0.046	–0.027	0.049	–0.101	–0.281	–0.243	0.305	0.114	–0.193
	p	0.266	0.651	0.795	0.634	0.317	**0.005**	**0.017**	**0.002**	0.268	0.055
B12 intake (µg/d)	r	–0.087	–0.048	–0.109	0.098	–0.029	–0.174	–0.153	0.227	0.073	–0.113
	p	0.389	0.639	0.302	0.334	0.774	0.089	0.134	0.025	0.474	0.262
Choline intake (mg/d)	r	–0.125	–0.111	–0.004	0.195	–0.027	–0.266	–0.260	0.348	0.042	–0.122
	p	0.219	0.272	0.973	0.053	0.792	**0.009**	**0.010**	**$4.8*10^{-4}$**	0.685	0.226
Betaine intake (mg/d)	r	–0.006	0.116	0.06	0.025	–0.198	–0.152	–0.178	0.082	0.284	–0.039
	p	0.952	0.262	0.573	0.809	0.052	0.137	0.081	0.423	**0.005**	0.699

Bonferroni Adj. $p = 0.007$.

3.4.7 PCA confirms the correlation between vitamin intakes and molecular hallmarks of aging

Due to the cross-correlation existing between the nutrients' intakes (Bartlett's test of sphericity; $p = 1.9*10^{-93}$), we performed a PCA as an unsupervised technique for dimensionality reduction (Table 4S) to confirm the association between these variables and the biological age. Two principal components (PCs), cumulatively explaining the 57.3% of the variance have been computed (Table 5S). Analysis of PCA loadings identified B1, B2, B6, B9 as major contributors for PC1, while B12, choline and betaine for PC2.

PC1 was negatively correlated with homocysteine levels in the whole sample (r = –0.187; $p = 0.009$), supporting the hypothesis that lower vitamins intake can impact the 1CC thus leading to homocysteine accumulation and *vice versa*. No association between circulating homocysteine levels and PC2 was detected ($p = 0.136$).

PC1 was positively associated with TL (r = 0.195; $p = 0.008$) and negatively with the 6CpG-EA (r = –0.158; $p = 0.028$). No associations were detected with LINE-1 methylation (r = 0.066; $p = 0.359$). PC2 was correlated with 6CpG-EA (r = –0.158; $p = 0.028$) and with LINE-1 methylation (r = 0.198; $p = 0.006$) but not with TL (r = 0.116; $p = 0.115$). Comparable results were obtained considering the AA. In particular, AA was correlated with PC1 (r = –0.158; $p = 0.030$) but not with PC2 (r = –0.94; $p = 0.199$). Overall, these findings suggest that higher intakes of these micronutrients can promote healthy aging measured with these molecular hallmarks.

Due to the differences concerning nutrient intakes measured between healthy and western diet groups (Table 3S), we evaluated the correlations between nutrient intakes and the molecular hallmarks of aging in the two groups separately (Figure 5, Figure 6).

As previously shown, these correlations mainly concern PC1 and are significant only in the western diet group (Figure 5). In particular, in the western diet group, PC1 was associated to significantly lower 6CpG-EA (r = –0.314; p = 0.003) and AA (r = –0.348; p = 0.001) and higher telomeres length (r = 0.381; $p = 4.5*10^{-5}$). Similar but weaker correlations were detected in the western diet group for the PC2 (Figure 6), which was also nominally correlated with LINE-1 methylation (r = 0.322; p = 0.002).

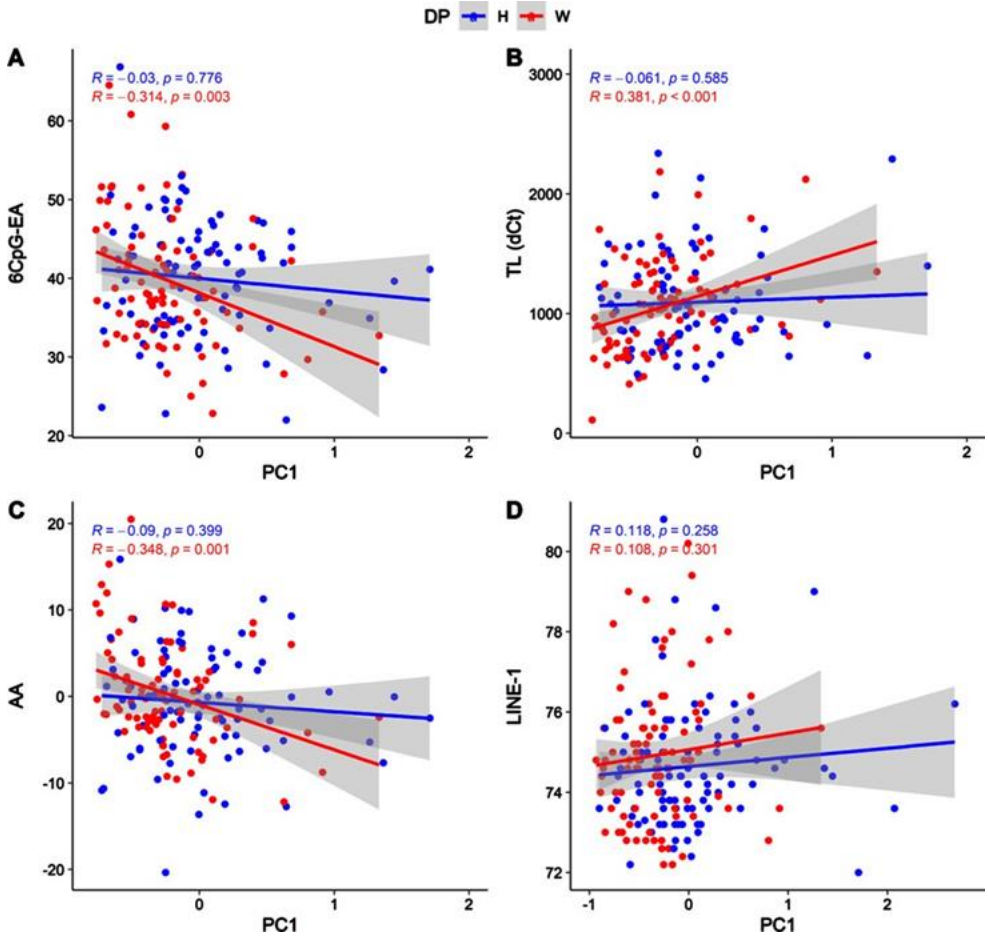


Figure 5. Scatter plot showing correlations between PC1 and 6CpG-EA (A), TL (B), AA (C), LINE-1 (D), in the two diet groups (H: healthy; W: western).

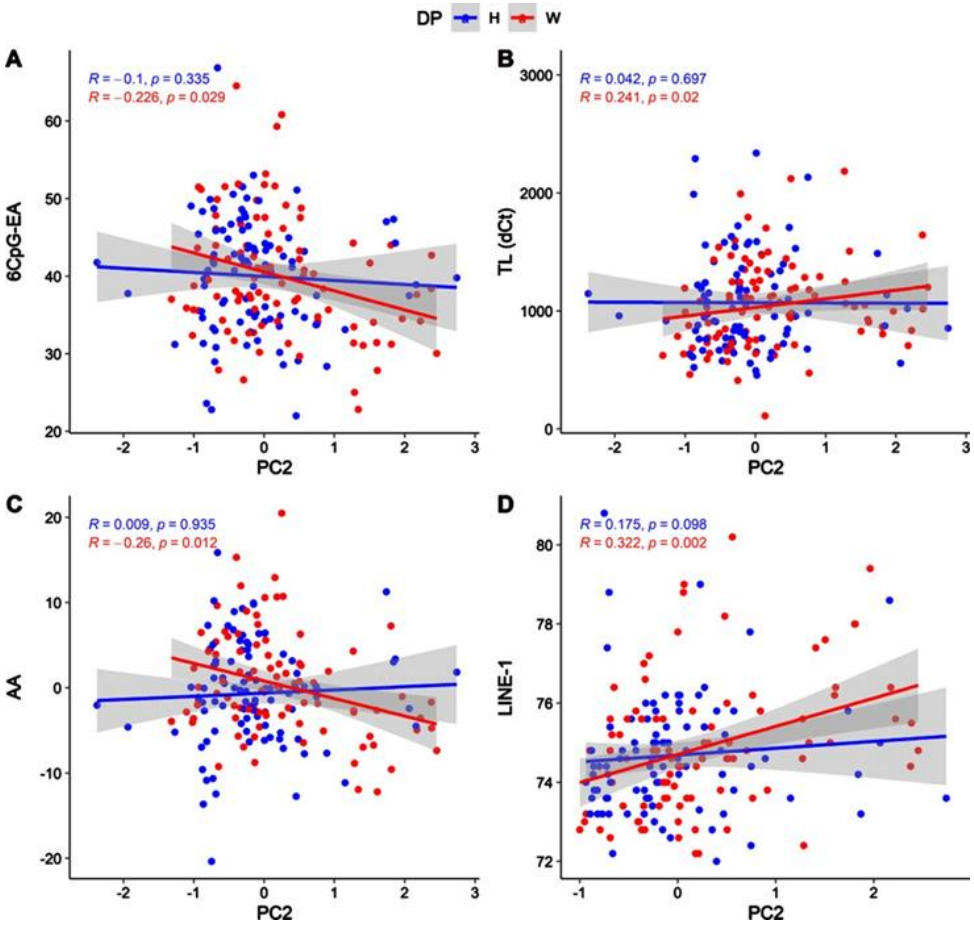


Figure 6. Scatter plot showing correlations between PC2 and 6CpG-EA (A), TL (B), AA (C), LINE-1 (D), in the two diet groups (H: healthy; W: western).

3.5 DISCUSSION AND CONCLUSIONS

Recent epigenetic biomarkers of aging based on the analysis of DNA methylation at specific sites of the genome have been proposed (i.e., epigenetic clocks). Among them, a simple and easily accessible way to measure the epigenetic age has been proposed by Han et al. by measuring 6 CpGs. In this study, we evaluated the validity of this 6CpG-based clock in association with other biomarkers of aging in an independent cohort of 200 subjects. The 6CpG-EA was significantly correlated with chronological age and negatively associated with telomeres' length and LINE-1 methylation levels. This evidence supports the validity of this tool to estimate the biological age of an individual, in accordance with other molecular hallmarks of aging. Since lifestyle factors have been related to a number of health outcomes as well as to molecular aging rates, it is of interest to understand if the epigenetic clock can be used to trace the effect of smoking, physical activity, body composition and dietary intakes on the biological age. Previous evidence (summarized in a recent systematic review [53]) showed that acceleration of epigenetic clocks is significantly related to

mortality, cardiovascular disease, cancer, and diabetes. Moreover, associations between the acceleration of one or more epigenetic clocks and BMI, HIV infection, or male sex have been identified. However, limited data are available about the impact of environmental factors, especially diet, on EA measured with targeted approaches (like the 6CpG-EA).

Despite cigarette smoking has been associated to alterations of DNA methylation patterns [54], we did not identify any association with smoking and the 6CpG-EA. This might be due to lack of quantitative data (number of cigarettes smoked per day) and limited number of smokers in the whole group. Moreover, the exclusion of the CpGs that are mostly affected by smoking in the selection process of the 6 CpG by Han et al. [35] might also explain the lack of this association. Physical activity was not associated to the 6CpG-EA in our sample, despite its correlation with epigenetic pathways has been described [55]. Although physical activity emerged as a modulator of the epigenetic age in some previous investigations based on other epigenetic clocks (e.g., [28]), this relation was not uniform among studies systematically reviewed by Oblak et al.[53]. A mild correlation between the 6CpG-EA with body composition was detected in our sample, but this was not confirmed adjusting the analysis for the chronological age. Even though previous studies identified a correlation between the EA and BMI [56,57], the exact connection between BMI and methylation levels remains poorly understood [53]. Moreover, in studies suggesting a correlation between these parameters, body composition was described using BMI and waist circumference. On the contrary, in our study we assessed body composition by the BodPod, which is a gold standard method using whole-body densitometry to determine FM%, less affected than BMI by confounders [58–61]

A body of evidence has very recently investigated the impact of diet on the epigenetic age measured with the Illumina-based epigenetic clocks [28,30,62–65]. Despite some associations between the overall dietary pattern and the EA has been detected, none of the studies provided insights on the role of dietary intake of all the major nutrients involved in the 1CC, nor of the role of some of their by-products such as TMA and TMAO. Interestingly, previous evidence showed an interaction between the methylenetetrahydrofolate reductase (MTHFR) C677T genotype and the impact of diet on the epigenetic age [64], suggesting a potential intermediary role of the 1CC underpinning the link between diet and epigenetic age. Also, Fitzgerald et al. have recently shown a reduction of the EA (measured from saliva DNA) in 43 males exposed to a healthy diet, which showed increased circulating levels of blood 5-methyltetrahydrofolate [30]. The hypothesis that boosting the 1C metabolism might promote health [16,66–68] and healthy aging [68–70] is supported by our findings, showing that lowering the intake of nutrients (especially vitamin B6 and choline) might promote AA if associated to a western dietary pattern.

Despite the intake of these micronutrients was associated with the 6CpG-EA in our sample, this finding does not support an unnecessary extra-dietary supplementation, also because the effect of

dietary supplements was not investigated in this study. Moreover, a long-term supplementation with methyl- donors has been associated to some risks [71–73], if not justified by specific physio-pathological conditions (i.e., pregnancy, dietary limitations, macrocytic anaemia or hyperhomocysteinemia) [30]. On the contrary, these data highlight with a new prospective a potential role for these vitamins in maintaining the epigenetic age, which otherwise might be accelerated by an unhealthy dietary pattern associated to a reduced dietary intake for these nutrients. Our findings also suggest that the epigenetic age might be negatively perturbed also by elevated TMA blood levels, even in case of healthy dietary habits. Thus, excess TMA in blood (and not elevated TMAO) may increase the discrepancy from the chronological age, also through epigenetic perturbations via the depletion of important epigenetic mediators of the 1CC. Indeed, one important methyl donor in the 1 CC is betaine, from which TMA is also produced (although at a lesser extent than choline and carnitine), and not only we found lower betaine level in people following a healthy dietary lifestyle, but previously published data [38] also found a positive correlation between circulating betaine and TMA levels (Table 6S).

A limitation of this study was that we did not consider socio-psychological aspects (e.g., education, stress, socio-economic status), that also have been shown to impact the epigenetic age [53,74]. However, we studied a cohort which is highly homogeneous for sex and ethnicity (which can influence the EA [75]), and where dietary habits, physical activity levels and body composition were accurately measured. Another apparent limitation is that we applied the epigenetic age calculation in a relatively young cohort quite homogeneous for age (38.2 4.9). However, whether this is a limitation for the discovery of new epigenetic clocks, it is an advantage when we want to measure the effect of environmental factors on the biological age, since the confounding effect of chronological aging is limited.

In conclusion, the 6CpG-EA appears as an interesting tool able to measure the impact of lifestyle factors (including dietary habits) on human health in an easy-accessible way. This supports its validation and usage in other independent and large cohorts, with the aim to quantify the impact of dietary habits on human health. Nevertheless, other epigenetic clocks (such as PhenoAge [76], GrimAge [77]], and DunedinPACE [78]) still remain better suitable and more informative tools to predict the effect of environmental exposures on the epigenome in case a more comprehensive analysis of the methylome can be performed.

Moreover, despite epigenetic clocks appear as interesting and promising tools to predict the biological age of an individual, it is still to be elucidated if methylation at the selected CpGs play a mechanistic role in the aging process. In this case, 6 CpGs located in genes having different biological roles have been studied. ELOVL2 is involved in elongation of long-chain polyunsaturated fatty acids and its methylation is one of the most robust biomarkers of human age [79]. Even though whether ELOVL2 has a functional role in molecular aging is still a critical question, it has been

reported that it regulates age-associated functional and anatomical aging in mice [80]. On the other hand, FHL2 is a scaffolding protein that modulates multiple signal transduction pathways, and it has been recently shown to be a novel regulator of obesity and energy expenditure [81]. However, whether an active role for some of the analysed genes in the aging process in supported by the literature, the role of others (i.e., IGSF11, CCDC102B, COL1A1 and MEIS1-AS3) in aging is still to be clarified. Interestingly, as discussed by Han et al., two of the CpGs included in the 6-CpG epigenetic clock (those in the ELOVL2 and FHL2 genes) were located in proximity of the binding site of CCCTC-binding factor (CTCF), which is involved in organization of chromatin structure. Since chromatin deregulation during aging is well documented [82], a functional role of methylation in this area in age promotion might be hypothesized. Definitely, an effort for future studies on epigenetic clocks is to describe if they are mere predictors of the biological age or if the studied CpGs have an active role in modulating aging. This is necessary to better support the usage of epigenetic clocks in clinical practice and to understand if the selected CpGs also represent a target for age rejuvenation and disease prevention.

Concerning the effects of diet on the selected epigenetic clock, our findings in particular suggest that dietary micronutrients supporting the 1CC might play a relevant role in maintaining a “young” epigenome. Remarkably, the possibility to monitor the epigenetic drift with epigenetic clocks suggests that personalized strategies aimed to prevent or even to reverse these age-related epigenetic changes to a “young” state might be developed in the near future [83]. Nevertheless, despite some interventional studies have been conducted [63,64], further evidence from independent cohorts is warranted to promote the translation of the epigenetic clock to the clinic as a tool for precision nutrition.

3.6 REFERENCES

1. Sen, P.; Shah, P.P.; Nativio, R.; Berger, S.L. Epigenetic Mechanisms of Longevity and Aging. *Cell* **2016**, *166*, 822–839, doi:10.1016/J.CELL.2016.07.050.
2. Teschendorff, A.E.; West, J.; Beck, S. Age-Associated Epigenetic Drift: Implications, and a Case of Epigenetic Thrift? *Hum Mol Genet* **2013**, *22*, doi:10.1093/HMG/DDT375.
3. Unnikrishnan, A.; Freeman, W.M.; Jackson, J.; Wren, J.D.; Porter, H.; Richardson, A. The Role of DNA Methylation in Epigenetics of Aging. *Pharmacol Ther* **2019**, *195*, 172–185, doi:10.1016/J.PHARMTHERA.2018.11.001.
4. Calvanese, V.; Lara, E.; Kahn, A.; Fraga, M.F. The Role of Epigenetics in Aging and Age-Related Diseases. *Ageing Res Rev* **2009**, *8*, 268–276, doi:10.1016/J.ARR.2009.03.004.
5. Erichsen, L.; Beermann, A.; Arauzo-Bravo, M.J.; Hassan, M.; Dkhil, M.A.; Al-Quraishy, S.; Hafiz, T.A.; Fischer, J.C.; Santourlidis, S. Genome-Wide Hypomethylation of LINE-1 and Alu Retroelements in Cell-Free DNA of Blood Is an Epigenetic Biomarker of Human Aging. *Saudi J Biol Sci* **2018**, *25*, 1220–1226, doi:10.1016/J.SJBS.2018.02.005.
6. Cho, Y.H.; Woo, H.D.; Jang, Y.; Porter, V.; Christensen, S.; Hamilton, R.F.; Chung, H.W. The Association of LINE-1 Hypomethylation with Age and Centromere Positive Micronuclei in Human Lymphocytes. *PLoS One* **2015**, *10*, e0133909, doi:10.1371/JOURNAL.PONE.0133909.
7. Byun, H.M.; Motta, V.; Panni, T.; Bertazzi, P.A.; Apostoli, P.; Hou, L.; Baccarelli, A.A. Evolutionary Age of Repetitive Element Subfamilies and Sensitivity of DNA Methylation to Airborne Pollutants. *Part Fibre Toxicol* **2013**, *10*, doi:10.1186/1743-8977-10-28.
8. Su, J.; Shao, X.; Liu, H.; Liu, S.; Wu, Q.; Zhang, Y. Genome-Wide Dynamic Changes of DNA Methylation of Repetitive Elements in Human Embryonic Stem Cells and Fetal Fibroblasts. *Genomics* **2012**, *99*, 10–17, doi:10.1016/J.YGENO.2011.10.004.
9. De Cecco, M.; Criscione, S.W.; Peterson, A.L.; Neretti, N.; Sedivy, J.M.; Kreiling, J.A. Transposable Elements Become Active and Mobile in the Genomes of Aging Mammalian Somatic Tissues. *Aging* **2013**, *5*, 867–883, doi:10.18632/AGING.100621.
10. Bourc'his, D.; Bestor, T.H. Meiotic Catastrophe and Retrotransposon Reactivation in Male Germ Cells Lacking Dnmt3L. *Nature* **2004**, *431*, 96–99, doi:10.1038/NATURE02886.
11. Ashapkin, V. V.; Kutueva, L.I.; Vanyushin, B.F. Epigenetic Clock: Just a Convenient Marker or an Active Driver of Aging? *Adv Exp Med Biol* **2019**, *1178*, 175–206, doi:10.1007/978-3-030-25650-0_10.
12. Horvath, S. DNA Methylation Age of Human Tissues and Cell Types. *Genome Biol* **2013**, *14*, 1–20, doi:10.1186/GB-2013-14-10-R115/COMMENTS.
13. Topart, C.; Werner, E.; Arimondo, P.B. Wandering along the Epigenetic Timeline. *Clin Epigenetics* **2020**, *12*, doi:10.1186/S13148-020-00893-7.
14. Bordoni, L.; Gabbianelli, R. Primers on Nutrigenetics and Nutri(Epi)Genomics: Origins and Development of Precision Nutrition. *Biochimie* **2019**, *160*, 156–171, doi:10.1016/J.BIOCHI.2019.03.006.
15. Anderson, O.S.; Sant, K.E.; Dolinoy, D.C. Nutrition and Epigenetics: An Interplay of Dietary Methyl Donors, One-Carbon Metabolism and DNA Methylation. *J Nutr Biochem* **2012**, *23*, 853–859, doi:10.1016/J.JNUTBIO.2012.03.003.
16. Mentch, S.J.; Locasale, J.W. One-Carbon Metabolism and Epigenetics: Understanding the Specificity. *Ann N Y Acad Sci* **2016**, *1363*, 91–98, doi:10.1111/NYAS.12956.
17. Ribarič, S. Diet and Aging. *Oxid Med Cell Longev* **2012**, *2012*, 20, doi:10.1155/2012/741468.
18. Jones, M.J.; Goodman, S.J.; Kobor, M.S. DNA Methylation and Healthy Human Aging. *Aging Cell* **2015**, *14*, 924–932, doi:10.1111/ACEL.12349.
19. Crott, J.W.; Choi, S.W.; Ordovas, J.M.; Ditelberg, J.S.; Mason, J.B. Effects of Dietary Folate and Aging on Gene Expression in the Colonic Mucosa of Rats: Implications for Carcinogenesis. *Carcinogenesis* **2004**, *25*, 69–76, doi:10.1093/CARCIN/BGG150.
20. Choi, S.W.; Friso, S. Epigenetics: A New Bridge between Nutrition and Health. *Adv Nutr* **2010**, *1*, 8–16, doi:10.3945/AN.110.1004.
21. Tang, W.H.W.; Wang, Z.; Levison, B.S.; Koeth, R.A.; Britt, E.B.; Fu, X.; Wu, Y.; Hazen, S.L. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. *N Engl J Med* **2013**, *368*, 1575–1584, doi:10.1056/NEJMOA1109400.
22. Wang, Z.; Klipfell, E.; Bennett, B.J.; Koeth, R.; Levison, B.S.; Dugar, B.; Feldstein, A.E.; Britt, E.B.; Fu, X.; Chung, Y.M.; et al. Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease. *Nature* **2011**, *472*, 57–65, doi:10.1038/NATURE09922.
23. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis. *Nat Med* **2013**, *19*, 576–585, doi:10.1038/NM.3145.
24. Romano, K.A.; Martinez-del Campo, A.; Kasahara, K.; Chittim, C.L.; Vivas, E.I.; Amador-Noguez, D.; Balskus, E.P.; Rey, F.E. Metabolic, Epigenetic, and Transgenerational Effects of Gut Bacterial Choline Consumption. *Cell Host Microbe* **2017**, *22*, 279, doi:10.1016/J.CHOM.2017.07.021.
25. Wang, J.; Wu, Z.; Li, D.; Li, N.; Dindot, S. V.; Satterfield, M.C.; Bazer, F.W.; Wu, G. Nutrition, Epigenetics, and Metabolic Syndrome. *Antioxid Redox Signal* **2012**, *17*, 282, doi:10.1089/ARS.2011.4381.
26. Guarner, V.; Veronica, G.; Maria Esther, R.-R. Aging, Metabolic Syndrome and the Heart. *Aging Dis* **2012**, *3*, 269.
27. Bonomini, F.; Rodella, L.F.; Rezzani, R. Metabolic Syndrome, Aging and Involvement of Oxidative Stress. *Aging Dis* **2015**, *6*, 109, doi:10.14336/AD.2014.0305.
28. 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.; et al. Epigenetic Clock Analysis of Diet, Exercise, Education, and Lifestyle Factors. *Aging* **2017**, *9*, 419–446, doi:10.18632/AGING.101168.
29. Fiorito, G.; Caini, S.; Palli, D.; Bendinelli, B.; Saieva, C.; Ermini, I.; Valentini, V.; Assedi, M.; Rizzolo, P.; Ambrogetti, D.; et al. DNA Methylation-Based Biomarkers of Aging Were Slowed down in a Two-Year Diet and Physical Activity Intervention Trial: The DAMA Study. *Aging Cell* **2021**, *20*, e13439, doi:10.1111/ACEL.13439.
30. Fitzgerald, K.N.; Hodges, R.; Hanes, D.; Stack, E.; Cheishvili, D.; Szyf, M.; Henkel, J.; Twedt, M.W.; Giannopoulou, D.; Herdell, J.; et al. Potential Reversal of Epigenetic Age Using a Diet and Lifestyle Intervention: A Pilot Randomized Clinical Trial. *Aging* **2021**, *13*, 9419–9432, doi:10.18632/AGING.202913.
31. Chamberlain, J.A.; Dugué, P.A.; Bassett, J.K.; Hodge, A.M.; Brinkman, M.T.; Joo, J.H.E.; Jung, C.H.; Makalic, E.; Schmidt, D.F.; Hopper, J.L.; et al. Dietary Intake of One-Carbon Metabolism Nutrients and DNA Methylation in Peripheral Blood. *Am J Clin Nutr* **2018**, *108*, 611–621, doi:10.1093/AJCN/NQY119.

32. Han, Y.; Eipel, M.; Franzen, J.; Sakk, V.; Dethmers-Ausema, B.; Yndriago, L.; Izeta, A.; de Haan, G.; Geiger, H.; Wagner, W. Epigenetic Age-Predictor for Mice Based on Three CpG Sites. *Elife* **2018**, *7*, doi:10.7554/ELIFE.37462.
33. Bekaert, B.; Kamalandua, A.; Zapico, S.C.; Van De Voorde, W.; Decorte, R. Improved Age Determination of Blood and Teeth Samples Using a Selected Set of DNA Methylation Markers. *Epigenetics* **2015**, *10*, 922–930, doi:10.1080/15592294.2015.1080413.
34. Zbieć-Piekarska, R.; Spólnicka, M.; Kupiec, T.; Makowska, Z.; Spas, A.; Parys-Proszek, A.; Kucharczyk, K.; Płoski, R.; Branicki, W. Examination of DNA Methylation Status of the ELOVL2 Marker May Be Useful for Human Age Prediction in Forensic Science. *Forensic Sci Int Genet* **2015**, *14*, 161–167, doi:10.1016/J.FSIGEN.2014.10.002.
35. Han, Y.; Franzen, J.; Stiehl, T.; Gobs, M.; Kuo, C.C.; Nikolić, M.; Hapala, J.; Koop, B.E.; Strathmann, K.; Ritz-Timme, S.; et al. New Targeted Approaches for Epigenetic Age Predictions. *BMC Biol* **2020**, *18*, 1–15, doi:10.1186/S12915-020-00807-2/FIGURES/5.
36. Malinowska, A.M. Easy Diet Screener: A Quick and Easy Tool for Determining Dietary Patterns Associated with Lipid Profile and Body Adiposity. *Journal of Human Nutrition and Dietetics* **2022**, *35*, 590–604, doi:10.1111/JHN.12973.
37. Malinowska, A.M.; Kok, D.E.; Steegenga, W.T.; Hooiveld, G.J.E.J.; Chmurzynska, A. Human Gut Microbiota Composition and Its Predicted Functional Properties in People with Western and Healthy Dietary Patterns. *Eur J Nutr* **2022**, *61*, 3887–3903, doi:10.1007/S00394-022-02928-6.
38. Bordoni, L.; Malinowska, A.M.; Petracci, I.; Szwengiel, A.; Gabbianelli, R.; Chmurzynska, A. Diet, Trimethylamine Metabolism, and Mitochondrial DNA: An Observational Study. *Mol Nutr Food Res* **2022**, *66*, e2200003, doi:10.1002/mnfr.202200003.
39. Craig, C.L.; Marshall, A.L.; Sjöström, M.; Bauman, A.E.; Booth, M.L.; Ainsworth, B.E.; Pratt, M.; Ekelund, U.; Yngve, A.; Sallis, J.F.; et al. International Physical Activity Questionnaire: 12-Country Reliability and Validity. *Med Sci Sports Exerc* **2003**, *35*, 1381–1395, doi:10.1249/01.MSS.0000078924.61453.FB.
40. Reedy, J.; Lerman, J.L.; Krebs-Smith, S.M.; Kirkpatrick, S.I.; Pannucci, T.E.; Wilson, M.M.; Subar, A.F.; Kahle, L.L.; Tooze, J.A. Evaluation of the Healthy Eating Index-2015. *J Acad Nutr Diet* **2018**, *118*, 1622–1633, doi:10.1016/j.jand.2018.05.019.
41. Krebs-Smith, S.M.; Pannucci, T.E.; Subar, A.F.; Kirkpatrick, S.I.; Lerman, J.L.; Tooze, J.A.; Wilson, M.M.; Reedy, J. Update of the Healthy Eating Index: HEI-2015. *J Acad Nutr Diet* **2018**, *118*, 1591–1602, doi:10.1016/j.jand.2018.05.021.
42. USDA Database for the Choline Content of Common Foods, Release 2 (2008) | Ag Data Commons Available online: https://data.nal.usda.gov/dataset/usda-database-choline-content-common-foods-release-2-2008 (accessed on 26 June 2023).
43. Gowacki, R.; Bald, E. Determination of N-Acetylcysteine and Main Endogenous Thiols in Human Plasma by HPLC with Ultraviolet Detection in the Form of Their S-Quinolinium Derivatives. *http://dx.doi.org/10.1080/10826070903249666* **2009**, *32*, 2530–2544, doi:10.1080/10826070903249666.
44. Głowacki, R.; Bald, E. Fully Automated Method for Simultaneous Determination of Total Cysteine, Cysteinylglycine, Glutathione and Homocysteine in Plasma by HPLC with UV Absorbance Detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **2009**, *877*, 3400–3404, doi:10.1016/J.JCHROMB.2009.06.012.
45. Johnson, D.W. A Flow Injection Electrospray Ionization Tandem Mass Spectrometric Method for the Simultaneous Measurement of Trimethylamine and Trimethylamine N-Oxide in Urine. *J Mass Spectrom* **2008**, *43*, 495–499, doi:10.1002/JMS.1339.
46. Koc, H.; Mar, M.H.; Ranasinghe, A.; Swenberg, J.A.; Zeisel, S.H. Quantitation of Choline and Its Metabolites in Tissues and Foods by Liquid Chromatography/Electrospray Ionization-Isotope Dilution Mass Spectrometry. *Anal Chem* **2002**, *74*, 4734–4740, doi:10.1021/AC025624X.
47. Vasilishina, A.; Kropotov, A.; Spivak, I.; Bernadotte, A. Relative Human Telomere Length Quantification by Real-Time PCR. *Methods Mol Biol* **2019**, *1896*, 39–44, doi:10.1007/978-1-4939-8931-7_5.
48. Tabish, A.M.; Baccarelli, A.A.; Godderis, L.; Barrow, T.M.; Hoet, P.; Byun, H.-M. Assessment of Changes in Global DNA Methylation Levels by Pyrosequencing® of Repetitive Elements. *Methods Mol Biol* **2015**, *1315*, 201–207, doi:10.1007/978-1-4939-2715-9_15.
49. IBM Corp. (2013) IBM SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY. - References - Scientific Research Publishing Available online: https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=2010524 (accessed on 26 June 2023).
50. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis.* ; 2nd ed.; Springer New York, 2009;
51. Malinowska, A.M.; Schmidt, M.; Kok, D.E.; Chmurzynska, A. Ex Vivo Folate Production by Fecal Bacteria Does Not Predict Human Blood Folate Status: Associations between Dietary Patterns, Gut Microbiota, and Folate Metabolism. *Food Res Int* **2022**, *156*, 111290, doi:10.1016/j.foodres.2022.111290.
52. Alisch, R.S.; Barwick, B.G.; Chopra, P.; Myrick, L.K.; Satten, G.A.; Conneely, K.N.; Warren, S.T. Age-Associated DNA Methylation in Pediatric Populations. *Genome Res* **2012**, *22*, 623–632, doi:10.1101/GR.125187.111.
53. Oblak, L.; van der Zaag, J.; Higgins-Chen, A.T.; Levine, M.E.; Boks, M.P. A Systematic Review of Biological, Social and Environmental Factors Associated with Epigenetic Clock Acceleration. *Ageing Res Rev* **2021**, *69*, doi:10.1016/J.ARR.2021.101348.
54. Gao, X.; Jia, M.; Zhang, Y.; Breitling, L.P.; Brenner, H. DNA Methylation Changes of Whole Blood Cells in Response to Active Smoking Exposure in Adults: A Systematic Review of DNA Methylation Studies. *Clin Epigenetics* **2015**, *7*, 1–10, doi:10.1186/S13148-015-0148-3/FIGURES/3.
55. Petracci, I.; Gabbianelli, R.; Bordoni, L. The Role of Nutri(Epi)Genomics in Achieving the Body's Full Potential in Physical Activity. *Antioxidants (Basel)* **2020**, *9*, 1–33, doi:10.3390/ANTIOX9060498.
56. Sun, D.; Zhang, T.; Su, S.; Hao, G.; Chen, T.; Li, Q.Z.; Bazzano, L.; He, J.; Wang, X.; Li, S.; et al. Body Mass Index Drives Changes in DNA Methylation: A Longitudinal Study. *Circ Res* **2019**, *125*, 824–833, doi:10.1161/CIRCRESAHA.119.315397.
57. McCartney, D.L.; Hillary, R.F.; Stevenson, A.J.; Ritchie, S.J.; Walker, R.M.; Zhang, Q.; Morris, S.W.; Bermingham, M.L.; Campbell, A.; Murray, A.D.; et al. Epigenetic Prediction of Complex Traits and Death. *Genome Biol* **2018**, *19*, 136, doi:10.1186/S13059-018-1514-1/TABLES/3.
58. Frankenfield, D.C.; Rowe, W.A.; Cooney, R.N.; Smith, J.S.; Becker, D. Limits of Body Mass Index to Detect Obesity and Predict Body Composition. *Nutrition* **2001**, *17*, 26–30, doi:10.1016/S0899-9007(00)00471-8.
59. Liu, P.; Ma, F.; Lou, H.; Liu, Y. The Utility of Fat Mass Index vs. Body Mass Index and Percentage of Body Fat in the Screening of Metabolic Syndrome. *BMC Public Health* **2013**, *13*, doi:10.1186/1471-2458-13-629.
60. Kyle, U.G.; Schutz, Y.; Dupertuis, Y.M.; Pichard, C. Body Composition Interpretation: Contributions of the Fat-Free Mass Index and the Body Fat Mass Index. *Nutrition* **2003**, *19*, 597–604, doi:10.1016/S0899-9007(03)00061-3.

61. Fosbøl, M.O.; Zerahn, B. Contemporary Methods of Body Composition Measurement. *Clin Physiol Funct Imaging* **2015**, *35*, 81–97, doi:10.1111/CPF.12152.
62. Kim, Y.; Ma, J.; Levy, D. Abstract MP06: Healthy Diet Quality Is Associated With Epigenetic Age Deceleration. *Circulation* **2021**, *143*, doi:10.1161/CIRC.143.SUPPL_1.MP06.
63. Gensous, N.; Garagnani, P.; Santoro, A.; Giuliani, C.; Ostan, R.; Fabbri, C.; Milazzo, M.; Gentilini, D.; di Blasio, A.M.; Pietruszka, B.; et al. One-Year Mediterranean Diet Promotes Epigenetic Rejuvenation with Country- and Sex-Specific Effects: A Pilot Study from the NU-AGE Project. *Geroscience* **2020**, *42*, 687, doi:10.1007/S11357-019-00149-0.
64. Sae-Lee, C.; Corsi, S.; Barrow, T.M.; Kuhnle, G.G.C.; Bollati, V.; Mathers, J.C.; Byun, H.M. Dietary Intervention Modifies DNA Methylation Age Assessed by the Epigenetic Clock. *Mol Nutr Food Res* **2018**, *62*, doi:10.1002/MNFR.201800092.
65. Kresovich, J.K.; Park, Y.M.M.; Keller, J.A.; Sandler, D.P.; Taylor, J.A. Healthy Eating Patterns and Epigenetic Measures of Biological Age. *Am J Clin Nutr* **2022**, *115*, 171–179, doi:10.1093/AJCN/NQAB307.
66. Bordoni, L.; Petracci, I.; Mlodzik-Czyzewska, M.; Malinowska, A.M.; Szwengiel, A.; Sadowski, M.; Gabbianelli, R.; Chmurzynska, A. Mitochondrial DNA and Epigenetics: Investigating Interactions with the One-Carbon Metabolism in Obesity. *Oxid Med Cell Longev* **2022**, *2022*, 9171684, doi:10.1155/2022/9171684.
67. Mlodzik-Czyzewska, M.A.; Malinowska, A.M.; Chmurzynska, A. Low Folate Intake and Serum Levels Are Associated with Higher Body Mass Index and Abdominal Fat Accumulation: A Case Control Study. *Nutr J* **2020**, *19*, doi:10.1186/S12937-020-00572-6.
68. Clare, C.E.; Brassington, A.H.; Kwong, W.Y.; Sinclair, K.D. One-Carbon Metabolism: Linking Nutritional Biochemistry to Epigenetic Programming of Long-Term Development. *Annu Rev Anim Biosci* **2019**, *7*, 263–287, doi:10.1146/ANNUREV-ANIMAL-020518-115206.
69. Lionaki, E.; Ploumi, C.; Tavernarakis, N. One-Carbon Metabolism: Pulling the Strings behind Aging and Neurodegeneration. *Cells* **2022**, *11*, doi:10.3390/CELLS11020214.
70. Annibal, A.; Tharyan, R.G.; Schonewolff, M.F.; Tam, H.; Latza, C.; Auler, M.M.K.; Antebi, A. Regulation of the One Carbon Folate Cycle as a Shared Metabolic Signature of Longevity. *Nature Communications 2021 12:1* **2021**, *12*, 1–14, doi:10.1038/s41467-021-23856-9.
71. Araghi, S.O.; Kiefte-De Jong, J.C.; Van Dijk, S.C.; Swart, K.M.A.; Van Laarhoven, H.W.; Van Schoor, N.M.; De Groot, L.C.P.G.M.; Lemmens, V.; Stricker, B.H.; Uitterlinden, A.G.; et al. Folic Acid and Vitamin B12 Supplementation and the Risk of Cancer: Long-Term Follow-up of the B Vitamins for the Prevention of Osteoporotic Fractures (B-PROOF) Trial. *Cancer Epidemiol Biomarkers Prev* **2019**, *28*, 275–282, doi:10.1158/1055-9965.EPI-17-1198.
72. Ebbing, M.; Bønaa, K.H.; Nygård, O.; Arnesen, E.; Ueland, P.M.; Nordrehaug, J.E.; Rasmussen, K.; Njølstad, I.; Refsum, H.; Nilsen, D.W.; et al. Cancer Incidence and Mortality after Treatment with Folic Acid and Vitamin B12. *JAMA* **2009**, *302*, 2119–2126, doi:10.1001/JAMA.2009.1622.
73. Figueiredo, J.C.; Grau, M. V.; Haile, R.W.; Sandler, R.S.; Summers, R.W.; Bresalier, R.S.; Burke, C.A.; McKeown-Eyssen, G.E.; Baron, J.A. Folic Acid and Risk of Prostate Cancer: Results from a Randomized Clinical Trial. *J Natl Cancer Inst* **2009**, *101*, 432–435, doi:10.1093/JNCI/DJP019.
74. Pavanello, S.; Campisi, M.; Tona, F.; Dal Lin, C.; Iliceto, S. Exploring Epigenetic Age in Response to Intensive Relaxing Training: A Pilot Study to Slow Down Biological Age. *Int J Environ Res Public Health* **2019**, *16*, doi:10.3390/IJERPH16173074.
75. Horvath, S.; Gurven, M.; Levine, M.E.; Trumble, B.C.; Kaplan, H.; Allayee, H.; Ritz, B.R.; Chen, B.; Lu, A.T.; Rickabaugh, T.M.; et al. An Epigenetic Clock Analysis of Race/Ethnicity, Sex, and Coronary Heart Disease. *Genome Biol* **2016**, *17*, doi:10.1186/S13059-016-1030-0.
76. 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.; et al. An Epigenetic Biomarker of Aging for Lifespan and Healthspan. *Aging* **2018**, *10*, 573–591, doi:10.18632/AGING.101414.
77. 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.; et al. DNA Methylation GrimAge Strongly Predicts Lifespan and Healthspan. *Aging* **2019**, *11*, 303–327, doi:10.18632/AGING.101684.
78. Belsky, D.W.; Caspi, A.; Corcoran, D.L.; Sugden, K.; Poulton, R.; Arseneault, L.; Baccarelli, A.; Chamarti, K.; Gao, X.; Hannon, E.; et al. DunedinPACE, A DNA Methylation Biomarker of the Pace of Aging. *Elife* **2022**, *11*, doi:10.7554/ELIFE.73420.
79. Garagnani, P.; Bacalini, M.G.; Pirazzini, C.; Gori, D.; Giuliani, C.; Mari, D.; Di Blasio, A.M.; Gentilini, D.; Vitale, G.; Collino, S.; et al. Methylation of ELOVL2 Gene as a New Epigenetic Marker of Age. *Aging Cell* **2012**, *11*, 1132–1134, doi:10.1111/ACEL.12005.
80. Chen, D.; Chao, D.L.; Rocha, L.; Kolar, M.; Nguyen Huu, V.A.; Krawczyk, M.; Dasyani, M.; Wang, T.; Jafari, M.; Jabari, M.; et al. The Lipid Elongation Enzyme ELOVL2 Is a Molecular Regulator of Aging in the Retina. *Aging Cell* **2020**, *19*, doi:10.1111/ACEL.13100.
81. Clemente-Olivo, M.P.; Habibe, J.J.; Vos, M.; Ottenhoff, R.; Jongejan, A.; Herrema, H.; Zelcer, N.; Kooijman, S.; Rensen, P.C.N.; van Raalte, D.H.; et al. Four-and-a-Half LIM Domain Protein 2 (FHL2) Deficiency Protects Mice from Diet-Induced Obesity and High FHL2 Expression Marks Human Obesity. *Metabolism* **2021**, *121*, doi:10.1016/J.METABOL.2021.154815.
82. Wang, K.; Liu, H.; Hu, Q.; Wang, L.; Liu, J.; Zheng, Z.; Zhang, W.; Ren, J.; Zhu, F.; Liu, G.H. Epigenetic Regulation of Aging: Implications for Interventions of Aging and Diseases. *Signal Transduction and Targeted Therapy 2022 7:1* **2022**, *7*, 1–22, doi:10.1038/s41392-022-01211-8.
83. Noroozi, R.; Ghafouri-Fard, S.; Pisarek, A.; Rudnicka, J.; Spólnicka, M.; Branicki, W.; Taheri, M.; Pośpiech, E. DNA Methylation-Based Age Clocks: From Age Prediction to Age Reversion. *Ageing Res Rev* **2021**, *68*, doi:10.1016/J.ARR.2021.101314.

3.7 SUPPORTING INFORMATION

Table 1S. Primers' sequences used for TL analysis and DNA methylation analyses for LINE-1 and 6CpG-EA calculation.

A) Primers for TL relative quantification

	Fw (5'-3')	Rv (5'-3')
TEL	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT
IFNB1	GGT TAC CTC CGA AAC TGA AGA	CCT TTC ATA TGC AGT ACA TTA GCC

B) Primers for amplification and DNA methylation assessment

	Fw (5'-3')	Rv (5'-3')	seq (5'-3')
LINE-1	TTT TGA GTT AGG TGT GGG ATA TA	Bio-AAA ATC AAA AAA TTC CCT TTC	AGT TAG GTG TGG ATA TAG T
FHL2	GTG TTT TTA GGG TTT TGG GAG TAT AGT AGT	Bio-CAC CTC CTA AAA CTT CTC CAA TCT CC	GGT TTT GGG AGT ATA GTA GTT
IGSF11	GTT GGA TAG TTT GTG GGT AGA AAA TTT A	Bio-ATT ATT CAT TCA TTA TTC TCC TTA AAA AAA TCT TAT T	AGA AGT TAA GAA GGT ATA GAT A
CCDC120	TGT TGA GGG AGG GGA ATG TTT GTA TTT AT	Bio-CCA ATA ATA TCT ATA TCA TCA ACA TTT CTA CAA CTT	GGA GGG GAA TGT TTG
MEIS1	TTG AAT AAT TAG TAA GAT TTT TGT TTG AAG GTT T	Bio-TTA CCT TTA AAA CAA CAA AAT AAA TCA CAC TAA CC	TTA GTA AGA TTT TTG TTT G
ELOVL	Bio-GGG AGG GGA GTA GGG TAA GTG A	CCA TCT AAA CAA CCA ATA AAT ATT CCT AAA AC	AAT AAA TAT TCC TAA AAC TC
COL1A2	TTG AAG GGA AGA GGT AAG GAA GAT TTT A	Bio-TAA CCC ATC TTT TTC CTT CTT CTC A	AAT TTG TAT AGA GAG TGT TTA TTG

Table 2S. CpG ID and associated genes for the selected 6 CpGs used for the EA calculation according to Han et al. are displayed. The equation proposed by Han et al. for the EA calculation according to the 6CpGs is shown at the bottom of the table.

Gene Name	CpG ID	Methylation level code in the algorithm
FHL2	cg22454769	α
IGSF11	cg00329615	β
CCDC102B	cg19283806	γ
MEIS1-AS3	cg11807280	ε
ELOVL2	cg16867657	δ
COL1A1	cg18618815	ϕ

$EA = 32.07 + 0.99*\alpha + (-0.12)*\beta + (-0.99)*\gamma + (-0.09)*\varepsilon + 0.05*\delta + 0.11*\phi$

Table 3S. Diet quality and nutrient intakes in the healthy and western diet groups. Circulating levels of homocysteine in the two groups are also shown.

	Healthy diet group				**Western diet group**				
	min	max	median	IQR	min	max	median	IQR	p
B1 intake (mg/d)	0.59	14.41	1.46	0.84	0.54	16.09	1.38	0.83	0.155
B2 intake (mg/d)	0.78	15.37	2.00	1.00	0.72	21.43	1.73	0.70	**0.006**
B6 intake (mg/d)	0.89	51.23	2.41	1.15	0.6	37.68	1.85	1.11	**$1.7*10^{-4}$**
B9 intake (µg/d)	170.25	1532.85	408.17	209.86	126.05	1032.33	312.07	121.94	**$1.84*10^{-7}$**
B12 intake (µg/d)	0.70	500.84	4.58	3.53	0.73	37.46	3.51	2.56	0.091
Choline intake (mg/d)	81.92	1687.81	410.24	273.71	92.03	1363.75	368.85	221.34	0.325
Betaine intake (mg/d)	8.40	482.46	62.08	79.98	6.70	422.38	111.05	118.76	**$5.9*10^{-6}$**
Plasma Homocysteine (µM)	4.82	19.43	10.09	3.63	7.17	21.32	10.97	4.16	0.034

Bonferroni P=0.006

Table 4S. Intakes of nutrients involved in 1CC in the two genders.

	M				**F**				
	Min	Max	Mean	SD	Min	Max	Mean	SD	p
B1 intake (mg/d)	0.54	16.09	2.0203	2.04466	0.59	11.21	1.6034	1.6844	**2.3*10-7**
B2 intake (mg/d)	0.85	17.47	2.4732	2.22171	0.72	21.43	2.1675	2.45831	**4.2*10-5**
B6 intake (mg/d)	0.64	37.68	3.2504	4.24812	0.6	51.23	3.0203	5.55344	**2.3*10-4**
B9 intake (µg/d)	139.56	1532.85	427.1592	201.6235	126.05	1140.86	354.4114	175.2046	**4.5*10-4**
B12 intake (µg/d)	0.7	500.84	11.1915	49.84499	0.73	12.27	3.7916	2.3545	**7.2*10-6**
Choline intake (mg/d)	81.92	1470.13	501.5905	236.017	83.83	1687.81	355.4558	230.6262	**3.04*10-8**
Betaine intake (mg/d)	9.39	422.38	131.0665	102.9698	6.7	482.46	87.207	69.94304	**0.005**

Table 5S. Description of PCs identified in the PCA. A) Principal Component Analysis (PCA). Eigenvalues for each PC and % of variance explained are shown. 2 PC have been selected, cumulatively explaining 57.5% of the variance. B) PCA Loadings defining which is the contribution of the original variables to the principal components. Varimax rotation with Kaiser normalization has been applied.

A

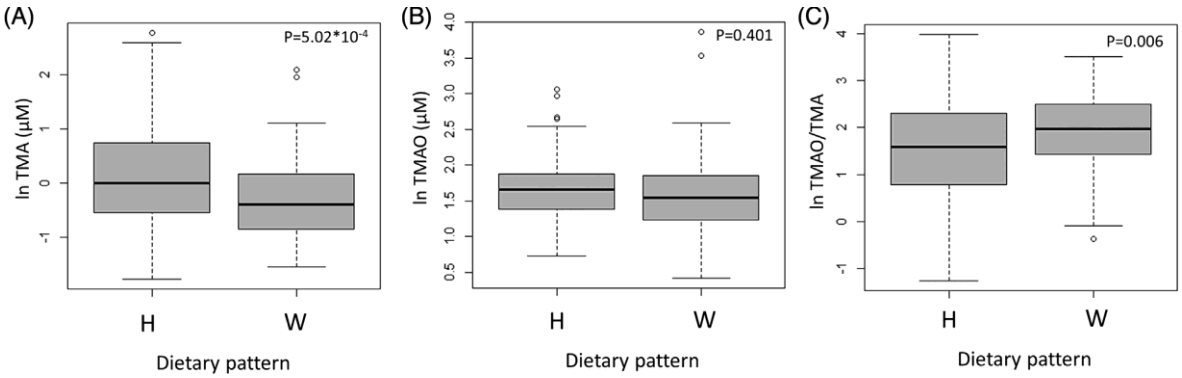
PC	Eigenvalues			Weight of rotated factors		
	Total	% variance	% cumulative	total	% variance	% cumulative
1	2.886	41.223	41.223	2.873	41.046	41.046
2	1.14	16.289	**57.512**	1.153	16.466	57.512
3	0.98	13.997	71.508			
4	0.88	12.571	84.079			
5	0.596	8.514	92.593			
6	0.442	6.308	98.902			
7	0.077	1.098	100			

B	Component	
	PC1	PC2
B1 intake	**0.934**	0.027
B2 intake	**0.923**	0.011
B6 intake	**0.723**	-0.2
B12 intake	0.086	**-0.468**
Choline intake	0.41	**0.564**
Betaine intake	-0.029	**0.747**
B9 intake	**0.671**	0.129

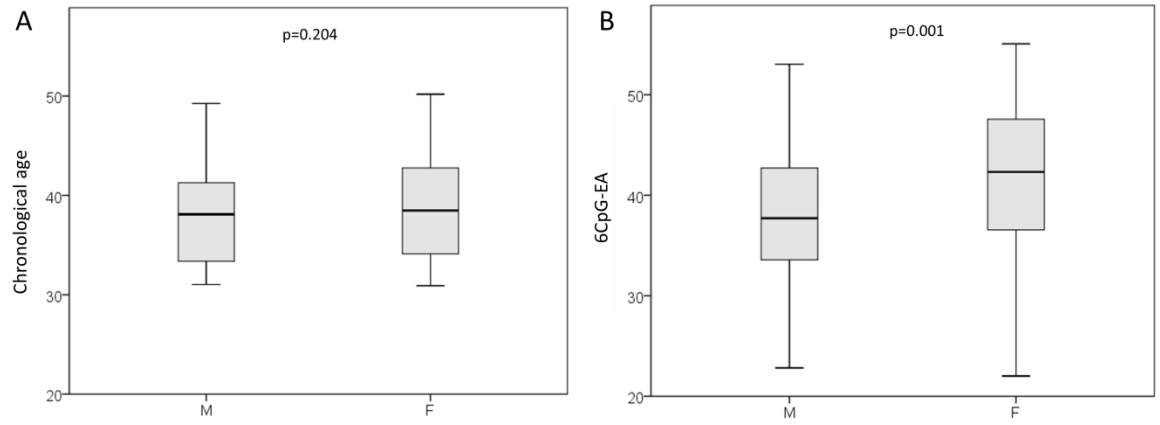
Table 6S. Correlations between circulating levels of dietary TMA precursors and TMA or TMAO in the healthy and western diet groups.

			Circulating choline levels	Circulating acetyl-carnitine levels	Circulating betaine levels	Circulating carnitine levels
HEALTHY DIET GROUP	TMA	Pearson's correlation	-0.059	-0.026	-0.051	0.310
		P	0.559	0.800	0.615	**0.002**
	TMAO	Pearson's correlation	-0.001	-0.097	0.260	-0.155
		P	0.993	0.336	**0.009**	0.123
WESTERN DIET GROUP	TMA	Pearson's correlation	-0.255*	0.201	-0.020	0.357
		P	0.010	0.045	0.844	0.**000**
	TMAO	Pearson's correlation	0.192	0.065	0.208	0.212
		P	0.055	0.519	0.**038**	0.034

Supplementary figure 1. Levels of plasma trimethylamine (TMA) A) trimethylamine-N-oxide (TMAO) B), and the TMAO/TMA ratio C) in groups with healthy (H) and western (W) dietary patterns.



Supplementary figure 2. Box plots showing mean difference of chronological age (A) and 6CpG-EA (B) between males and females in this cohort.



Chapter 4

Gut-derived trimethylamine promotes inflammation and perturbs epigenetic and mitochondrial homeostasis on colon cells

4.1 STATE OF ART

The correlation between diet, gut microbiota and human health has been widely recognized. In fact, diet provides molecules that the intestinal bacteria will further metabolize for energetic purposes. The metabolites deriving from bacterial metabolism may be either beneficial to human health, or not [1]. Among the gut metabolites, there is trimethylamine (TMA), whose effect on human health is still debated.

TMA is an amine produced by anaerobic bacteria in in the caecum and colon (belonging mainly to *Firmicutes, Actinobacteria, and Proteobacteria*) from choline, betaine, L-carnitine, dimethylglycine, and their precursors (e.g., phosphatidylcholine, cronobetaine, γ-butyrobetaine), which are abundant in red meat, fish, and eggs [2]. Once produced, TMA is absorbed via passive diffusion by the human enterocytes and conveyed to the liver by the portal circulation, where is oxidized to trimethylamine-N-Oxide (TMAO) by the flavin monooxygenase 1 and 3 (FMO1 and FMO3). Most of the produced TMA is either oxidized to TMAO or excreted in urines and feces.

Despite TMA is a well-known uremic toxin and its harmful effects have been described by several clinical and experimental studies from the early 20th century [3], up to date most of the scientific attention has been conveyed onto the harmful effects of TMAO and its role in several complex diseases. However, recent studies have reproposed a potential effect of TMA in boosting the pro-inflammatory response. TMA-induced genotoxicity and cytotoxicity on cardiomyocytes and intestinal epithelial cells have been documented [4,5]. The pro-inflammatory effects of TMA on the intestinal epithelium have been confirmed also by *in vivo* studies, in which the intrarectal and intraperitoneal injection of TMA resulted in a marked infiltration of inflammatory cells in the colon and rectal epithelium [5].

However, the precise mechanisms by which TMA promotes inflammation remain to be established and a few studies have investigated it. Recent evidence suggests that the perturbation of mitochondrial homeostasis could be one of the underlying causes [5]. Indeed, mitochondrial dynamics are influenced by both intrinsic and extrinsic factors, including diet, and they also play a key role in inflammation. Through the release of reactive oxygen species during aerobic respiration, mitochondria contribute to oxidative stress, which sustains inflammation and cell damage. In turn, perpetuated inflammatory stimuli (like it could be an excessive production of TMA) might lead to alteration of the mitochondrial dynamics and function [6,7], further contributing to cellular damage [8]. The maintenance of mitochondrial function is dependent on the normal expression of mitochondrial proteins, which are encoded by mitochondrial DNA (mtDNA).

Indeed, the inflammatory cascade may induce mitochondrial damage leading to changes of mitochondrial DNA copy number (mtDNAcn), which is the number of mtDNA molecules per cell. Therefore, mtDNAcn has been proposed as a marker of inflammation [9]. mtDNAcn can be also

influenced by mtDNA methylation. Indeed, it has recently been demonstrated that DNA methylation can also occur in the mtDNA, especially in the displacement loop or D-loop, a region involved in mtDNA replication and transcription [10]. Moreover, alterations of mtDNA methylation have been associated with multifactorial diseases such as obesity [11], and with environmental exposures, such as diet [12]. For this reason, also abnormal mtDNA methylation is attracting increasing attention as potential biomarker of mitochondrial health.

The mitochondrial damage caused by proinflammatory mediators may also lead to a decreased ATP production (through down regulation of complex I of the electron transport chain) and alterations of mitochondrial membrane potential (Δψm) [13–15]. Changes in the mitochondrial membrane potential trigger apoptotic signaling pathways that culminate with the release of mitochondrial components, including mtDNA, into the extracellular environment. Cell free mtDNA (mt-cfDNA) is known to function as damage-associated molecular pattern (DAMP) and to be a potent stimulator of the immune system [16]. For this reason, mt-cfDNA is also under investigation for its clinical significance and it has been suggested as another biomarker of inflammation [17,18].

Lastly, several studies have well described the crosstalk between inflammation and epigenetics. Indeed, while epigenetic mechanisms are crucial in the regulation of inflammatory genes' expression) [19], also pro-inflammatory signals may establish positive and negative feedback circuits with chromatin and epigenetic enzymes that may alter the global epigenetic landscape [20].

4.2 AIM OF THE STUDY

Because colonic epithelial cells face the large intestine lumen, where TMA is formed, they are most accessible to this bacterial product and thus are the first target of its effect. For this reason, the present study aims to better elucidate the impact of excess TMA on intestinal cells, using colon adenocarcinoma Caco-2 cells as an *in vitro* model. Given the prominent role of mitochondria in inflammation, we investigated the effect of TMA on mitochondrial dynamics, focusing on perturbation of ATP production and cellular membrane potential, as well as on the markers of mitochondrial damage (i.e., mtDNAcn, mt-cfDNA). Moreover, considering that a well-established link between inflammation and epigenetic perturbations exists, we also investigated the effect of TMA on the expression levels and activity of DNA methyltransferases (DNMTs) and sirtuins (SIRTs), key enzymes in the epigenetic landscape.

Lastly, we did set up a model of intestinal epithelium in order to preliminary evaluate the effect of TMA on the intestinal permeability.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture

To study the effect of TMA on intestinal cells, Caco-2 cells, a human colonic epithelial cell line (ATCC, Rockville, MD), were chosen. Caco-2 cells were also grown in multilayers to mimic the intestinal epithelium as much as possible. The experimental conditions and results relative to the intestinal epithelium study are always specified in the text.

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM 1X) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 1% L-glutamine; 1% Non-Essential Amino Acids (NEAA) and 1% penicillin/streptomycin (all from Euroclone, Milano, Italy). The cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 2 days and cells were passaged at 80% confluence.

To set up the intestinal epithelium model, the protocol by Kampfer et. Al.[21], was followed, although a few modifications were made. Briefly, Caco-2 cells were seeded on non-coated transwell inserts (0.4 µm pore size, ThinCert®, Cat. GR657641, Greiner Bio-one, Germany) at a density of $1.8x10^5$cells/cm^2 and maintained in DMEM 1X (as described above) both in apical (AP) and basal (BL) compartments for 5 days to allow the formation of a differentiated intestinal epithelium. On the days 2, 4, and 5 of culture, spent medium was replaced with fresh medium in both AP and BL compartments, to avoid nutrient depletion.

4.3.2 Viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate the cytotoxic effect of TMA. Briefly, Caco-2 cells were seeded in 96-well plates ($1x10^4$ cell/well) in DMEM 1X, cultured for 24h and treated with several concentrations of TMA (1nM, 10nM, 0,1µM, 1µM, 10µM, 100µM, 1mM, 10mM,100mM). At the end of the incubation period, 5 mg/ml MTT solution was added to the cells and incubated for 4h. The insoluble formazan salt product was solubilized by adding dimethyl sulfoxide (DMSO) and its amount was determined by measuring the optical density at 540 nm using a microplate reader. Cell viability was calculated according to the equation (T/C) × 100%, where T and C represent respectively the mean optical density of the treated group and the control group.

4.3.3 Cell Treatments

Intestinal TMA concentrations vary considerably from one individual to another, since, as already mentioned, several factors may regulate TMA production. In this study Caco-2 cells were treated with 10 µM, 400 µM and 1 mM TMA for 24h. The lowest TMA concentration was chosen based on

the average physiological faecal TMA concentrations [22]. The other two concentrations were selected based on the MTT results: 1 mM TMA was the highest not cytotoxic concentration, while 400 µM TMA was chosen because approximately halfway between the value of 10 µM and 1 mM. A negative control (vehicle only) and two positive controls (lipopolysaccharide (LPS) 100 ng/mL and 10 µg/mL) were also added. Experiments were run in triplicate.

After 24h of incubation, both cells and medium were collected. Medium was centrifuged at 12000xrpm for 5 min and clear supernatant was aliquoted into new nuclease-free conical tubes and stored at -80°C until further use. Cells were detached with trypsin and centrifuged at 300xg for 10 min; the supernatant was removed, and cell pellets were immediately frozen in liquid nitrogen and stored at -80°C until further use.

For the intestinal epithelium model, Caco-2 cells were treated with TMA (10 µM, 400 µM, 1 mM) for 24h. The treatments were added on the 3rd day of Caco-2 cells culture when the insert membrane was covered with a dense and uniform cell layer of cells. A negative control and a positive control (LPS 10 µg/mL) were also added. Experiments were run in duplicate. 48h after treatments, cells were mechanically detached from the insert membrane, centrifuged at 300xg for 10 min; the supernatant was removed, and cell pellets were immediately frozen in liquid nitrogen and stored at -80°C until further use.

4.3.4 Nuclear Extraction

The nuclear protein fraction was isolated from Caco-2 cells using the Nuclear Extraction kit (Cat. ab113474, Abcam, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, untreated Caco-2 cells were first lysed and then the nuclear protein fraction was isolated form the cytoplasmic fraction. Lastly, the protein concentration of the nuclear extract was quantified by Bradford Assay, using bovine serum albumin (BSA) as a calibrator.

4.3.5 Quantification of DNMTs and SIRTs Activities

The nuclear extracts were used to assess DNMTs and SIRTs activity. For the assessment of DNMTs activity the EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Kit (Cat. P-3009, EpigenTek, Farmingdale, NY, USA) was used according to the manufacturer's instructions. Briefly, 5 µg of nuclear proteins were transferred to a 96-well plate and incubated with several TMA concentrations (1mM, 400µM, 100µM, 50µM, 10µM, 1µM, 100nM, 10nM, 1nM, 0,01nM, 0,001nM, 0,000001nM) for 120 minutes at 37°C. Negative and positive controls were also included. The absorbance was read on a microplate reader at 450 nm, and data were used to calculate DNMTs activity (OD/h/mg).

For the assessment of SIRTs activity the Universal SIRT Activity Assay Kit (Cat. ab156915, Abcam, Waltham, MA, USA) was used according to the manufacturer's instructions. Briefly, 4µg of nuclear proteins were transferred to a 96-well plate and incubated with several TMA concentrations (1mM, 400µM, 100µM, 50µM, 10µM, 1µM, 100nM, 1nM, 0,01nM, 0,001nM, 0,000001nM) for 90 minutes. Negative and positive controls were also included. The absorbance was read at 450 nm and data were used to calculate SIRT activity (OD/min/mg).

4.3.6 mtDNA quantification

Pellets from treated Caco-2 cells were used for the extraction of genomic DNA, while culture medium from treated Caco-2 cells was used for the extraction of circulating cell-free DNA (cf-DNA). Genomic DNA was extracted using the DNAzol Reagent (Cat. 10503027, Invitrogen). Briefly, the cell pellets were lysed with DNAzol. 100% ethanol was added to the lysate to precipitate genomic DNA. Lastly, several washes in 75% ethanol were performed to remove any contaminants from the isolated DNA. Concentration and purity of DNA were assessed by UV spectrophotometer (NanoDrop, Thermo Fisher Scientific, Italy). Genomic DNA was used to quantify mtDNAcn in the samples.

Cf-DNA was extracted from culture medium using the Plasma/Serum Cell-Free Circulating DNA Purification Kit (Cat. 55100, Norgen Biotek, Thorold, ON, Canada). Concentration of cf-DNA was assessed fluorometrically (Qubit Fluorometer, Thermo Fisher Scientific, Italy). The isolated total cf-DNA was used to quantify cf-DNA of mitochondrial origin (cf-mtDNA). Relative quantification by quantitative PCR (Biorad CFX96) was chosen to quantify mtDNAcn, considering nuclear DNA as a normalizer, as previously reported [23]. The mitochondrial primers have been previously validated for their specificity (unique amplification of mtDNA) and the absence of coamplified nuclear insertions of mitochondrial origin (NUMTs). For relative quantification of mtDNAcn, *mtDNA-tRNALeu* (fw: CACCCAAGAACAGGGTTTGT; rv: TGGCCATGGGTATGTTGT), and *Beta-2-Microglobulin* (*B2M*) (fw: TGCTGTCTCCATGTTTGATGTATCT; rv: TCTCTGCTCCCCACCTCTAAGT) primers were chosen to amplify mtDNA and nDNA, respectively. Instead, for relative quantification of cf-mtDNA, *mtDNA-tRNALeu* and *18S ribosomal RNA* (fw: GCAATTATTCCCCATGAACG; rv: GGGACTTAATCAACGCAAGC) primers were chosen to amplify cf-mtDNA and cf-nDNA, respectively. The amplification conditions were: 30 seconds at 95°C followed by 5 seconds at 95°C and 30 seconds at 60°C, these latter repeated for 40 cycles. To check the specificity of each amplification, a melting curve was also performed. Relative mtDNAcn or relative cf-mtDNA were determined using the $2^{-\Delta\Delta Ct}$ method. Each analysis was run in duplicate. An inter-run calibrator sample was applied to adjust the results obtained from different amplification plates. The absolute quantification of cf-mtDNA was also performed by digital PCR (QIAcuity One, 2plex Device, Qiagen,

Milano, Italia) and Poisson distribution was applied to yield the absolute quantitation of the target sequence.

4.3.7 DNA methylation

Since mtDNA methylation might differ between the two strands [24], mitochondrial DNA methylation was assessed in two areas of the D-loop region, corresponding to the promoter of heavy strand (HSP), and to the promoter of the light strand (LSP). Methylation analysis was carried out by bisulphite pyrosequencing on mtDNA of treated Caco-2 cells. Prior to the methylation analysis, mtDNA was purified from the nuclear DNA (nDNA) [25], even though the D-loop region is one of the fewest regions on mtDNA that is not included in the nDNA as NUMTs [26]. Briefly, this mtDNA isolation protocol is based on alternated steps of nDNA-specific enzymatic digestion and nDNA removal by means of magnetic beads (Agencourt AMPure XP) that selectively bind to mtDNA. Since the circular structure of mtDNA could affect the bisulphite conversion, the purified mtDNA was linearized through BamHI enzymatic digestion. The linearized mtDNA was converted with bisulphite using the EZ-96 DNA Methylation-Gold kit (Zymo Research, Orange, USA). Then, PCR amplification was performed using PyroMark PCR kits (Qiagen Inc., Venlo, the Netherlands) in a standard thermal cycling device (2720 Thermal cycler, Applied Biosystem, Waltham, USA). The amplification accuracy was checked by gel electrophoresis. Amplicons where then pyrosequenced using the PyroMark Q24 device (Qiagen Inc., Venlo, the Netherlands). The efficiency of the bisulphite conversion was assessed using non-CpG cytosine residues within the analyzed sequence. The percentage of methylated cytosines over the sum of methylated and unmethylated cytosines was used to define the degree of methylation.

4.3.8 Gene expression analysis

Total RNA was extracted from treated Caco-2 cells (also from the intestinal epithelium model) with the Total RNA Purification Plus Kit (Cat. 48300, Norgen Biotek, Thorold, ON, Canada), according to the manufacturer's instructions. Concentration of RNA and purity were assessed by UV spectrophotometer (NanoDrop, Thermo Fisher Scientific, Italy). Then 1µg RNA was retrotranscribed to cDNA using the PrimeScript RT-PCR Kit (Cat. RR037A, Takara Bio, Göteborg, Sweden) according to the manufacturer's instructions. Gene expression analyses were carried out by quantitative real-time PCR (Biorad CFX96), using the TB Green® Premix Ex Taq™ (Cat. RR420A, Takara Bio, Göteborg, Sweden). The amplification conditions were: 30 seconds at 95°C followed by 5 seconds at 95°C and 30 seconds at 60°C, these latter repeated for 40 cycles. To check the specificity of each

amplification, a melting curve was also performed. The expression levels of the target genes were normalized relative to β-actin, using the $2^{-\Delta\Delta Ct}$ method.

Each analysis was run in duplicate. An inter-run calibrator sample was applied to adjust the results obtained from different amplification plates. The target genes analysed in Caco-2 cells were the epigenetic *DNMT1, DNMT3A, DNMT3B, SIRT1, SIRT6,* and *SIRT7*, the pro-inflammatory *IL-6* and *IL-1β* and the mitochondrial *ND6*, *CYTB*, *CO1*, *ATP6*. Instead, cDNA from Caco-2 cells from the intestinal epithelium model, was used to assess the expression levels of the tight junctions' major proteins *Claudin1, Occludin* and *Zonulin1*). The sequences of the primers used in the study are listed below: *DNMT1*, *DNMT3A* and *DNMT3B* (Cat. 10025636, Bio-Rad, Milan, Italy), *SIRT1* (fw: ACGCTGGAACAGGTTGCGGG; rv: AGCGGTTCATCAGCTGGGCAC), *SIRT6* (fw: AGTTCGACACCACCTTTGAG; rv: CGTACTGCGTCTTACACTTG), *SIRT7* (fw: CGTCCGGAACGCCAAATAC; rv: GACGCTGCCGTGCTGATT), *IL-6* (fw: TGCAATAACCACCCCTGACC; rv: GTGCCCATGCTACATTTGCC), *IL-1β* (fw: AGATGATAAGCCCACTCTACAG; rv: ACATTCAGCACAGGACTCTC), *ND6* (fw: GCTTTGTATGATTATGGGCGT; rv: CACCAACAAACAATGTTCAACC), *CYTB* (fw: ATCACTCGAGACGTAAATTATGGCT; rv: TGAACTAGGTCTGTCCCAATGTATG), *CO1* (fw: GACGTAGACACACGAGCATATTTCA; rv: AGGACATAGTGGAAGTGAGCTACAAC), *ATP6* (fw: TAGCCATACACAACACTAAAGGACGA; rv: GGGCATTTTTAATCTTAGAGCGAAA), *Claudin1*(fw: TGGTCAGGCTCTCTTCACTG; rv: TTGGATAGGGCCTTGGTGTT), *Occludin* (fw: GGGCATTGCTCATCCTGAAG; rv: GCCTGTAAGGAGGTGGACTT), and *Zonulin1* (fw: TTCACGCAGTTACGAGCAAG; rv: TTGGTGTTTGAAGGCAGAGC).

4.3.9 ATP quantification

The ATP content from treated Caco-2 cells was quantified using the ATP Colorimetric Assay Kit (Cat. MAK190, Sigma-Aldrich, Germany) according to the manufacturer's instructions. Briefly, pellets from treated Caco-2 cells (10μM, 400μM and 1mM TMA, 10μg/ml LPS) were lysed, and the ATP content was determined by phosphorylating glycerol. Negative controls were also included. The absorbance was read at 570 nm and data an ATP calibration curve was used for accurate ATP quantification. All analyses were run in triplicate.

4.3.10 Mitochondrial membrane potential

For the assessment of the mitochondria membrane potential (ΔΨm) in treated Caco-2 cells, the Mitochondrial Membrane Potential Kit (Cat. MAK159, Sigma-Aldrich, Germany) was used according to manufacturer's instructions. This assay uses cationic, lipophilic dye JC-10 that can discriminate between living cells and apoptotic cells through differences in their ΔΨm. Briefly, $8x10^4$ cells were

seeded into a 96-well plate. Different concentrations of TMA (0.01 µM, 0.1 µM, 1 µM, 10 µM, 100 µM, 200 µM, 400 µM and 1 mM) were added to the cells. Two positive controls (100 ng/mL and 10 µg/mL LPS) and a negative control (vehicle only) were also included. Treated cells were incubated 24h at 37°C. Afterword, treated cells were incubated with the JC-10 dye at 37°C for 60 minutes and the fluorescence was red at λ_{ex}=490/λ_{em}=525 nm and at λ_{ex}=540/λ_{em}=590 nm) through a fluorometer. Each experimental condition was set up in duplicate.

4.3.11 Permeability assay

In the intestinal epithelium model, Lucifer Yellow (LY, Sigma-Aldrich Life Science, USA) was used to monitor if the permeability of the Caco-2 multilayer had been modified by the TMA treatments. Briefly, after 24h treatments the medium from both AP and BL compartments was removed and Caco-2 cells on the AP side were gently washed twice with Phosphate Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} (Corning, AZ, USA). LY 100 µM (in PBS without Ca^{2+} and Mg^{2+}) was added to the AP compartment, while PBS (without Ca^{2+} and Mg^{2+}) was added to the BL compartment. 150 µL of the solution from both AP and BL compartments were immediately transferred to a 96-well plate and the fluorescence was read with a fluorometer at $\lambda_{Ex}/\lambda_{Em}$= 485/520 nm. The readings were repeated after 30, 60 and 120 minutes from the addition of the LY solution in the AP. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2 between each reading. All analysis were run in duplicate. Data were then used to calculate the Apparent permeability App.

4.3.12 Statistics analysis

Statistical analysis was performed by using SPSS (IBM SPSS Statistics for Windows, Version 24.0, USA) and R version 3.5.3 (R Core Team, Vienna, Austria). The ANOVA test was used to compare the difference between group means. A *p*-value < 0.05 was considered significant throughout the study.

4.4 RESULTS

4.4.1 Cell viability

The analysis showed that TMA 100mM and 10mM are both significantly cytotoxic (100 mM $p < 0.01$; 10 mM $p < 0.05$). In fact, cell viability is 10.01% ± 4.6% for cells treated with 100 mM TMA and 72.66% ± 16.3% for those treated with 10 mM TMA (Figure 1). No significant reductions of the cell viability were detected for the other tested concentrations; thus, the lower concentrations were considered acceptable for the treatments.

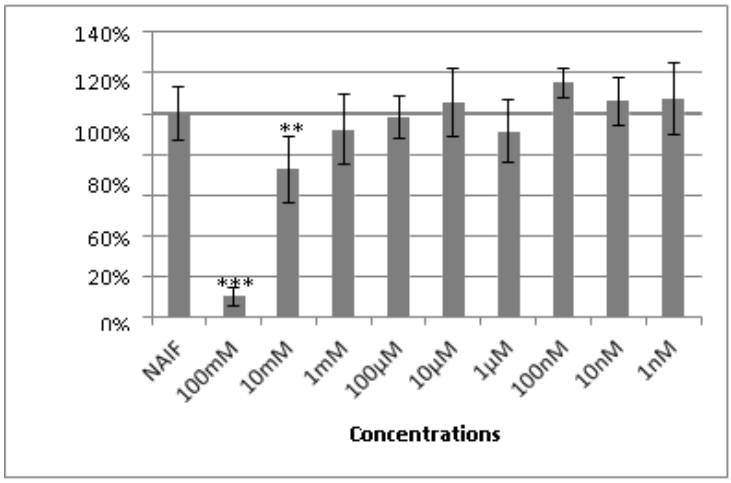


Figure 1. Cell viability percentages with standard deviation for each condition: naive and treatments at different concentrations of TMA.

4.4.2 Expression levels of pro-inflammatory genes

The expression of the pro-inflammatory *IL-6* and *IL-1β* was investigated in Caco-2 cells upon treatments with LPS 100 ng/mL and TMA 10 µM, TMA 400 µM and TMA 1 mM.

A statistically significant increase in the expression levels of all the analysed genes was observed in cells treated with TMA 1mM (*IL-6, p* < 0.001; *IL-1β, p* < 0.001) (Figure 2A,B).

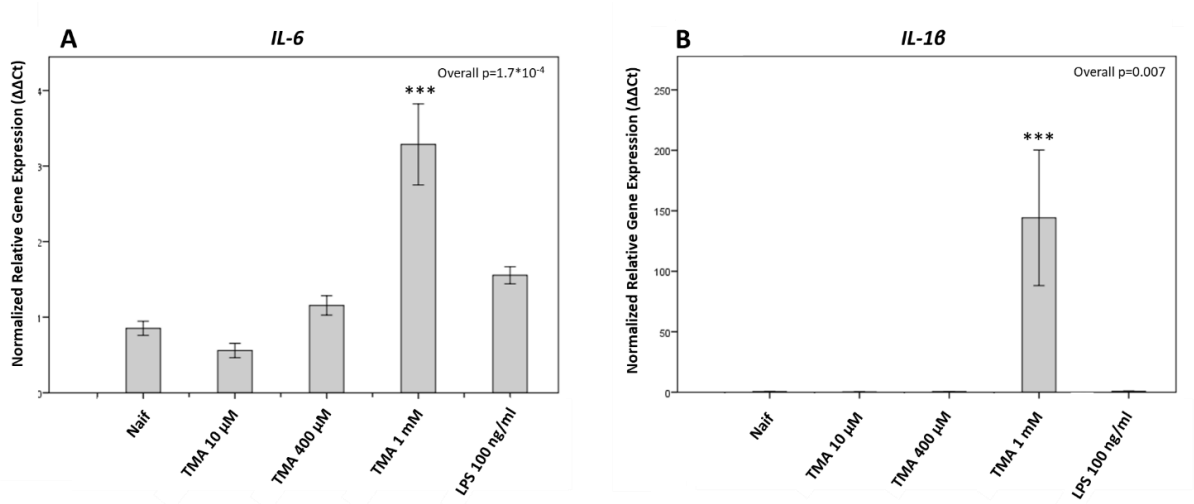


Figure 2. Expression levels of *IL-6* (A) and *IL-1β* (B) on Caco-2 cells.

4.4.3 DNMTs and SIRTs activity

The activity of SIRTS and DNMTs was investigated in Caco-2 cells exposed to increasing TMA concentrations.

At the tested experimental conditions, TMA did not induce any significant variations of DNMTs activity (overall $p > 0.05$) (Figure 3A). On the contrary, results show that TMA inhibits sirtuins' activity in a dose dependent manner. The inhibition of the activity was significant in a concentration range between 0.1 µM and 1mM (overall $p=9.7*10^{-6}$) (Figure 3B).

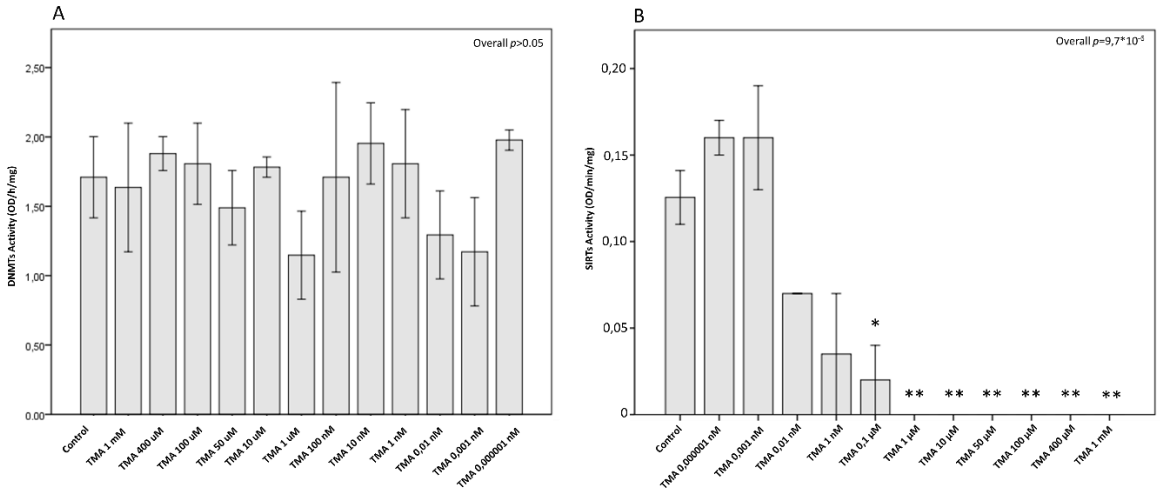


Figure 3. DNMTs activity (A) and SIRTs activity (B) of Caco-2 cells after TMA treatments.

4.4.4 Expression levels of DNMT1, DNMT3A, DNMT3B and SIRT1, SIRT6, SIRT7

Since DNMTs and SIRTs activities were investigated, it felt appropriate to also investigate their expression levels. *DNMT1, DNMT3A, DNMT3B* expression was measured in Caco-2 cells upon treatments with LPS 100 ng/mL and TMA 10 µM, TMA 400 µM and TMA 1 mM. For *SIRT1, SIRT6,* and *SIRT7* also a higher LPS concentration was tested (LPS 10 µg/ml).

A significant increase in *DNMT1* expression was observed with TMA 10 µM and TMA 400 µM ($p < 0.05$) (Figure 4A). Instead, a statistically relevant decrease of *DNMT3A* expression was observed with TMA 1mM and LPS 100 ng/mL treatments ($p < 0.05$) (Figure 4B). Lastly, *DNMT3B* expression was not affected by neither TMA not LPS treatments (overall, $p > 0.05$) (Figure 4C).

A significant increase of *SIRT1* in Caco-2 cells treated with all concentrations tested was measured (TMA 10 µM, $p < 0.05$; TMA 400 µM $p < 0.01$; TMA 1 mM $p < 0.01$) (Figure 4D). Instead, the expression of *SIRT6* and *SIRT7* was affected only by LPS 10 µg/ml, as showed by the significant decrease of their levels ($p < 0.05$ for both treatments) (Figure 4E,F).

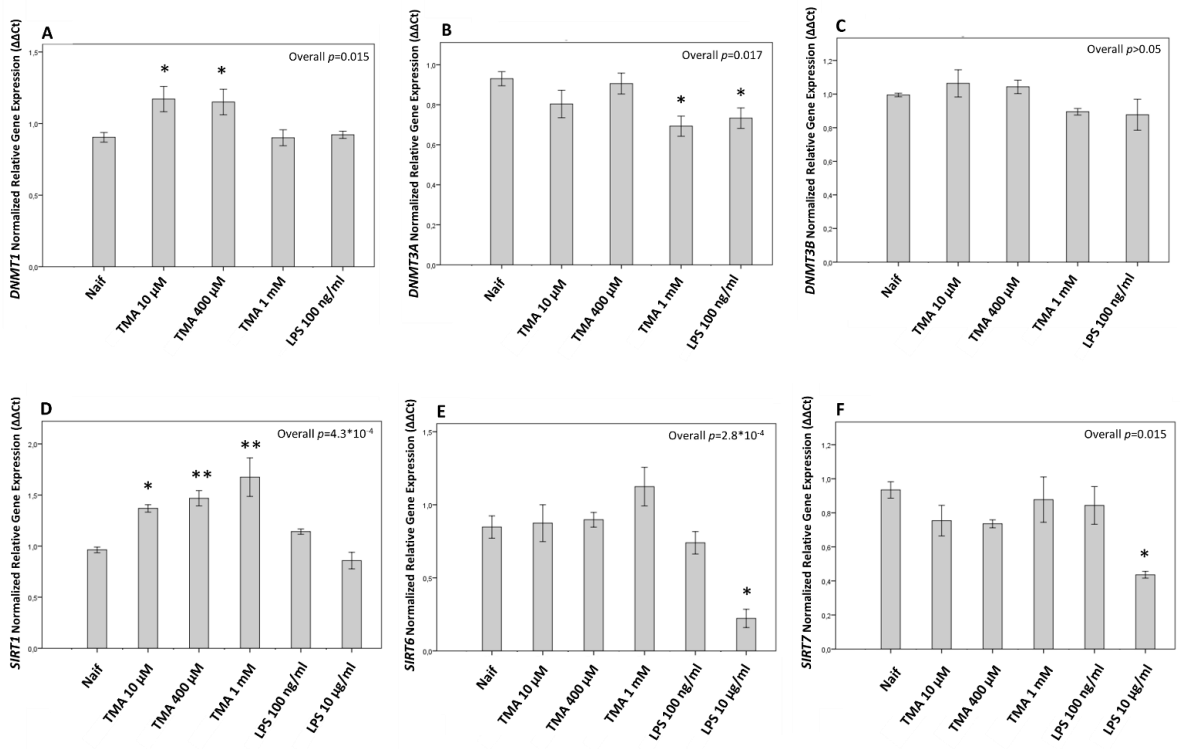


Figure 4. *DNMT1*(A)*, DNMT3A* (B)*, DNMT3B* (C) and *SIRT1* (D), *SIRT6* (E), *SIRT7* (F) expression levels on Caco-2 cells after TMA treatments.

4.4.5 mtDNA quantification

Significant variations of mtDNAcn were observed in one of the five experimental conditions tested. In fact, only the treatment with TMA 1 mM resulted in a significant decrease in mtDNAcn ($p < 0.05$) while neither lower TMA concentrations nor LPS 100 ng/ml were able to affect mtDNAcn (Figure 5A). At the same time, the relative quantification analysis showed a significant increase of cf-mtDNA released in the medium by Caco-2 cells after TMA 1 mM ($p < 0.05$), and after LPS 100 ng/ml ($p < 0.01$) (Figure 5B). However, the absolute quantification analysis confirmed a significant increase of cf-mtDNA released in the medium only upon TMA 1 mM treatment ($p < 0.05$). (Figure 5C).

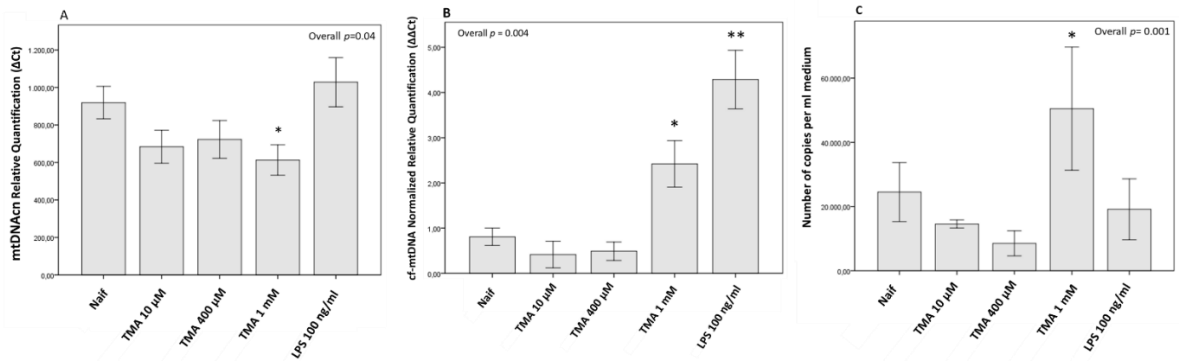


Figure 5. MtDNAcn (A) and cf-mtDNA released in the culture medium (B,C) by Caco-2 cells.

4.4.6 D-loop methylation

The methylation levels were investigated in 3 distinct CpGs in either the HSP or LSP D-loop promoters. The methylation levels in the HSP D-loop were not affected by any treatments. Conversely, a significant increase in the degree of methylation was observed in the LSP D-Loop area after TMA 1 mM treatment ($p < 0.01$) (Figure 6A), with the second CpG (CpG2) of the LSP D-Loop area being the major contributor ($p < 0.001$) (Figure 6B).

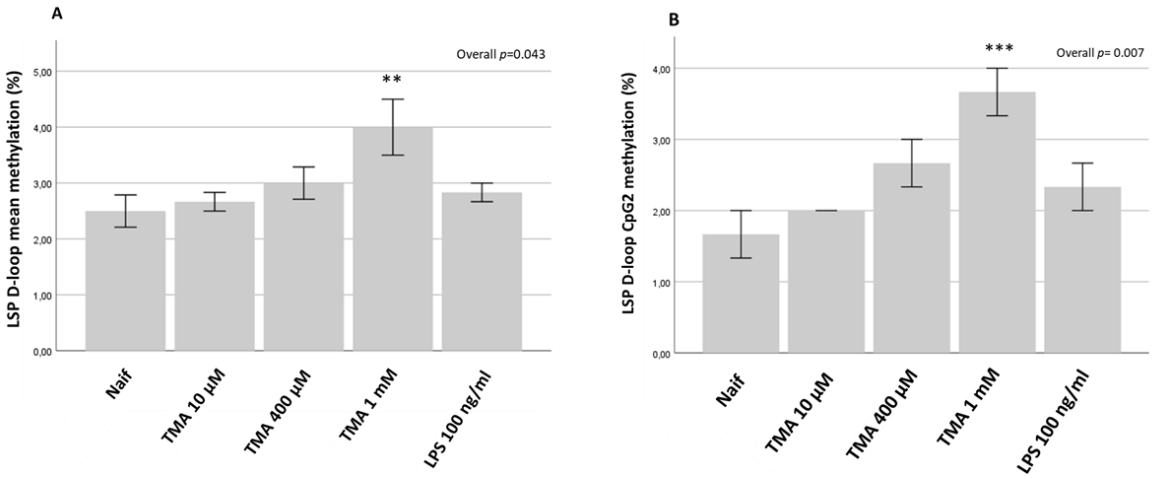


Figure 6. LSD D-loop methylation % in Caco-2 cells after TMA and LPS treatments. LSD D-loop mean methylation % (6A) and LSD D-loop CpG2 methylation % (6B).

4.4.7 Expression of mitochondrial genes

In this study, the capacity of TMA to modulate the expression of the mitochondrial genes correlated to ATP formation was investigated. Interestingly, apart from *MT-ATP6* and *MT-CYB* that were not affected by TMA treatments but only by LPS 10 µg/mL ($p = 0.05$ for both genes) (Figure 7A,D), the expression levels of *MT-ND6* significantly decreased with the highest TMA concentration (TMA 1 mM, $p < 0.05$), as well as both the LPS treatments ($p < 0.05$) (Figure 7B). Instead, *MT-CO1* showed a significant downregulation after treatments with the lowest TMA concentration (TMA 10 µM, $p < 0.05$) and LPS 10 µg/mL ($p < 0.05$) (Figure 7D).

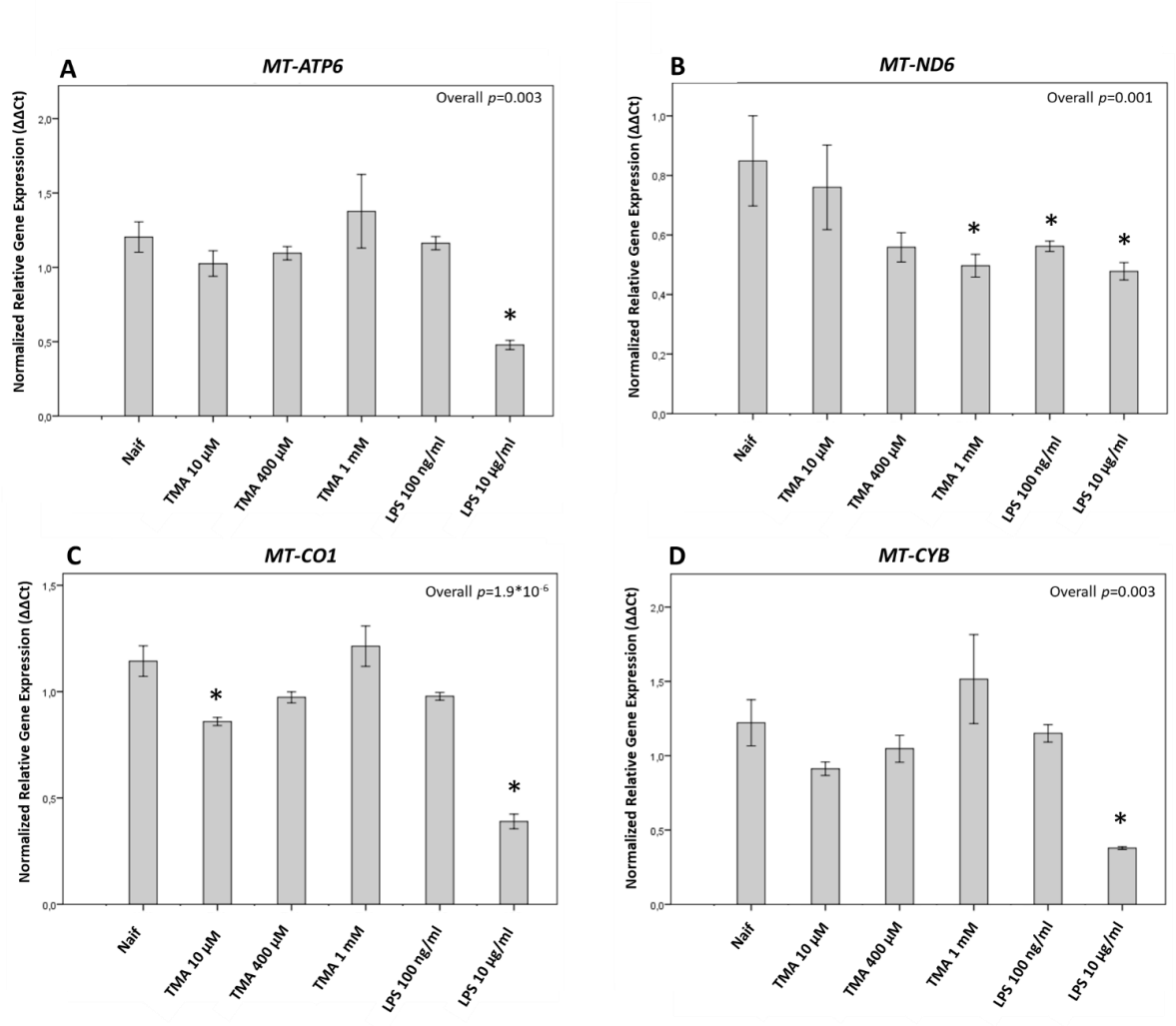


Figure 7. Expression levels of mitochondrial genes *MT-ATP6 (A)*, *MT-ND6 (B), MT-CO1 (C),* and *MT-CYB (D)* in Caco-2 cells.

4.4.8 ATP quantification

To determine the effect of TMA on intracellular ATP levels, we cultured Caco-2 cells in the presence of different concentrations of TMA (10 µM, 400 µM, and 1 mM) for 24h and intracellular ATP was quantified on lysed cells. In the present study, ATP contents is expressed as nmoles ATP per 1 million cells. As ATP content reflects the metabolic activity of cells, here we confirm that TMA slows down the cellular metabolism in Caco-2 cells. Indeed, after 24h exposure of TMA, a decrease in ATP content was observed for all the tested TMA concentration (10 µM TMA, $p < 0.001$; 400 µM TMA, $p < 0.01$ and 1mM TMA, $p < 0.001$), as well as following treatment with 100 ng/ml LPS ($p < 0.01$) (Figure 8).

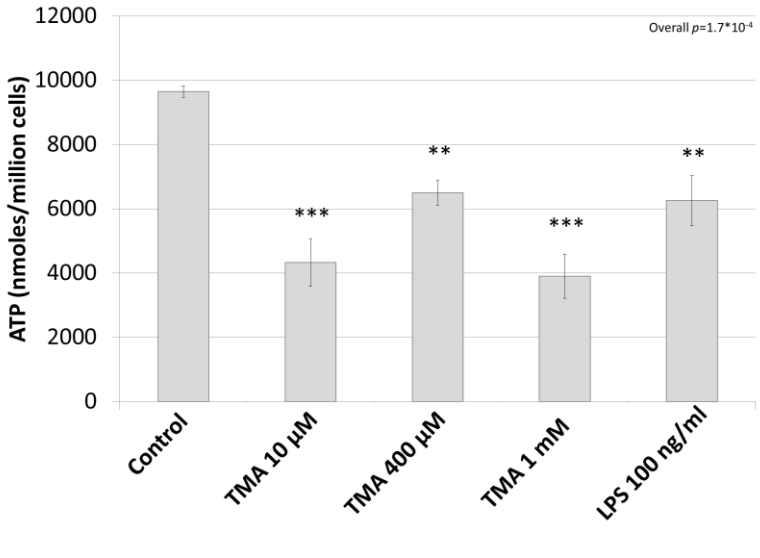


Figure 8. Intracellular ATP levels in Caco-2 cells.

4.4.9 Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm), which refers to the difference in the electrical potentials of the mitochondrial membrane, is not only a key indicator of mitochondrial activity, because it reflects ATP production, but also of good cellular health. In this study, the effects of several doses of TMA on mitochondrial membrane potential were investigated. After 24h treatment with various TMA concentrations, LPS 100 ng/mL and LPS 10 µg/mL, no significant variation of the mitochondrial membrane potential was observed at the tested concentrations (overall $p > 0.05$) (Figure 9).

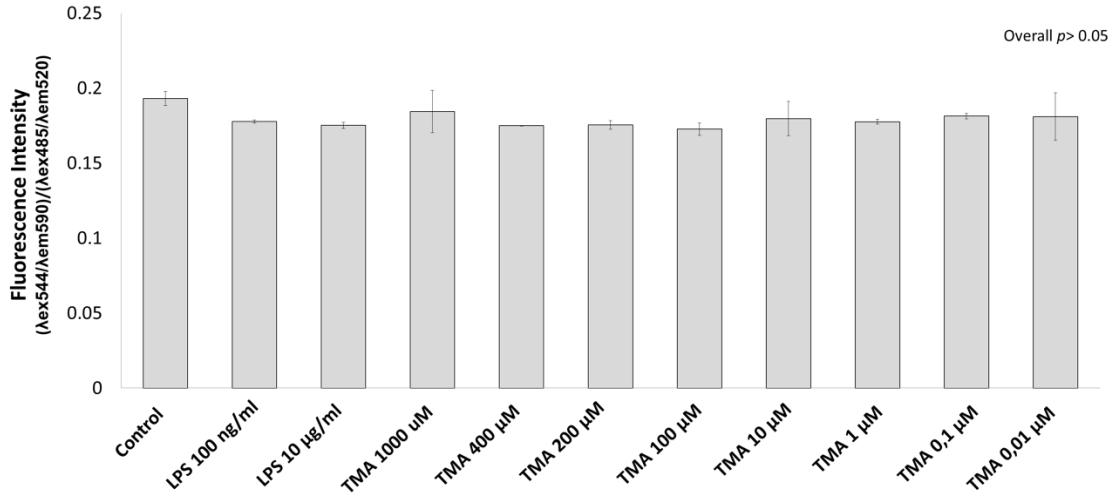


Figure 9. Mitochondrial membrane potential (ΔΨm) of Caco-2 cells was not affected by the tested TMA treatments.

4.4.10 Intestinal permeability assay

The effects of TMA on the intestinal permeability were evaluated in a Caco-2 cells epithelium model. At the tested experimental conditions, no significant variations of permeability of the intestinal epithelium were observed upon neither TMA nor LPS treatments (overall $p > 0.05$) (Figure 10).

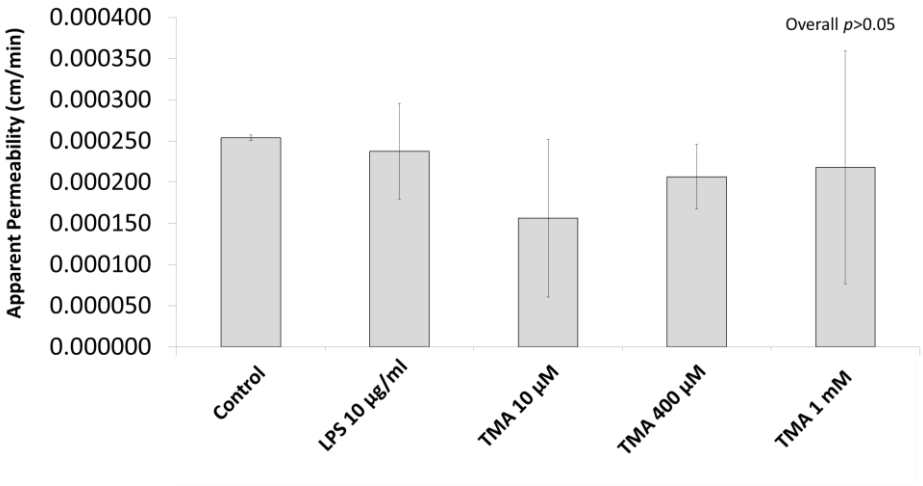


Figure 10. Intestinal permeability of a Caco-2 cells epithelium model.

4.4.11 Expression levels of tight junctions

No significant changes in the expression levels of genes involved in the formation of tight junctions emerged after none of the tested TMA and LPS treatments (overall $p > 0.05$) (Figure 11).

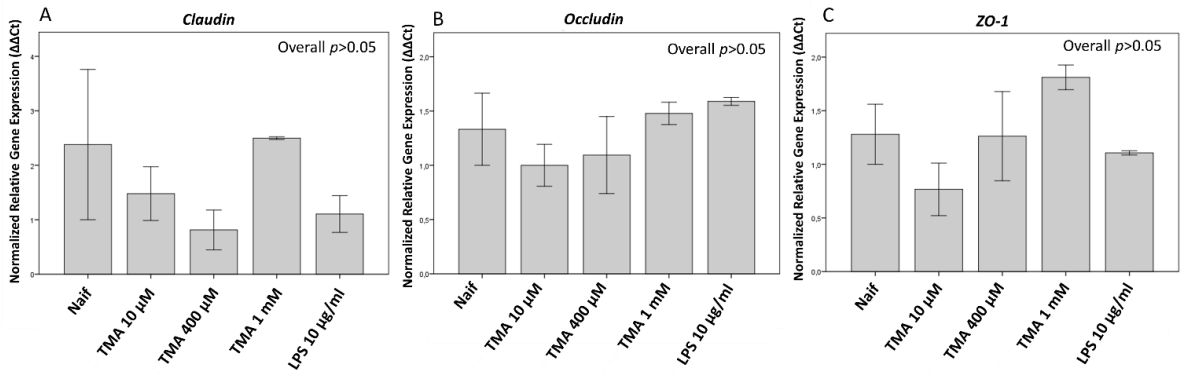


Figure 11. Expression levels of *Claudin, Occludin* and *ZO*-1 in a Caco-2 cells epithelium model.

4.5 DISCUSSION AND CONCLUSIONS

A growing number of studies is supporting the link between nutrition, gut microbiota, and human diseases. Gut metabolites derived from the bacterial metabolism of dietary precursors use both passive and active transport to reach the intracellular space first, and the systemic circulation later. Once inside the cell, they may perturb cellular homeostasis and interfere with vital physiological functions.

Most of the scientific attention has been converged towards the effects of TMAO on human health. Indeed, the fact that circulating TMAO derives from the almost total oxidation of its TMA precursor, explains why TMA has rarely been the target of choice for most studies. However also TMA is a well-known uremic toxin, and it has been implicated in various chronic health conditions [4,27], including trimethylaminuria and cardiovascular disease [4,28]. Moreover, since the intestine is the site of TMA production, the study of its effects on the intestinal environment is particularly interesting. Indeed, the pro-inflammatory action of TMA on the colon cells and epithelium have been recently documented [5]. In our study, excess TMA was associated with pro-inflammatory signalling, as demonstrated by the increased expression of pro-inflammatory cytokines IL-6 and IL-1β. The increased levels of pro-inflammatory cytokines alone warn against the potentially harmful effect of TMA. Indeed, elevated levels of pro-inflammatory mediators have been associated with disease evolution and unfavourable outcomes in several pathological scenarios, such as chronic heart failure, and cancer, just to mention a few [29,30]. TMA-induced inflammation is coherent with previous investigations by Jalandra et al., although the reported study did not investigate inflammatory gene expression but focused on other molecular aspects of inflammation both *in vitro* and *in vivo* [5].

The existence of a strict crosstalk between inflammation and epigenetics is well established. Indeed, epigenetic mechanisms are crucial for the regulation of inflammatory genes [19,31], and conversely activated inflammatory signaling pathways may induce epimutations [32], which sustain inflammation. Changes in the methylation status, as well as perturbations of sirtuins activity and levels, have been linked to various biological conditions and human diseases, including inflammatory responses [33,34]. In the present study we investigated expression levels and activity of DNMTs and SIRTs, two of the most important classes of epigenetic enzymes, in response to intestinal TMA. In fact, DNMTs and SIRTs are susceptible to inflammatory stimuli and to dietary factors, and an altered SIRTs and DNMTs expression in response to TMAO, the TMA oxidation product, has been already documented [35,36]. In our study, TMA treatment did not disturb DNMTs activity, but it resulted in a significant impairment of SIRTs activity, and the effect was dose dependent. The reduction of SIRTs activity may be due to the pro-inflammatory effect of TMA, since the downregulation of SIRTs as part of the acute inflammatory response has been already

documented both *in vivo* and *in vitro* [37,38]. The massive decline of NAD^+ that cells experience during the inflammatory response [39] could explain the reduction of SIRTs activity, which relies on intracellular NAD^+ as cofactor. Interestingly, we measured increased expression levels of *SIRT1*, most likely as a compensatory mechanism to counteract the decreased enzymatic activity. Similarly, *DNMT1* was upregulated by high TMA doses in our study, most likely because of the TMA-induced inflammation. The increased expression levels of IL-6 and IL-1β cytokines above discussed, which is a clear sign of the activated inflammatory response, may justify the detected *DNMT1* upregulation. Indeed, IL-6-induced DNMT1 upregulation has been already documented in colon cancer [40]. On the contrary, our results showed a significant downregulation of *de novo DNMT3A*. Although to the best of our knowledge there are not studies that investigate the relation between TMA and DNMTs, alterations of *DNMTs* expression levels have been reported for TMAO, which suggests that a similar mechanism for TMA may also exist.

Considering that mitochondria have a significant role in inflammation (mitochondria are a key source of DMAPs) and *vice versa* pro-inflammatory mediators may alter mitochondrial function [6], we chose to investigate the mitochondrial dynamics of intestinal cells in response to excess TMA. Our results showed that cells treated with a high TMA dose had reduced mtDNAcn, a biomarker of mitochondrial health. TMA-induced mitochondrial damage and impairment of the replication machinery may explain the observed reduction of mtDNAcn. Interestingly, our results showed elevated methylation levels in the LSP D-loop area; since the D-loop region is important for mitochondrial genes transcription and mtDNA replication, the increased methylation may compromise mtDNA replication and justify the reduced mtDNAcn measured in the study [41,42]. Indeed, an inverse correlation between D-loop methylation levels and the mtDNA copy number has been already reported in literature in human cell cultures [43,44], even though this has not always been confirmed [45,46].

Moreover, in our study significantly higher levels of cf-mtDNA in the culture medium of cells treated with high TMA doses were detected and have been confirmed by both relative and absolute quantification methods. According to our hypothesis, in response to high TMA concentrations, inflamed intestinal cells release mtDNA in the extracellular milieu, in this way contributing to the detected reduction of mtDNAcn even further.

The alteration of mitochondrial functions was further confirmed by the TMA-induced impairment of the energy production system. Indeed, our results showed a decreased intracellular ATP content in cells treated with TMA, most likely due to the inflammation-induced downregulation of *MT-ND6* and *MT-CO1*, two important components of the electron transport chain. Indeed, the decrease in ATP production because of pro-inflammatory mediators-driven downregulation of mitochondrial complex I has already been described [13–15]. Moreover, our data are consistent with those by Jalandra et al., who recently described a decreased ATP content in intestinal cells after acute TMA

exposure. The reduction in ATP levels indicates a compromised mitochondrial metabolic integrity and is also linked to cell death [47]. Since the driving force for ATP production is provided by the mitochondrial membrane potential (Δψm), we also investigated whether the observed ATP drop was due to the TMA-induced collapse of the Δψm. However, our results did not show any significant alteration of the Δψm after TMA treatment, thus, other molecular mechanisms are probably responsible for the compromised energy production.

Lastly, bacterial metabolites may compromise the integrity of the intestinal barrier, gain access to circulation, and reach distant organs. Indeed, gut barrier leakage has been associated to the pathophysiology of gastrointestinal as well as extra-intestinal diseases, such as heart failure, metabolic syndrome, diabetes, and psychiatric disorders [48,49]. For this reason, we set up a simplified model of intestinal epithelium to investigate the effect of TMA on the gut-blood barrier. Our results did not show any significant alteration of intestinal permeability after 24 h TMA exposure at the tested concentrations, also confirmed by the expression levels of the tight junctions that remain unchanged compared to controls. Our results are consistent with those by Jalandra et al., who, despite reporting impaired intestinal membrane integrity after exposure to high TMA doses, also stated that no significant alterations were observed below 5 mM TMA concentration [5]. However, they studied the integrity of intestinal cell membranes and not the integrity of the intestinal epithelium, as we did with our model. Nonetheless, our model of intestinal epithelium needs further implementations, and the set-up of co-culture systems with other cell lines (i.e., macrophages) will certainly represent a more reliable model to elucidate the effect of TMA on intestinal barrier integrity.

In conclusion, based on what seen on our cellular model, our study reveals that excess TMA in the intestinal environment may induce inflammation in intestinal cells and perturb both epigenetic and mitochondrial homeostasis. As a result of the activated pro-inflammatory status, overwhelmed mitochondria may experience impaired mtDNA replication (as evidenced by the altered D-loop methylation and reduction of the mtDNAcn), which results in compromised respiration (as proved by the downregulation of some respiratory chain components and decreased ATP content). Even though we didn’t detect changes in the intestinal permeability, our study certainly contributes to reinforcing the hypothesis that TMA (at least at high doses tested in the study) is not a harmless metabolite and should not be forgotten when trying to elucidate the impact of dietary molecules on human health because it may have a contributing role in microbiota-induced intestinal diseases. Further efforts are necessary to better elucidate the mechanistic pathways of TMA-induced inflammation; in addition, the setup of a proper co-culture model may help evaluate the role of TMA as a possible modulator of intestinal permeability and inflammation, also in view of personalized therapies to address intestinal inflammation.

4.6 REFERENCES

1. Gentile, C.L.; Weir, T.L. The Gut Microbiota at the Intersection of Diet and Human Health. *Science* **2018**, *362*, 776–780, doi:10.1126/SCIENCE.AAU5812.
2. Rath, S.; Heidrich, B.; Pieper, D.H.; Vital, M. Uncovering the Trimethylamine-Producing Bacteria of the Human Gut Microbiota. *Microbiome* **2017**, *5*, 54, doi:10.1186/s40168-017-0271-9.
3. Brieger, H.; Hodes, W.A. Toxic Effects of Exposure to Vapors of Aliphatic Amines. *Arch. Indust. Hyg. & Occupational Med.* **1951**, *3*, 287–291.
4. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kaplon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins (Basel)* **2019**, *11*, doi:10.3390/TOXINS11090490.
5. Jalandra, R.; Makharia, G.K.; Sharma, M.; Kumar, A. Inflammatory and Deleterious Role of Gut Microbiota-Derived Trimethylamine on Colon Cells. *Front Immunol* **2023**, *13*, doi:10.3389/FIMMU.2022.1101429.
6. López-Armada, M.J.; Riveiro-Naveira, R.R.; Vaamonde-García, C.; Valcárcel-Ares, M.N. Mitochondrial Dysfunction and the Inflammatory Response. *Mitochondrion* **2013**, *13*, 106–118, doi:10.1016/J.MITO.2013.01.003.
7. Tolstik, T. V.; Bogatyreva, A.I.; Grechko, A. V.; Oishi, Y.; Markin, A.M. Features of Mitochondrial Dynamics in Monocytes in Inflammatory and Metabolic Disorders. *Vessel Plus* **2022**, *6*, 58, doi:10.20517/2574-1209.2022.22.
8. Li, Y.; Yang, S.; Jin, X.; Li, D.; Lu, J.; Wang, X.; Wu, M. Mitochondria as Novel Mediators Linking Gut Microbiota to Atherosclerosis That Is Ameliorated by Herbal Medicine: A Review. *Front Pharmacol* **2023**, *14*, doi:10.3389/FPHAR.2023.1082817.
9. Castellani, C.A.; Longchamps, R.J.; Sun, J.; Guallar, E.; Arking, D.E. Thinking Outside the Nucleus: Mitochondrial DNA Copy Number in Health and Disease. *Mitochondrion* **2020**, *53*, 214–223, doi:10.1016/J.MITO.2020.06.004.
10. Mposhi, A.; Van Der Wijst, M.G.P.; Faber, K.N.; Rots, M.G. Regulation of Mitochondrial Gene Expression, the Epigenetic Enigma. *Front Biosci (Landmark Ed)* **2017**, *22*, 1099–1113, doi:10.2741/4535.
11. Bordoni, L.; Smerilli, V.; Nasuti, C.; Gabbianelli, R. Mitochondrial DNA Methylation and Copy Number Predict Body Composition in a Young Female Population. *J Transl Med* **2019**, *17*, 1–11, doi:10.1186/S12967-019-02150-9/FIGURES/6.
12. Corsi, S.; Iodice, S.; Shannon, O.; Siervo, M.; Mathers, J.; Bollati, V.; Byun, H.-M. Mitochondrial DNA Methylation Is Associated with Mediterranean Diet Adherence in a Population of Older Adults with Overweight and Obesity. *Proceedings of the Nutrition Society* **2020**, *79*, E95, doi:10.1017/S0029665120000439.
13. Zell, R.; Geck, P.; Werdan, K.; Boekstegers, P. TNF-Alpha and IL-1 Alpha Inhibit Both Pyruvate Dehydrogenase Activity and Mitochondrial Function in Cardiomyocytes: Evidence for Primary Impairment of Mitochondrial Function. *Mol Cell Biochem* **1997**, *177*, 61–67, doi:10.1023/A:1006896832582.
14. López-Armada, M.J.; Caramés, B.; Martín, M.A.; Cillero-Pastor, B.; Lires-Dean, M.; Fuentes-Boquete, I.; Arenas, J.; Blanco, F.J. Mitochondrial Activity Is Modulated by TNFα and IL-1β in Normal Human Chondrocyte Cells. *Osteoarthritis Cartilage* **2006**, *14*, 1011–1022, doi:10.1016/j.joca.2006.03.008.
15. Stadler, J.; Bentz, B.G.; Harbrecht, B.G.; Di Silvio, M.; Curran, R.D.; Billiar, T.R.; Hoffman, R.A.; Simmons, R.L. Tumor Necrosis Factor Alpha Inhibits Hepatocyte Mitochondrial Respiration. *Ann Surg* **1992**, *216*, 539, doi:10.1097/00000658-199211000-00003.
16. Riley, J.S.; Tait, S.W. Mitochondrial DNA in Inflammation and Immunity. *EMBO Rep* **2020**, *21*, e49799, doi:10.15252/embr.201949799.
17. Rosa, H.S.; Ajaz, S.; Gnudi, L.; Malik, A.N. A Case for Measuring Both Cellular and Cell-Free Mitochondrial DNA as a Disease Biomarker in Human Blood. *FASEB J* **2020**, *34*, 12278–12288, doi:10.1096/FJ.202000959RR.
18. Nakahira, K.; Kyung, S.Y.; Rogers, A.J.; Gazourian, L.; Youn, S.; Massaro, A.F.; Quintana, C.; Osorio, J.C.; Wang, Z.; Zhao, Y.; et al. Circulating Mitochondrial DNA in Patients in the ICU as a Marker of Mortality: Derivation and Validation. *PLoS Med* **2013**, *10*, 1–12, doi:10.1371/JOURNAL.PMED.1001577.
19. Bayarsaihan, D. Epigenetic Mechanisms in Inflammation. *J Dent Res* **2011**, *90*, 9, doi:10.1177/0022034510378683.
20. Tan, S.Y.X.; Zhang, J.; Tee, W.W. Epigenetic Regulation of Inflammatory Signaling and Inflammation-Induced Cancer. *Front Cell Dev Biol* **2022**, *10*, 1232, doi:10.3389/FCELL.2022.931493/BIBTEX.
21. Kämpfer, A.A.M.; Urbán, P.; Gioria, S.; Kanase, N.; Stone, V.; Kinsner-Ovaskainen, A. Development of an in Vitro Co-Culture Model to Mimic the Human Intestine in Healthy and Diseased State. *Toxicol In Vitro* **2017**, *45*, 31–43, doi:10.1016/J.TIV.2017.08.011.
22. Borrel, G.; McCann, A.; Deane, J.; Neto, M.C.; Lynch, D.B.; Brugère, J.F.; O'Toole, P.W. Genomics and Metagenomics of Trimethylamine-Utilizing Archaea in the Human Gut Microbiome. *ISME J* **2017**, *11*, 2059–2074, doi:10.1038/ISMEJ.2017.72.
23. Fazzini, F.; Schöpf, B.; Blatzer, M.; Coassin, S.; Hicks, A.A.; Kronenberg, F.; Fendt, L. Plasmid-Normalized Quantification of Relative Mitochondrial DNA Copy Number. *Scientific Reports 2018 8:1* **2018**, *8*, 1–11, doi:10.1038/s41598-018-33684-5.
24. Vos, S.; Nawrot, T.S.; Martens, D.S.; Byun, H.M.; Janssen, B.G. Mitochondrial DNA Methylation in Placental Tissue: A Proof of Concept Study by Means of Prenatal Environmental Stressors. *Epigenetics* **2021**, *16*, 121, doi:10.1080/15592294.2020.1790923.
25. Balagurumoorthy, P.; Adelstein, S.J.; Kassis, A.I. Method to Eliminate Linear DNA from Mixture Containing Nicked-Circular, Supercoiled, and Linear Plasmid DNA. *Anal Biochem* **2008**, *381*, 172, doi:10.1016/J.AB.2008.06.037.
26. Tsuji, J.; Frith, M.C.; Tomii, K.; Horton, P. Mammalian NUMT Insertion Is Non-Random. *Nucleic Acids Res* **2012**, *40*, 9073–9088, doi:10.1093/NAR/GKS424.
27. Pignanelli, M.; Just, C.; Bogiatzi, C.; Dinculescu, V.; Gloor, G.B.; Allen-Vercoe, E.; Reid, G.; Urquhart, B.L.; Ruetz, K.N.; Velenosi, T.J.; et al. Mediterranean Diet Score: Associations with Metabolic Products of the Intestinal Microbiome, Carotid Plaque Burden, and Renal Function. *Nutrients* **2018**, *10*, 779, doi:10.3390/NU10060779.
28. Messenger, J.; Clark, S.; Massick, S.; Bechtel, M. A Review of Trimethylaminuria: (Fish Odor Syndrome). *J Clin Aesthet Dermatol* **2013**, *6*, 45.
29. Hiller, D.J.; Meschonat, C.; Kim, R.; Li, B.D.L.; Chu, Q.D. Chemokine Receptor CXCR4 Level in Primary Tumors Independently Predicts Outcome for Patients with Locally Advanced Breast Cancer. *Surgery* **2011**, *150*, 459–465, doi:10.1016/J.SURG.2011.07.005.
30. Yndestad, A.; Finsen, A.V.; Ueland, T.; Husberg, C.; Dahl, C.P.; Øie, E.; Vinge, L.E.; Sjaastad, I.; Sandanger, Ø.; Ranheim, T.; et al. The Homeostatic Chemokine CCL21 Predicts Mortality and May Play a Pathogenic Role in Heart Failure. *PLoS One* **2012**, *7*, doi:10.1371/JOURNAL.PONE.0033038.

31. Chen, S.; Yang, J.; Wei, Y.; Wei, X. Epigenetic Regulation of Macrophages: From Homeostasis Maintenance to Host Defense. *Cell Mol Immunol* **2020**, *17*, 36–49, doi:10.1038/S41423-019-0315-0.
32. Tan, S.Y.X.; Zhang, J.; Tee, W.W. Epigenetic Regulation of Inflammatory Signaling and Inflammation-Induced Cancer. *Front Cell Dev Biol* **2022**, *10*, doi:10.3389/FCELL.2022.931493.
33. Kim, J.H.; Yoo, B.C.; Yang, W.S.; Kim, E.; Hong, S.; Cho, J.Y. The Role of Protein Arginine Methyltransferases in Inflammatory Responses. *Mediators Inflamm* **2016**, *2016*, doi:10.1155/2016/4028353.
34. Serrano-Marco, L.; Chacón, M.R.; Maymó-Masip, E.; Barroso, E.; Salvadó, L.; Wabitsch, M.; Garrido-Sánchez, L.; Tinahones, F.J.; Palomer, X.; Vendrell, J.; et al. TNF-α Inhibits PPARβ/δ Activity and SIRT1 Expression through NF-KB in Human Adipocytes. *Biochim Biophys Acta* **2012**, *1821*, 1177–1185, doi:10.1016/J.BBALIP.2012.05.006.
35. Ke, Y.; Li, D.; Zhao, M.; Liu, C.; Liu, J.; Zeng, A.; Shi, X.; Cheng, S.; Pan, B.; Zheng, L.; et al. Gut Flora-Dependent Metabolite Trimethylamine-N-Oxide Accelerates Endothelial Cell Senescence and Vascular Aging through Oxidative Stress. *Free Radic Biol Med* **2018**, *116*, 88–100, doi:10.1016/J.FREERADBIOMED.2018.01.007.
36. Luo, T.; Liu, D.; Guo, Z.; Chen, P.; Guo, Z.; Ou, C.; Chen, M. Deficiency of Proline/Serine-Rich Coiled-Coil Protein 1 (PSRC1) Accelerates Trimethylamine N-Oxide-Induced Atherosclerosis in ApoE-/- Mice. *J Mol Cell Cardiol* **2022**, *170*, 60–74, doi:10.1016/J.YJMCC.2022.05.013.
37. Bai, X.; He, T.; Liu, Y.; Zhang, J.; Li, X.; Shi, J.; Wang, K.; Han, F.; Zhang, W.; Zhang, Y.; et al. Acetylation-Dependent Regulation of Notch Signaling in Macrophages by SIRT1 Affects Sepsis Development. *Front Immunol* **2018**, *9*, doi:10.3389/FIMMU.2018.00762.
38. Li, T.; Zhang, J.; Feng, J.; Li, Q.; Wu, L.; Ye, Q.; Sun, J.; Lin, Y.; Zhang, M.; Huang, R.; et al. Resveratrol Reduces Acute Lung Injury in a LPS-induced Sepsis Mouse Model via Activation of Sirt1. *Mol Med Rep* **2013**, *7*, 1889–1895, doi:10.3892/MMR.2013.1444.
39. Covarrubias, A.J.; Kale, A.; Perrone, R.; Lopez-Dominguez, J.A.; Pisco, A.O.; Kasler, H.G.; Schmidt, M.S.; Heckenbach, I.; Kwok, R.; Wiley, C.D.; et al. Senescent Cells Promote Tissue NAD+ Decline during Ageing via the Activation of CD38+ Macrophages. *Nat Metab* **2020**, *2*, 1265–1283, doi:10.1038/S42255-020-00305-3.
40. Foran, E.; Garrity-Park, M.M.; Mureau, C.; Newell, J.; Smyrk, T.C.; Limburg, P.J.; Egan, L.J. Upregulation of DNA Methyltransferase-Mediated Gene Silencing, Anchorage-Independent Growth, and Migration of Colon Cancer Cells by Interleukin-6. *Mol Cancer Res* **2010**, *8*, 471–481, doi:10.1158/1541-7786.MCR-09-0496.
41. Xu, Y.Y.; Xu, L.L.; Han, M.; Liu, X.T.; Li, F.; Zhou, X.Y.; Wang, Y.; Bi, J.Z. Altered Mitochondrial DNA Methylation and Mitochondrial DNA Copy Number in an APP/PS1 Transgenic Mouse Model of Alzheimer Disease. *Biochem Biophys Res Commun* **2019**, *520*, 41–46, doi:10.1016/J.BBRC.2019.09.094.
42. Cui, D.; Xu, X. DNA Methyltransferases, DNA Methylation, and Age-Associated Cognitive Function. *Int J Mol Sci* **2018**, *19*, doi:10.3390/IJMS19051315.
43. Stoccoro, A.; Mosca, L.; Carnicelli, V.; Cavallari, U.; Lunetta, C.; Marocchi, A.; Migliore, L.; Coppedè, F. Mitochondrial DNA Copy Number and D-Loop Region Methylation in Carriers of Amyotrophic Lateral Sclerosis Gene Mutations. *Epigenomics* **2018**, *10*, 1431–1443, doi:10.2217/EPI-2018-0072.
44. Bianchessi, V.; Vinci, M.C.; Nigro, P.; Rizzi, V.; Farina, F.; Capogrossi, M.C.; Pompilio, G.; Gualdi, V.; Lauri, A. Methylation Profiling by Bisulfite Sequencing Analysis of the MtDNA Non-Coding Region in Replicative and Senescent Endothelial Cells. *Mitochondrion* **2016**, *27*, 40–47, doi:10.1016/J.MITO.2016.02.004.
45. Dragoni, F.; Garau, J.; Orcesi, S.; Varesio, C.; Bordoni, M.; Scarian, E.; Di Gerlando, R.; Fazzi, E.; Battini, R.; Gjurgjaj, A.; et al. Comparison between D-Loop Methylation and MtDNA Copy Number in Patients with Aicardi-Goutières Syndrome. *Front Endocrinol (Lausanne)* **2023**, *14*, 656, doi:10.3389/FENDO.2023.1152237/BIBTEX.
46. Byun, H.M.; Panni, T.; Motta, V.; Hou, L.; Nordio, F.; Apostoli, P.; Bertazzi, P.A.; Baccarelli, A.A. Effects of Airborne Pollutants on Mitochondrial DNA Methylation. *Part Fibre Toxicol* **2013**, *10*, doi:10.1186/1743-8977-10-18.
47. Braissant, O.; Astasov-Frauenhoffer, M.; Waltimo, T.; Bonkat, G. A Review of Methods to Determine Viability, Vitality, and Metabolic Rates in Microbiology. *Front Microbiol* **2020**, *11*, doi:10.3389/FMICB.2020.547458.
48. Bischoff, S.C.; Barbara, G.; Buurman, W.; Ockhuizen, T.; Schulzke, J.D.; Serino, M.; Tilg, H.; Watson, A.; Wells, J.M. Intestinal Permeability – a New Target for Disease Prevention and Therapy. *BMC Gastroenterol* **2014**, *14*, doi:10.1186/S12876-014-0189-7.
49. Lopetuso, L.R.; Scaldaferri, F.; Bruno, G.; Petito, V.; Franceschi, F.; Gasbarrini, A. The Therapeutic Management of Gut Barrier Leaking: The Emerging Role for Mucosal Barrier Protectors. *Eur Rev Med Pharmacol Sci* **2018**, *9*, 1068–1076.

Chapter 5

Mitochondrial DNA copy number and trimethylamine levels in the blood: new insights on cardiovascular disease biomarkers

5.1 STATE OF ART

Cardiovascular disease (CVD) is a major cause of death and disability worldwide[1]. The primary prevention of CVD relies on the identification of high-risk individuals before the manifestation of the event. Thus, the need for new methods for accurate risk stratification is drawing attention, and several biomarkers have been proposed to predict cardiovascular events[2].Biomarkers play a critical role in the definition, prognosis, and decision-making in cardiovascular disease management. Nevertheless, despite increasing efforts, the possibility to identify high-risk individuals is still limited. Among the novel candidates, trimethylamine N-oxide (TMAO) has attracted a growing attention as a potential promoter of atherosclerosis in humans [3–7]. TMAO is the oxidative metabolite of trimethylamine (TMA), which is produced by the gut microbiome from dietary precursors (i.e., choline, betaine, and carnitine). The TMA is absorbed via the intestinal epithelium, and it is further oxidized in the liver to TMAO, which is then excreted in urine. Several hypotheses have linked the TMAO to the development of atherosclerosis and CVD risk via promotion of platelet hyperreactivity [8], pro-inflammatory changes in the artery wall [9], enhanced macrophage cholesterol accumulation and foam cell formation [3], increased levels of pro-inflammatory monocytes [10]. Nevertheless, contrasting evidence on the association of TMAO and CVD emerged [11–16], and a clear mechanistic explanation of this association is still missing.

Another interesting peripheral biomarker that has been recently proposed for CVD prediction is the mitochondrial DNA copy number (mtDNAcn) [17]. Human mitochondrial DNA (mtDNA) is a small, circular, and multi-copy genome, located in the inner matrix of mitochondria. It incorporates 37 mitochondrial genes (13 coding for essential components of the mitochondrial electron transport chain and of the ATP synthase complex, 22 for mitochondrial transfer RNAs and 2 for ribosomal RNAs). Mitochondrial DNA content reflects the energy demand of a cell [18] and is disturbed by imbalanced energy metabolism and reactive oxygen species overproduction. Thus, mtDNAcn changes have been proposed as an early biomarker of damage and mitochondrial dysfunction [19,20]. Remarkably, since mtDNAcn changes have been associated with both intrinsic and extrinsic factors [21,22], mtDNAcn has been proposed as a potential biomarker for complex diseases, which are linked to both genetics and environmental exposures[23]. Circulating mtDNAcn has been investigated in cardiovascular diseases [24], mainly analysing peripheral blood cells. Interestingly, it has been recently demonstrated that blood also contains circulating cell-free respiratory competent mitochondria [25], suggesting that the measure of mtDNAcn in the whole blood might better represent the health status. Moreover, despite the involvement of TMAO in mitochondrial

metabolism has been presented [26–29], none of the previous investigations examined the association between blood mtDNAcn and TMA or TMAO levels in humans.

5.2 AIM OF THE STUDY

This study aims to investigate, in a population of 540 subjects of coronary artery disease (CAD) patients and controls, (a) if the mtDNA copy number measured in the whole blood is a marker of CAD; (b) if any association between mtDNAcn and TMAO or TMA levels exists; (c) if mtDNAcn is associated with other risk factors for CVD that are linked to metabolic alterations (i.e., hypertension [30], diabetes [31], glomerular filtration rate [GFR]) or environmental exposures (i.e., smoking [32], BMI [33]; (d) provide further insights on the effects of TMA and TMAO levels changes in CVD. The final goal is to identify relevant factors that can be used for CVD prognosis through adequate prediction models.

5.3 MATERIALS AND METHODS

5.3.1 Study cohort recruitment and sample collection

CAD patients were consecutively recruited in Wejherowo Cardiovascular Center with angiographically confirmed CAD or with angina referred to elective or urgent coronary angiography as inclusion criteria. To characterize the severity of CAD, all patients were classified into 1-, 2- or 3-vessel disease groups based on the presence of stenosis in major coronary arteries or their branches. CAD patients were further divided in subgroups based on the severity of disease: mild severity (1 vessel involved) (MS) or high severity (two or three vessels involved) (HS). Moreover, CAD patients were divided into stable angina (SA), and acute coronary syndrome (ACS) patients. SA patients were treated with statins for a secondary prevention of cardiovascular morbidity. For this reason, despite dyslipidaemia being an established risk factor for CAD, blood lipid measurements (Table S1) were excluded from the analysis. Control subjects without a self-reported medical history of CVD were recruited in the same region. The study was approved by the Regional Bioethical Committee (RBC) in Gdansk (KB-27/16 and KB 32-17) and registered at clinicaltrials.gov (NCT03899389). All methods were carried out in accordance with relevant guide- lines and regulations approved by RBC. Informed consent was obtained from all subjects. Venous blood samples were collected in EDTA-containing tubes. The plasma samples were prepared by centrifugation at 1300 g for 10 min at 18-25°C, and were kept frozen at −80°C.

5.3.2 Assessment of variables related to cardiovascular risk

Plasma TMA and TMAO were determined by the Ultra-Performance Liquid Chromatography (UHPLC) tandem mass spectrometry method, as previously described [34]. Troponin, as an established biomarker of ACS [35,36], was determined in hospital diagnostic laboratory on Dimension EXL with LOCI Module Integrated Chemistry System (Siemens Healthcare GmbH, Erlangen, Germany) using high sensitivity cardiac troponin I test (Dimension EXL hs-cTnI assay, Siemens Healthcare GmbH, Erlangen, Germany) [37].

Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Hypertension and diabetes were diagnosed by medical doctors in diagnostic processes and were reported by subjects, as well as smoking habits (present or past), in self-reported questionnaires. GFR was calculated by Cockcroft-Gault Equation[38].

5.3.3 MtDNAcn assessment

Genomic DNA was extracted from whole blood using the kit for genomic DNA purification (A&A Biotechnology, Gdynia, Poland). All samples have been processed under the same conditions and with the same DNA extraction method because variation of the DNA extraction method might affect the evaluation of mtDNAcn[39]. Relative mtDNAcn quantification [40] (considering nDNA as a normalizer), which is the current method of choice for mtDNAcn assessment [19], was performed by real-time PCR (Biorad CFX96). Briefly, the cycle threshold (C_t) values of a mitochondrial-specific and nuclear-specific target were determined in triplicate for each sample. The difference in C_t values (ΔC_t) between the mitochondrial and nuclear gene for each sample is calculated and $2^{-\Delta Ct}$ represents a relative measure of mtDNAcn. The following genes have been amplified for the detection of mitochondrial and nuclear DNA, respectively, using the listed primers: mtDNA-tRNALeu (f w:5'-CACCCAAGAACAGGGTTTGT-3'; rv: 5'-TGGCCATGGGTATGTTGTTA-3') for mitochondrial DNA, and beta-2-microglobulin (B2M) (fw: 5'-TGCTGTCTCCATGTTTGATGTATCT-3'; rv: 5'-TCTCTGCTCCCCACCTCTAAGT-3') for the nuclear DNA. These primers have been verified by Fazzini and colleagues [39] for their specificity (unique amplification of mtDNA) and for the absence of co-amplified nuclear insertions of mitochondrial origin (NUMTs). An inter-run calibrator sample was used to adjust the results obtained from different amplification plates. All samples were anonymized for laboratory personnel.

5.3.4 Statistical analysis

Power analysis for studying mtDNAcn in this population was performed according to the effect size reported by the meta-analysis from Yue and colleagues [24] and revealed a power > 0.99. The

Shapiro-Wilk test was used for the analysis of the normality of data distribution. Spearman correlation or linear regression (adjusting for confounding variables) was used for testing the correlation among continuous variables. Bonferroni's correction was applied to confirm statistical significance in multiple correlations. Chi-square test, Kruskal-Wallis test, and Generalized Linear Model (GLM) were used to test differences in the analyzed variables among groups adjusting for covariates. A stepwise logistic regression model was applied to identify (according to Wald statistics) which of the variables predicted the cardiovascular risk. Precision-Recall (PR) curves were used to evaluate the performance of the prediction model when considering a database with unbalanced classes, while Receiver Operating Characteristic (ROC) curves were considered when classes were numerically balanced. For completeness of information, we reported both the models in the figures.

For testing the predictive biomarkers, the dataset has been divided into two parts: the training set and the testing set. The former contains 75% of the whole dataset while the latter contains the remaining 25%. We used the training set for training the logistic classifier (discovery stage) and then the testing set to evaluate it by computing the PR curve (validation stage). Precision and recall have been calculated as follows:

$$\textbf{Precision} = TP/(TP + FP)$$

$$\textbf{Recall} = TP/(TP + FN)$$

where TP, True Positives; FN, False Negatives.

Technical replicates are described in each specific material and methods section and are not considered for the inference statistics (no inflation of units of analysis was performed). Two-sided *p* values have been calculated, and significant differences were attributed to $p < 0.05$.

5.4 RESULTS

5.4.1 Descriptive statistics

Five hundred and forty recruited subjects (65.7% male, 34.3% female) were analyzed in this study. Mean age of the population was 65 (±10) years old. Among the subjects, 46.7% (n = 252) were smokers, 66.5% (n = 359) were diagnosed with hypertension, and 25.4% (n = 137) were diagnosed with diabetes. Among the recruited subjects, 151 were controls (28%), 389 were CAD patients (72%). Descriptive statistics for BMI, TMA, TMAO, TMAO/TMA ratio, and GFR in the total population are shown in Table 1. A detailed comparison of TMA, TMAO, BMI, and GFR in the CAD population vs controls in this population has been previously published [34] and summarized in Table S2.

As expected, smoking (Pearson's chi-square = 4.86; p = 0.028), hypertension (Pearson's chi-square = 60.79; $p = 6.3*10^{-15}$), and diabetes (Pearson's chi-square = 14.55; $p = 1.3*10^{-4}$) are confirmed as risk factors for CAD development (Figure S1). An increased BMI was also measured in CAD patients with respect to controls (controls: 27.8 ± 4.1; CAD: 28.8 ± 4.5; p = 0.023).

TABLE 1 Descriptive statistics. Descriptive statistics on the whole population (n = 540) for the analyzed variables.

	n	Min	Max	Mean	SD
BMI (kg/m2)	540	18.00	45.00	28.50	4.40
TMAO (µM)	540	0.01	37.50	5.37	4.50
TMA (µM)	540	0.34	1.15	0.60	0.11
TMAO/TMA	540	0.01	64.87	8.86	7.35
GFR (mL/ min/1.73 m^2)	540	9.71	289.96	88.17	33.76

Abbreviation: SD, standard deviation

5.4.2 Association between mtDNAcn, CAD, and CVD risk factors

In the studied population, blood mtDNAcn decreased with age (Spearman's Rho = −0.101; p = .019) (Figure S2). A significantly lower level of mtDNAcn was measured in the CAD group compared to controls (median values in controls vs CAD: 76.9 vs 51.5; $p = 2.6*10^{-12}$) (Figure 1). Analysis of covariates (age, sex, TMA, TMAO, GFR) revealed that this association is modulated by TMA (p = 0.0001) and GFR (p = 0.026). Moreover, mtDNAcn was significantly lower in hypertensive than in normotensive individuals (median values, control = 59.8; CAD = 53.6; p = 0.002) (Figure 2A). This association was modulated by TMA (p = 0.0001) and GFR (p = 0.002), but not by TMAO (p = 0.549), sex (p = 0.987), BMI (p = 0.821) or age (p = 0.231). However, it was no more significant when adjusting the analysis for CVD status (p = 0.830), suggesting that CAD is the major driver of this association. Adjusting the analysis for age, sex, BMI, GFR, TMA and TMAO, the mtDNAcn did not differ in individuals diagnosed with diabetes (p = 0.795) (Figure 2C), nor in smokers with respect to controls (p = 0.082) (Figure 2B). However, the association with smoking was significant (p = 0.006) when adjusting the analysis for CAD, that contribute to explain the model (p = 0.002), as well as GFR (p = 0.016) and TMA (p = 0.0001).

MtDNAcn was directly correlated with GFR (Spearman's Rho = 0.103; p = 0.017), suggesting that higher mtDNAcn can be associated with a better glomerular filtration capacity (Figure 2D). Adjusting the analysis for age, sex and BMI, the correlation is even stronger (B = 0.216; p = 0.001).

It remains significant also after adjusting the analysis for CAD status (B = 0.155; p = 0.010). No significant direct association was measured between mtDNAcn and BMI in the whole population (Spearman's Rho = 0.044; p = 0.304), neither adjusting the analysis for sex and age (p = 0.310).

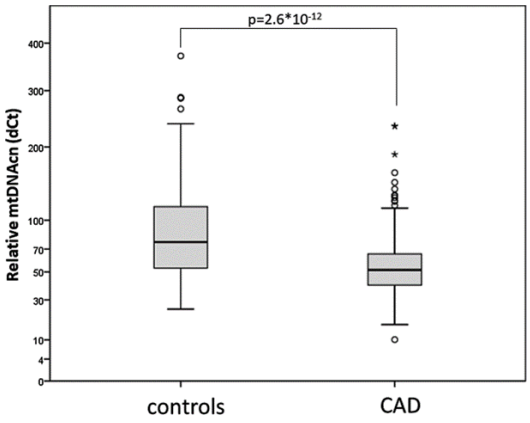


Figure 1. Relative mtDNAcn in controls and CAD group.

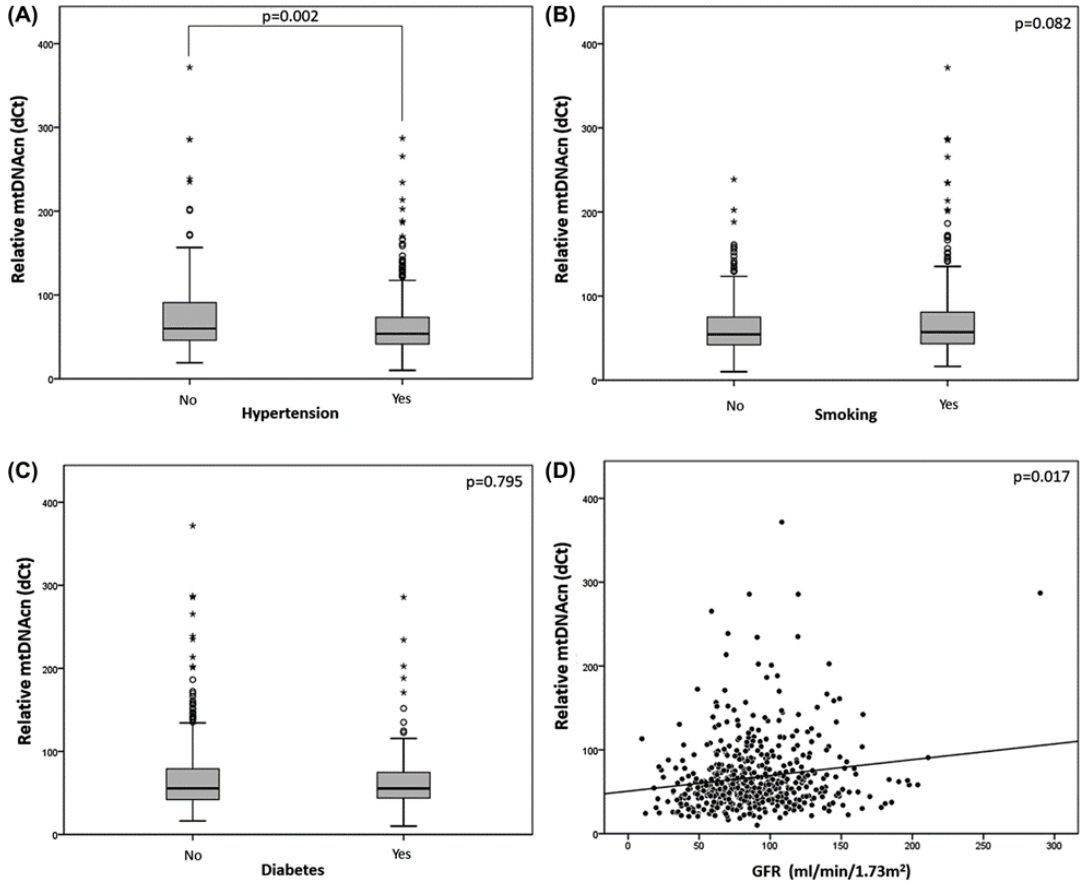


Figure 2. Relative mtDNAcn and CAD risk factors exposures in the analyzed population: hypertension (A), smoking (B), diabetes (C), GFR (D). Row P values are shown in the figures. Adjusted analysis and covariates are described in the main text.

5.4.3 MtDNAcn correlates with TMA but not TMAO

A direct correlation between mtDNAcn and TMA levels was measured (Spearman's Rho = 0.166; p = 0.0004) (Figure 3A). Linear regression adjusting the analysis for age, sex, GFR, and BMI confirmed a strong association between TMA and mtDNAcn ($B = 0.227$; $p = 6.5*10^{-7}$), with GFR (B = 0.206; p = 0.002) contributing to explain the mtDNAcn variance. This association remains significant even adjusting the analysis for the CAD status ($p = 2.3*10^{-5}$). No significant associations were detected between mtDNAcn and TMAO (Spearman's Rho = 0.020; p = 0.643) (Figure 3B), neither adjusting the analysis for the previously mentioned variables (p = 0.928). Similarly, no significant association was measured with TMAO/TMA (Spearman's Rho = −0.033; p = 0.441) (Figure 3C), neither adjusting the analysis for confounding variables (p = 0.598).

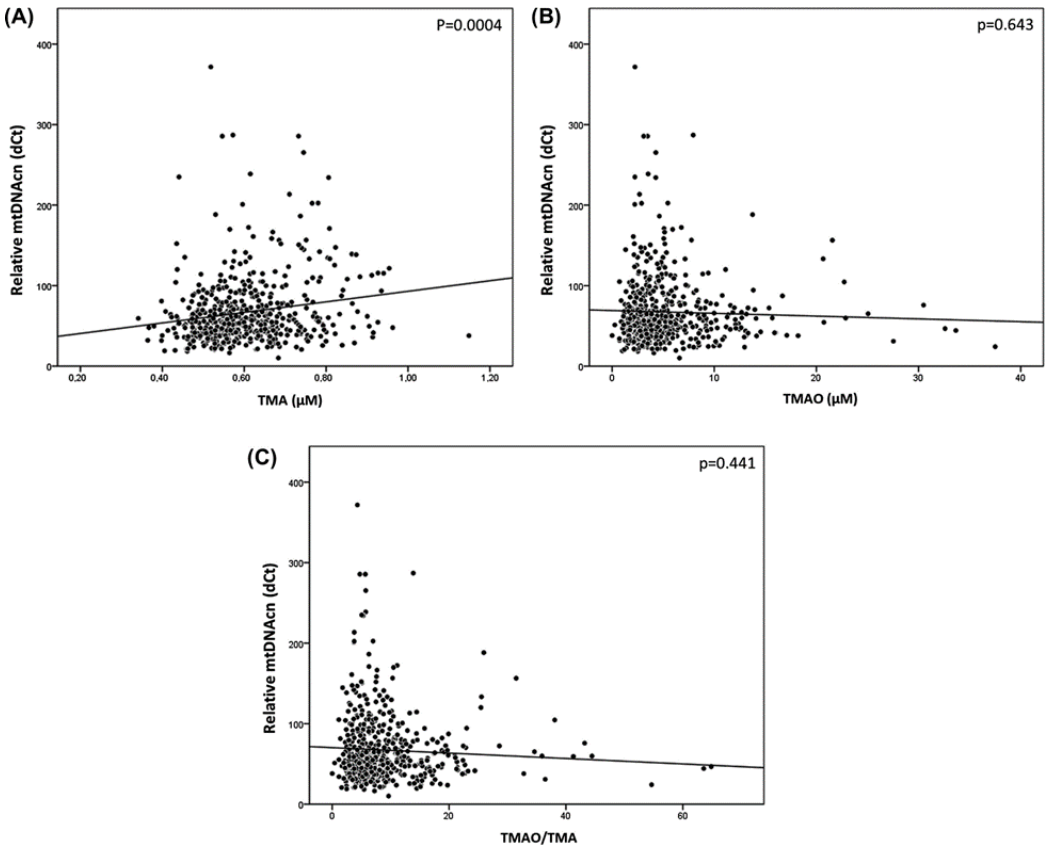


Figure 3. Correlations between mtDNAcn and TMA (A), TMAO (B) or TMAO/TMA (C) in the analyzed population. TMA levels correlates with mtDNAcn (p < 0.0001, adjusted for age and sex). The association remains significant also correcting the analysis for GFR as well (p < 0.0001). No significant correlations were detected between TMAO and mtDNA or TMA/TMAO ratio and mtDNAcn.

5.4.4 MtDNAcn, TMA, TMAO and troponin levels in CAD groups

Troponin, a marker of acute myocardial injury, was higher in HS than MS (p = 0.003) (Figure S3A) and in ACS than in SA (p = $1.7*10^{-6}$) (Figure S3B). On the other hand, mtDNAcn decreased with respect to controls in both MS (p = 0.0001) and HS (p = 0.0001) but did not significantly differ between MS and HS groups (p = 0.666) (Figure S3C). Moreover, mtDNAcn was lower in both SA (p = 0.0001) and ACS groups (p = 0.0001) compared to controls, but no significant differences were measured between ACS and SC (p = 0.186) for this biomarker (Figure S3D). Interestingly, a significant inverse correlation between mtDNAcn and troponin was measured in the CAD group (Spearman's Rho = −0.218; p = $1.5*10^{-5}$) (Figure S4). This association was confirmed also after adjusting the analysis for sex and age (B = −0.451 ± 0.218; p = 0.039) and can be considered reliable among acute cases (that are those that display higher troponin levels). No significant correlations were detected between troponin and TMA (p = 0.554), TMAO (p = 0.946), or TMA/TMAO (p = 0.985) in the CAD group.

5.4.5 Correlations between TMA, TMAO, GFR, and mtDNAcn in controls and CAD patients

To clarify the role of TMA in this complex picture, an exploratory analysis was performed to evaluate the correlations between TMA and TMAO in the control and CAD groups separately. Results showed that high TMA levels were not accompanied by increased TMAO in controls (Spearman's Rho = 0.065; p = 0.429), but high TMA levels were associated with high TMAO levels in CAD patients (Spearman's Rho = 0.338; p = $7.2*10^{-5}$) (Figure 4A). This correlation is even more significant adjusting the analysis for sex, age, BMI and GFR (B = 0.246; p = $1.4*10^{-6}$) in the CAD group, but not in the control group (p = 0.964). This evidence corroborates the hypothesis that TMA levels are not *per se* linked to high TMAO. Higher TMA levels are positively correlated to GFR in controls (Spearman's Rho = 0.257; p = 0.001), while TMA is negatively associated to GFR levels in the CAD group (Spearman's Rho = −0.260; p = $2.1*10^{-7}$) (Figure 4B). By adjusting the analysis for sex, age, and BMI, only the inverse correlation between GFR and TMA in the CAD group was confirmed (B = −0.454; p = $7.8*10^{-7}$) after Bonferroni correction. This suggests a different metabolism of TMA and TMAO in the presence of cardiovascular disease, with respect to healthy conditions. Moreover, increased TMA levels correlated to higher mtDNAcn only in the controls (Spearman's Rho = 0.280, p = $7.8*10^{-5}$), and not in the CAD group (Spearman's Rho = 0.076, p = 0.133). However, this correlation between TMA and mtDNAcn in the controls was not significant when adjusting the analysis for sex, age, and GFR (p = 0.707).

By observing the TMAO/TMA ratio in the population divided by the cardiovascular health status in respect to mtDNAcn and GFR, we found that the TMAO/TMA ratio shows a significant inverse

correlation with GFR in the CAD group (Spearman's Rho = −0.307; $p = 5.8*10^{-6}$), which, on the other hand, is not significant in controls (Spearman's Rho = −0.176; $p = 0.180$) after Bonferroni correction (Figure 4C). This inverse correlation in the CAD group was confirmed also adjusting the analysis for sex and age ($B = -1.032$; $p = 0.0001$). Additionally, the TMAO/TMA ratio was inversely correlated to mtDNAcn in the control group (Spearman's Rho = −0.161; $p = 0.049$), but not in the CAD group (Spearman's Rho = −0.019; $p = 0.712$). However, this correlation was not significant when adjusting the analysis for sex, age and GFR ($p = 0.292$).

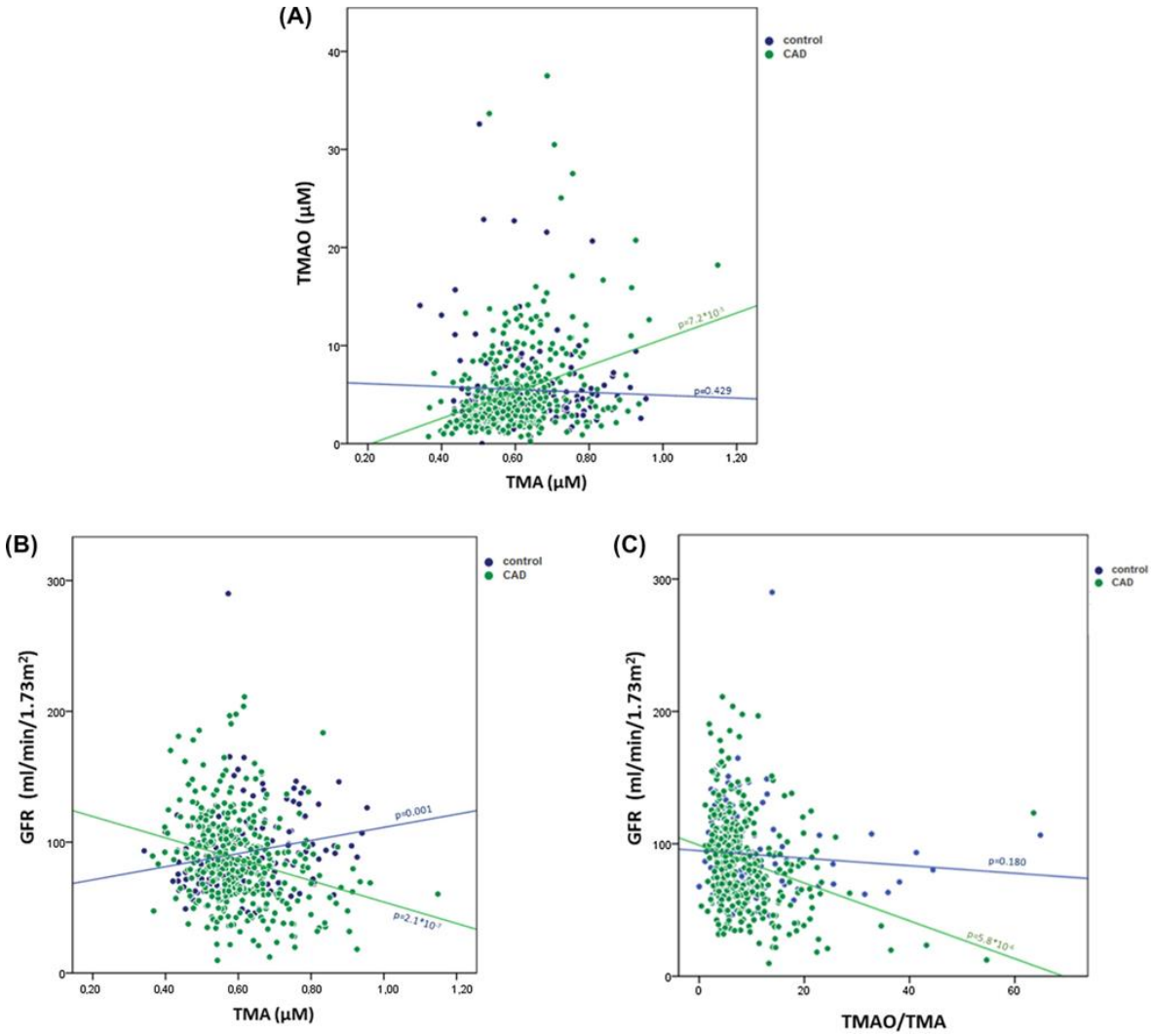


Figure 4. Correlations between TMA and TMAO (A), TMA and GFR (B), TMAO/TMA ratio and GFR (C) in the population divided for groups (controls vs CAD).

5.4.6 Identification of CAD prediction models and comparison with troponin

A stepwise logistic regression analysis was applied to identify significant predictors of CAD. The following variables, all potential risk factors for CAD, were tested: age, sex, mtDNAcn, TMA, TMAO, diagnosis of hypertension and diabetes, smoking habits, GFR. A forward selection criterion was applied, and Wald statistics was used to identify significant predictors. The variables that emerged as significant predictors of CAD are the following: mtDNAcn (OR = 0.972; 95% CI = [0.965-0.979]; $p = 7.6*10^{-9}$); hypertension (OR = 4.131; 95% CI = [2.582-6.609]; $p = 6.3*10^{-6}$); sex (OR = 0.574; 95% CI = [0.355-0.929]; $p = 0.024$); smoking (OR = 1.754; 95% CI = [1.083-2.841]; $p = 0.022$); diabetes (OR = 1.861; 95% CI = [1.011-3.425]; $p = 0.045$). This 5-step model correctly categorized 81.7% of cases. Good performances were obtained also by a 3-step model (including mtDNAcn, hypertension, and sex), that successfully categorized 80.3% of cases. TMAO and TMA are not significant CAD predictors according to this model. On the contrary, mtDNAcn is the first element to enter in the stepwise forward analysis, thus affecting the goodness of the prediction and the information carried by the other variables. This evidence suggests that mtDNAcn might significantly improve the population risk stratification for CAD, in addition to other known risk factors.

To confirm this hypothesis, the PR curves were calculated (Figure 5) to evaluate the performance of both prediction models previously identified by the stepwise forward logistic regression. PR curve is used to evaluate the performance of a classifier (to compare classifiers, too) for unbalanced classes through the computation of the area under the curve (AUC). This value is a number between 0 and 1: the bigger the value, the better the model. PR curve, as the name suggests, is obtained using precision and recall, two evaluation metrics that can deal with unbalanced classes [41] as in this case. PR shows an AUC equal to 0.894 for the 3-step model, with mtDNAcn, hypertension and sex applied as predictors. For the 5-step model (with mtDNAcn, hypertension, sex, smoking, and diabetes applied as predictors), the resulting AUC was 0.901. Figure 5 shows the PR curves and the confusion matrices for both models, concluding that their performances are good and similar. In addition, we tested the ability of these two models to distinguish between acute or stable CAD patients (Figure 6), or between different degrees of CAD severity (Figure 7), and we compared them to the performance of troponin. Results confirmed that troponin can distinguish between stable and acute cases (ROC AUC = 0.79), while none of the new proposed biomarkers (3-steps, ROC AUC = 0.58; 5-steps, ROC AUC = 0.53) were able to confidently predict the acute or stable status of the dis- ease (Figure 6D-F respectively). Remarkably, our 5-step (PR AUC = 0.703), and 3-step model (PR AUC = 0.778) showed to be better predictors of severity than troponin (PR AUC = 0.667). (Figure 7A-C respectively). Therefore, the proposed 3-step model, including mtDNAcn, sex, and hypertension, was confirmed as an interesting predictor of cardiovascular health in this population.

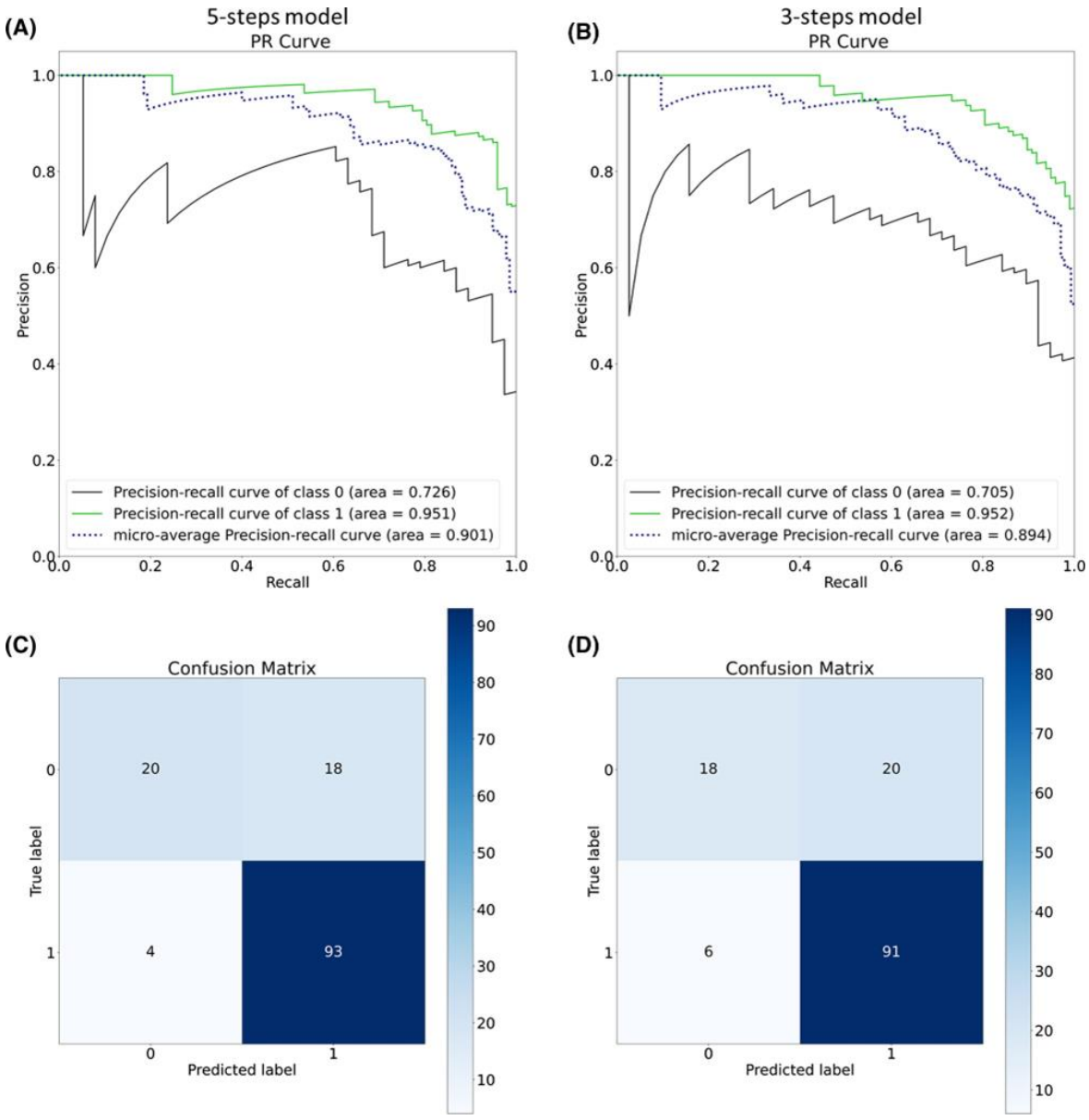


Figure 5. PR curves for CAD prediction with 5 features (A, 5-step model: including mtDNAcn, hypertension, sex, smoking, diabetes) or 3 features (B, 3-step model: including mtDNAcn, hypertension, sex). Panel C and D show the confusion matrices for the two models (5-step and 3-step model, respectively). 5-step model, Accuracy: 0.837; Sensitivity: 0.526; Specificity: 0.959. 3-step model, Accuracy: 0.807; Sensitivity: 0.474; Specificity: 0.938.

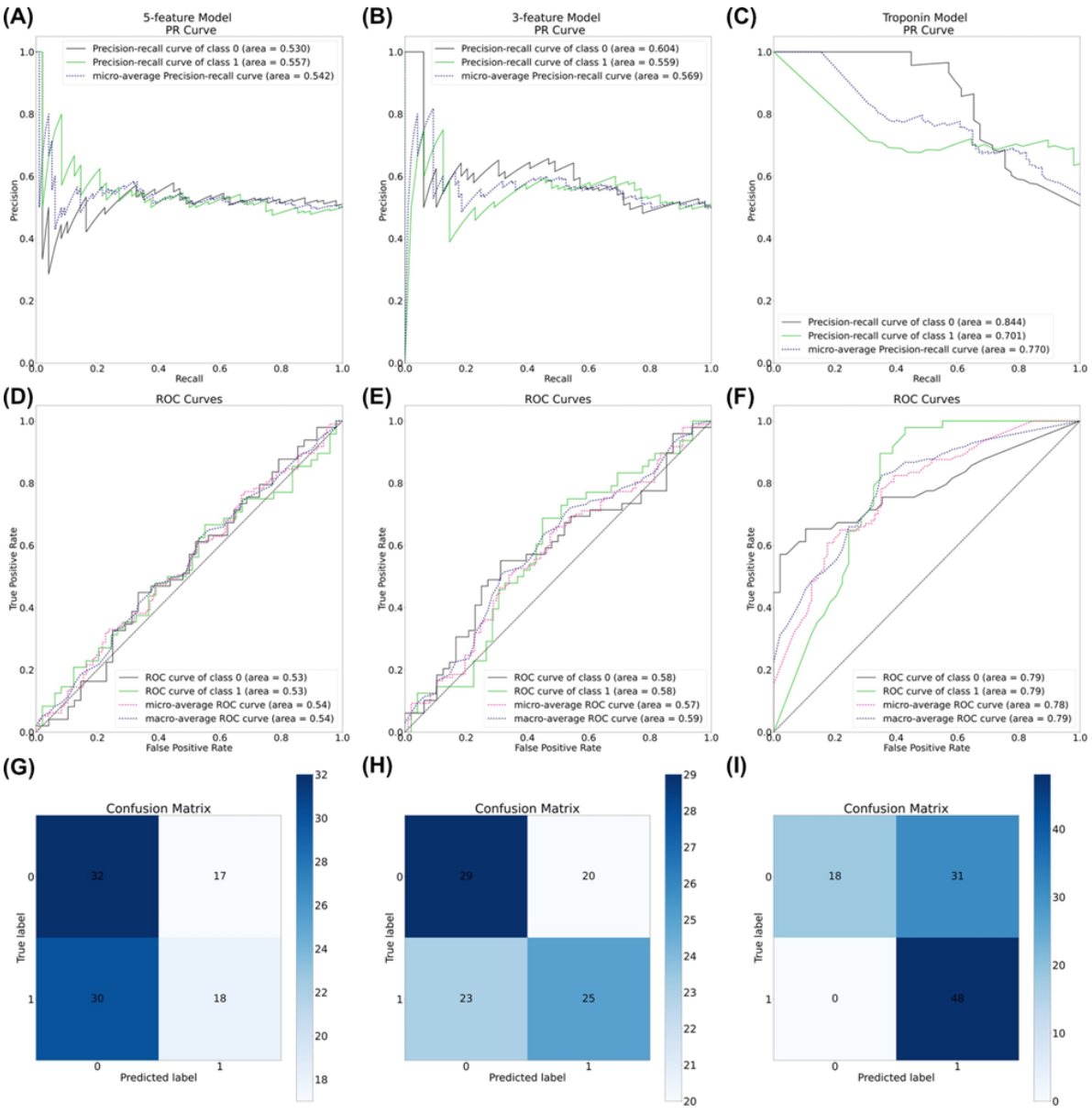


Figure 6. PR curves, ROC curves and confusion matrices for prediction of the disease state (SA vs ACS) in the CAD group. A, D, G panels are referred to the 5-step model; B, E, H panels are referred to the 3-step model; C, F, I panels are referred to the troponin. 5-steps model, Accuracy: 0.515; Sensitivity: 0.653; Specificity: 0.375; 3-step model, Accuracy: 0.557, Sensitivity: 0.592, Specificity: 0.521; troponin, Accuracy: 0.681, Sensitivity: 0.367, Specificity: 1.0.

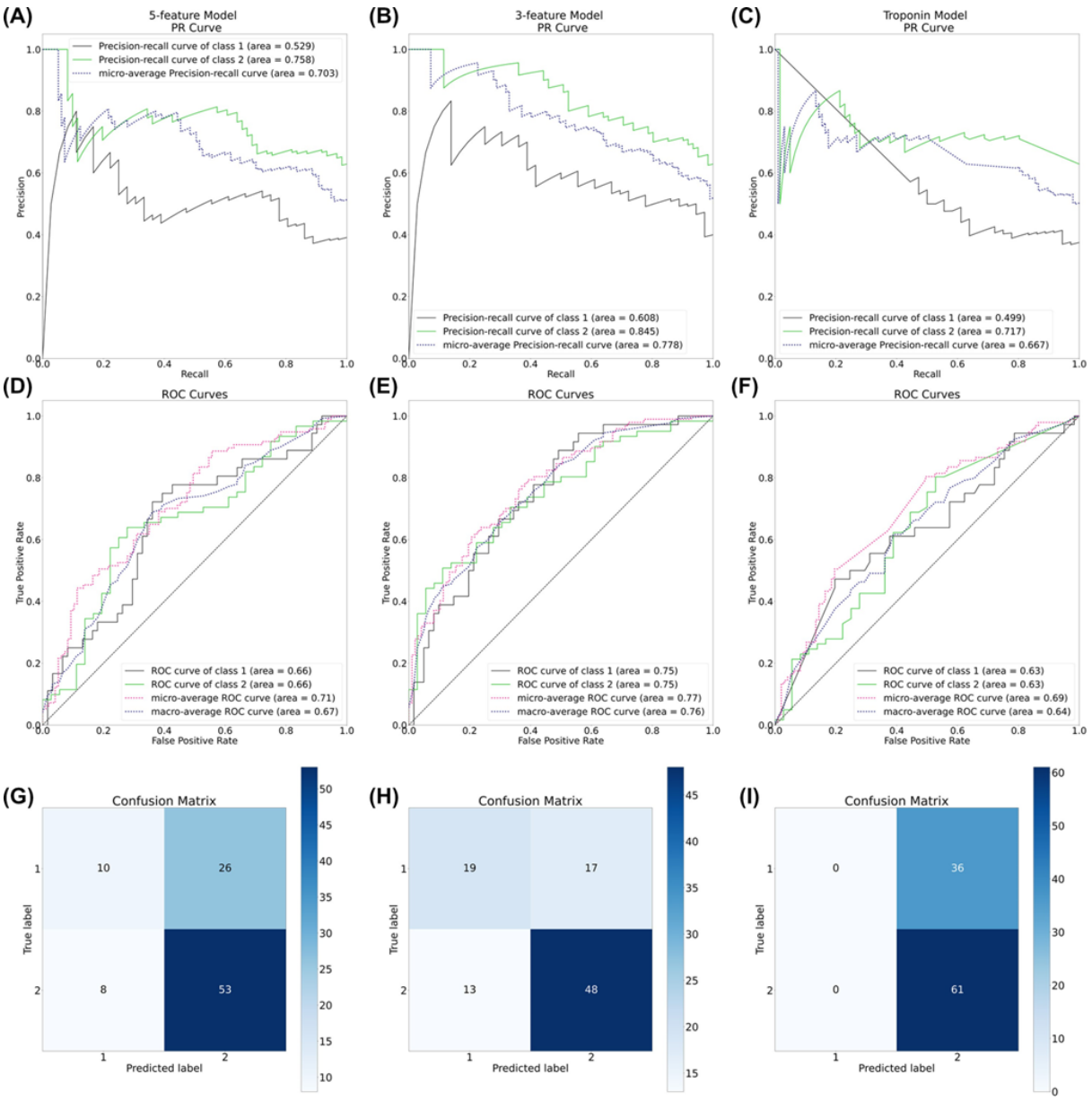


Figure 7. PR curves, ROC curves and confusion matrices for prediction of disease severity in the CAD group. A, D, G panels are referred to the 5-step model; B, E, H panels are referred to the 3-step model; C, F, I panels are referred to the troponin. 5-step model, Accuracy: 0.650; Sensitivity: 0.279; Specificity: 0.869. 3-steps model, Accuracy: 0.691, Sensitivity: 0.528, Specificity: 0.787. Troponin, Accuracy: 0.628, Sensitivity: 0.0, Specificity: 1.0.

5.5 DISCUSSION AND CONCLUSIONS

Alterations in TMAO metabolism [42] and mitochondrial dynamics [43] have been previously independently identified as risk factors for cardiovascular disease development. We have found that mtDNA copy number, measured in the whole blood, is lower in CAD patients than in controls. This is coherent with previous investigations reviewed by Yue et al. [24], although the reported

studies had determined mtDNAcn in buffy coat/circulating leukocytes. Recently, it has been demonstrated that blood contains circulating cell-free respiratory competent mitochondria [25], and a reduction of mtDNAcn was also detected in the whole blood of peripheral arterial disease male patients compared to controls [44]. Therefore, our findings about the reduction of mtDNAcn in the whole blood of CAD patients support the interest towards mtDNAcn measurement as an easily accessible biomarker for cardiovascular disease risk stratification.

To explain the observed decrease of mtDNAcn in CAD patients, we tested the correlation of this biomarker with other established risk factors for CVD. In our population, mtDNAcn decreased with age, according to previous evidence examining mtDNAcn in blood cells [45]. In addition, we found a correlation between mtDNAcn, hypertension and GFR, which are established risk factors for CVD[46–48]. In particular, mtDNAcn was decreased in hypertensive subjects. Even if the presence of CAD is a confounder for this association in our population, previous hypothesis linked mtDNAcn and hypertension [49], which is also interesting considering the role of mitochondrial oxidative stress in CAD[50]. Nevertheless, only a few data on this topic have been collected until now, and a clear mechanistic explanation is missing. Lei et al. [51] measured mtDNAcn in white blood cells of a coal mining group located in northern China and reported no differences between hypertensive and healthy controls. An elevated urinary mtDNAcn was detected in hypertensive patients, and it was correlated with markers of renal injury and dysfunction [52,53]. The authors explained this observation considering that fragments of the mitochondrial genome released from dying cells might be considered surrogate markers of mitochondrial injury. Prestes et al. [49] also suggested that alterations of mtDNAcn in a cell can impair mitochondrial respiration and a reduction of mtDNAcn might represent a surrogate measure for reduced mitochondrial function, which is coherent with what we observed in our CAD group. In addition, elevation of mtDNAcn in urine samples has been shown in progressive acute kidney injury patients [54], and this parameter has been associated with glomerular hyperfiltration in obese African American hypertensive patients [53]. All these data support the involvement of mitochondrial dynamics in both the alteration of kidney functions and hypertension. In our population, mtDNAcn decreased proportionally with GFR, in accordance with previous evidence, suggesting that a higher blood mtDNA copy number is associated with a lower risk of incident chronic kidney disease [55]. The reduction of mtDNAcn in individuals with lower GFR might be explained by the mitochondrial dysfunctions in kidney cells implicated in the pathogenesis of chronic kidney diseases [56–58], and by the exposure to reactive oxygen species that can damage the mitochondrial DNA replication enzyme (polymerase γ), leading to a reduction in mtDNA copy number [59]. On the other hand, diabetes, that has been previously related to both the increase and decrease of mtDNAcn [60,61], was not directly associated with mtDNAcn in our study. This evidence cannot exclude an influence of diabetes on mtDNAcn levels, but the findings are still inconclusive to demonstrate a direct association between these two

variables. Recently, a meta-analysis has shown that metabolic parameters (waist circumference, BMI and triglycerides) are major mediators of this association [62]. Nevertheless, the diagnosis of diabetes is still informative in this context, as it was one of the significant predictors of CAD in our precision-recall analysis if considered together with mtDNAcn, sex, hypertension, and smoking. Dyslipidemia, which is also an established risk factor for CVD, was not considered in this study because CAD patients were treated with statins for secondary prevention of cardiovascular events. Thus, data on blood lipids were not considered reliable in this population. Interestingly, mtDNAcn showed an inverse correlation with troponin levels, corroborating the hypothesis that lower mtDNAcn is predictive of worse cardiovascular health. Troponin was confirmed as a good biomarker of CAD in the acute phase. On the contrary, mtDNAcn increases both in acute and stable conditions and was predictive of disease severity when considered with sex and hypertension diagnosis.

This is in accordance with recent evidence suggesting that mtDNAcn is linked to cardiovascular disease patient phenotypes [63]. In addition to classical risk factors for CVD, for the first time, we also tested correlations between mtDNAcn and TMAO metabolism. While no associations with TMAO were measured, mtDNAcn correlated with TMA levels in the whole population. This finding suggests that a reduction of mitochondrial functions might occur in individuals that display lower TMA. This is in accordance with our previously published data describing a subtle but significant reduction of TMA in CAD patients with respect to controls [34], while no significant differences were measured for TMAO between the two groups. A definite explanation of this phenomenon is still to be identified. In our cohort, a direct correlation between TMA and TMAO can be measured only in the CAD group, while we did not observe a proportional increase of TMAO in controls with higher TMA levels. Preliminary evidence suggested that TMA might have a role per se in CVD [64–66]. Jaworska *et al*. showed that TMA was inversely correlated with the estimated glomerular filtration rate in humans; moreover, TMA reduced cardiomyocyte viability *in vitro*, while TMAO was even protective against TMA-induced cytotoxicity [64]. The authors hypothesized a detrimental effect of TMA due to its role in triggering the degradation of protein structures (i.e., albumin). The possibility of stable protein-TMA complexes (especially in the condition of increased oxidative stress, as occurring in inflammatory conditions [67–69]), together with the increased ratio of conversion of TMA into TMAO might provide a possible explanation for the low TMA levels measured in CAD patients and suggest a different fate and metabolism of TMA in CVD respect to healthy people. Studies further investigating this hypothesis are ongoing in our laboratories.

A limitation of this study is the absence of data on urinary TMA and TMAO excretion. Moreover, although a positive correlation between the number of mitochondria and mtDNAcn has been demonstrated [70], mtDNAcn is not always a reliable predictor for mitochondrial abundance [71–74], probably because of the existence of compensatory effects. Thus, our study supports a potential involvement of TMA (but not TMAO) in CVD pathogenesis; nonetheless, further

mechanistic investigations are necessary to clarify the real role of this metabolite in CVD and its usage as a clinical biomarker. In this regard, we tested the possibility to predict CAD by using all measured variables, including mtDNAcn and other classical CVD risk factors. A logistic regression model identified decreased mtDNAcn, male gender, diagnosis of hypertension, smoke habits, and diagnosis of diabetes as significant risk factors for CAD development in this population; conversely, neither TMA nor TMAO have been included as significant predictors. The precision-recall analysis showed that it is possible to have a good prediction of CAD by including information limited to mtDNAcn, sex, and hypertension diagnosis. Indeed, our 3-step prediction model (including mtDNAcn, sex, and hypertensions) displayed the best performance in distinguishing not only healthy subjects from CAD patients (even in stable conditions and with a very high true negative rate), but also patients with different degrees of disease. This confirms the hypothesis that mtDNAcn (considered together with other risk factors for CVD) could be an informative parameter describing cardiovascular health. Thus, validation in other cohorts of these 3 easy-to-measure parameters could pro- mote their usage in the risk stratification for CVD at population level.

In conclusion, although we cannot infer any causal direction of the relationship between mtDNAcn and CAD, we consider unlikely that mtDNAcn has a direct role in increasing the risk for cardiovascular events (despite some animal studies have detected mtDNA damage before arterial wall lesions occurred [75,76]. The precise mechanism underlying the association between mtDNA damage and atherosclerosis is still poorly understood. However, it is well-known that mtDNA is a major target of oxidative stressors [22] and mitochondrial dysfunctions promote the development of pathological conditions; on the other hand, caloric restriction and physical activity have been shown to limit mtDNA damage [77,78]. Indeed, this biomarker appears to vary in accordance with several risk factors for CVD. Thus, mtDNAcn reduction might represent an alarm signal for impaired mitochondrial homeostasis. Of note, cardiometabolic risk factors such as high blood pressure, obesity, and dyslipidemia have been previously shown to modify mtDNAcn [79,80]. Thus, the hypothesis that environment and lifestyle factors might have major effects on mtDNAcn has been raised. Several pollutants (e.g., benzene [81], particulate matter [82], and polycyclic aromatic hydrocarbons [83]) have been associated with changes in mtDNAcn. In addition, dietary factors [84,85], especially salt intake [86], might modify the mtDNAcn in blood. This is particularly interesting in the context of CVD prevention. Thus, mtDNAcn appears as a plastic biomarker that might change in response to environmental and dietetic factors. Further research on modifiable CVD risk factors influencing mtDNA copy number may improve the prevention and treatment of this multifactorial pathology, making mtDNAcn a new tool to monitor complex disorders related to both metabolic imbalances and environmental insults at the molecular level [21].

5.6 REFERENCES

1. Roth, G.A.; Johnson, C.; Abajobir, A.; Abd-Allah, F.; Abera, S.F.; Abyu, G.; Ahmed, M.; Aksut, B.; Alam, T.; Alam, K.; et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *J Am Coll Cardiol* **2017**, *70*, 1–25, doi:10.1016/J.JACC.2017.04.052.
2. Wang, J.; Tan, G.J.; Han, L.N.; Bai, Y.Y.; He, M.; Liu, H. Bin Novel Biomarkers for Cardiovascular Risk Prediction. *J Geriatr Cardiol* **2017**, *14*, 135–150, doi:10.11909/J.ISSN.1671-5411.2017.02.008.
3. Wang, Z.; Klipfell, E.; Bennett, B.J.; Koeth, R.; Levison, B.S.; Dugar, B.; Feldstein, A.E.; Britt, E.B.; Fu, X.; Chung, Y.M.; et al. Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease. *Nature 2011 472:7341* **2011**, *472*, 57–63, doi:10.1038/nature09922.
4. Tang, W.H.W.; Wang, Z.; Levison, B.S.; Koeth, R.A.; Britt, E.B.; Fu, X.; Wu, Y.; Hazen, S.L. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. *N Engl J Med* **2013**, *368*, 1575–1584, doi:10.1056/NEJMOA1109400.
5. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis. *Nature Medicine 2013 19:5* **2013**, *19*, 576–585, doi:10.1038/nm.3145.
6. Senthong, V.; Wang, Z.; Li, X.S.; Fan, Y.; Wu, Y.; Tang, W.H.W.; Hazen, S.L. Intestinal Microbiota-Generated Metabolite Trimethylamine-N-Oxide and 5-Year Mortality Risk in Stable Coronary Artery Disease: The Contributory Role of Intestinal Microbiota in a COURAGE-Like Patient Cohort. *Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease* **2016**, *5*, doi:10.1161/JAHA.115.002816.
7. Kanitsoraphan, C.; Rattanawong, P.; Charoensri, S.; Senthong, V. Trimethylamine N-Oxide and Risk of Cardiovascular Disease and Mortality. *Curr Nutr Rep* **2018**, *7*, 207–213, doi:10.1007/S13668-018-0252-Z.
8. Zhu, W.; Gregory, J.C.; Org, E.; Buffa, J.A.; Gupta, N.; Wang, Z.; Li, L.; Fu, X.; Wu, Y.; Mehrabian, M.; et al. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. *Cell* **2016**, *165*, 111–124, doi:10.1016/J.CELL.2016.02.011.
9. Seldin, M.M.; Meng, Y.; Qi, H.; Zhu, W.F.; Wang, Z.; Hazen, S.L.; Lusis, A.J.; Shih, D.M. Trimethylamine N-Oxide Promotes Vascular Inflammation Through Signaling of Mitogen-Activated Protein Kinase and Nuclear Factor-KB. *J Am Heart Assoc* **2016**, *5*, doi:10.1161/JAHA.115.002767.
10. Haghikia, A.; Li, X.S.; Liman, T.G.; Bledau, N.; Schmidt, D.; Zimmermann, F.; Kränkel, N.; Widera, C.; Sonnenschein, K.; Haghikia, A.; et al. Gut Microbiota-Dependent Trimethylamine N-Oxide Predicts Risk of Cardiovascular Events in Patients With Stroke and Is Related to Proinflammatory Monocytes. *Arterioscler Thromb Vasc Biol* **2018**, *38*, 2225–2235, doi:10.1161/ATVBAHA.118.311023.
11. Olek, R.A.; Samulak, J.J.; Sawicka, A.K.; Hartmane, D.; Grinberga, S.; Pugovics, O.; Lysiak-Szydlowska, W. Increased Trimethylamine N-Oxide Is Not Associated with Oxidative Stress Markers in Healthy Aged Women. *Oxid Med Cell Longev* **2019**, *2019*, doi:10.1155/2019/6247169.
12. Samulak, J.J.; Sawicka, A.K.; Hartmane, D.; Grinberga, S.; Pugovics, O.; Lysiak-Szydlowska, W.; Olek, R.A. L-Carnitine Supplementation Increases Trimethylamine-N-Oxide but Not Markers of Atherosclerosis in Healthy Aged Women. *Ann Nutr Metab* **2019**, *74*, 11–17, doi:10.1159/000495037.
13. Bordoni, L.; Sawicka, A.K.; Szarmach, A.; Winklewski, P.J.; Olek, R.A.; Gabbianelli, R. A Pilot Study on the Effects of L-Carnitine and Trimethylamine-N-Oxide on Platelet Mitochondrial DNA Methylation and CVD Biomarkers in Aged Women. *Int J Mol Sci* **2020**, *21*, doi:10.3390/IJMS21031047.
14. Yin, J.; Liao, S.X.; He, Y.; Wang, S.; Xia, G.H.; Liu, F.T.; Zhu, J.J.; You, C.; Chen, Q.; Zhou, L.; et al. Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide Level in Patients With Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack. *J Am Heart Assoc* **2015**, *4*, doi:10.1161/JAHA.115.002699.
15. Collins, H.L.; Drazul-Schrader, D.; Sulpizio, A.C.; Koster, P.D.; Williamson, Y.; Adelman, S.J.; Owen, K.; Sanli, T.; Bellamine, A. L-Carnitine Intake and High Trimethylamine N-Oxide Plasma Levels Correlate with Low Aortic Lesions in ApoE(-/-) Transgenic Mice Expressing CETP. *Atherosclerosis* **2016**, *244*, 29–37, doi:10.1016/J.ATHEROSCLEROSIS.2015.10.108.
16. Landfald, B.; Valeur, J.; Berstad, A.; Raa, J. Microbial Trimethylamine- N-Oxide as a Disease Marker: Something Fishy? *Microb Ecol Health Dis* **2017**, *28*, 1327309, doi:10.1080/16512235.2017.1327309.
17. Ashar, F.N.; Zhang, Y.; Longchamps, R.J.; Lane, J.; Moes, A.; Grove, M.L.; Mychaleckyj, J.C.; Taylor, K.D.; Coresh, J.; Rotter, J.I.; et al. Association of Mitochondrial DNA Copy Number With Cardiovascular Disease. *JAMA Cardiol* **2017**, *2*, 1247–1255, doi:10.1001/JAMACARDIO.2017.3683.
18. Melser, S.; Lavie, J.; Bénard, G. Mitochondrial Degradation and Energy Metabolism. *Biochim Biophys Acta* **2015**, *1853*, 2812–2821, doi:10.1016/J.BBAMCR.2015.05.010.
19. Malik, A.N.; Czajka, A. Is Mitochondrial DNA Content a Potential Biomarker of Mitochondrial Dysfunction? *Mitochondrion* **2013**, *13*, 481–492, doi:10.1016/J.MITO.2012.10.011.
20. Clay Montier, L.L.; Deng, J.J.; Bai, Y. Number Matters: Control of Mammalian Mitochondrial DNA Copy Number. *J Genet Genomics* **2009**, *36*, 125–131, doi:10.1016/S1673-8527(08)60099-5.
21. Alejandro Alegría Torres, J. The Mitochondrial DNA Copy Number Used as Biomarker. *International Journal of Molecular Biology: Open Access* **2018**, *Volume 3*, doi:10.15406/IJMBOA.2018.03.00063.
22. Bordoni, L.; Gabbianelli, R. Mitochondrial DNA and Neurodegeneration: Any Role for Dietary Antioxidants? *Antioxidants 2020, Vol. 9, Page 764* **2020**, *9*, 764, doi:10.3390/ANTIOX9080764.
23. Castellani, C.A.; Longchamps, R.J.; Sun, J.; Guallar, E.; Arking, D.E. Thinking Outside the Nucleus: Mitochondrial DNA Copy Number in Health and Disease. *Mitochondrion* **2020**, *53*, 214, doi:10.1016/J.MITO.2020.06.004.
24. Yue, P.; Jing, S.; Liu, L.; Ma, F.; Zhang, Y.; Wang, C.; Duan, H.; Zhou, K.; Hua, Y.; Wu, G.; et al. Association between Mitochondrial DNA Copy Number and Cardiovascular Disease: Current Evidence Based on a Systematic Review and Meta-Analysis. *PLoS One* **2018**, *13*, doi:10.1371/JOURNAL.PONE.0206003.
25. Al Amir Dache, Z.; Otandault, A.; Tanos, R.; Pastor, B.; Meddeb, R.; Sanchez, C.; Arena, G.; Lasorsa, L.; Bennett, A.; Grange, T.; et al. Blood Contains Circulating Cell-Free Respiratory Competent Mitochondria. *FASEB J* **2020**, *34*, 3616–3630, doi:10.1096/FJ.201901917RR.
26. Vallance, H.D.; Koochin, A.; Branov, J.; Rosen-Heath, A.; Bosdet, T.; Wang, Z.; Hazen, S.L.; Horvath, G. Marked Elevation in Plasma Trimethylamine-N-Oxide (TMAO) in Patients with Mitochondrial Disorders Treated with Oral l-Carnitine. *Mol Genet Metab Rep* **2018**, *15*, 130–133, doi:10.1016/J.YMGMR.2018.04.005.

27. Schneider, J.; Girreser, U.; Havemeyer, A.; Bittner, F.; Clement, B. Detoxification of Trimethylamine N-Oxide by the Mitochondrial Amidoxime Reducing Component MARC. *Chem Res Toxicol* **2018**, *31*, 447–453, doi:10.1021/ACS.CHEMRESTOX.7B00329.
28. Makrecka-Kuka, M.; Volska, K.; Antone, U.; Vilskersts, R.; Grinberga, S.; Bandere, D.; Liepinsh, E.; Dambrova, M. Trimethylamine N-Oxide Impairs Pyruvate and Fatty Acid Oxidation in Cardiac Mitochondria. *Toxicol Lett* **2017**, *267*, 32–38, doi:10.1016/J.TOXLET.2016.12.017.
29. Chen, M.L.; Zhu, X.H.; Ran, L.; Lang, H.D.; Yi, L.; Mi, M.T. Trimethylamine-N-Oxide Induces Vascular Inflammation by Activating the NLRP3 Inflammasome Through the SIRT3-SOD2-MtROS Signaling Pathway. *J Am Heart Assoc* **2017**, *6*, doi:10.1161/JAHA.117.006347.
30. Fuchs, F.D.; Whelton, P.K. High Blood Pressure and Cardiovascular Disease. *Hypertension* **2020**, *75*, 285–292, doi:10.1161/HYPERTENSIONAHA.119.14240.
31. BM, L.; TM, M. Diabetes and Cardiovascular Disease: Epidemiology, Biological Mechanisms, Treatment Recommendations and Future Research. *World J Diabetes* **2015**, *6*, 1246, doi:10.4239/WJD.V6.I13.1246.
32. Messner, B.; Bernhard, D. Smoking and Cardiovascular Disease. *Arterioscler Thromb Vasc Biol* **2014**, *34*, 509–515, doi:10.1161/ATVBAHA.113.300156.
33. Khan, S.S.; Ning, H.; Wilkins, J.T.; Allen, N.; Carnethon, M.; Berry, J.D.; Sweis, R.N.; Lloyd-Jones, D.M. Association of Body Mass Index With Lifetime Risk of Cardiovascular Disease and Compression of Morbidity. *JAMA Cardiol* **2018**, *3*, 280–287, doi:10.1001/JAMACARDIO.2018.0022.
34. Bordoni, L.; Samulak, J.J.; Sawicka, A.K.; Pelikant-Malecka, I.; Radulska, A.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Trimethylamine N-Oxide and the Reverse Cholesterol Transport in Cardiovascular Disease: A Cross-Sectional Study. *Sci Rep* **2020**, *10*, 18675, doi:10.1038/s41598-020-75633-1.
35. Hoff, J.; Wehner, W.; Nambi, V. Troponin in Cardiovascular Disease Prevention: Updates and Future Direction. *Curr Atheroscler Rep* **2016**, *18*, 1–9, doi:10.1007/S11883-016-0566-5.
36. Garg, P.; Morris, P.; Fazlanie, A.L.; Vijayan, S.; Dancso, B.; Dastidar, A.G.; Plein, S.; Mueller, C.; Haaf, P. Cardiac Biomarkers of Acute Coronary Syndrome: From History to High-Sensitivity Cardiac Troponin. *Intern Emerg Med* **2017**, *12*, 147, doi:10.1007/S11739-017-1612-1.
37. Calderón, J.L.M.; Pérez, J.M.V.; Bustos, F.; Gómez, J.C.S. Performance Characteristics of Loci Method for Measuring Cardiac Troponin I on the Dimension EXL. *Pract Lab Med* **2015**, *1*, 42–47, doi:10.1016/J.PLABM.2015.03.006.
38. Cockcroft, D.W.; Gault, M.H. Prediction of Creatinine Clearance from Serum Creatinine. *Nephron* **1976**, *16*, 31–41, doi:10.1159/000180580.
39. Fazzini, F.; Schöpf, B.; Blatzer, M.; Coassin, S.; Hicks, A.A.; Kronenberg, F.; Fendt, L. Plasmid-Normalized Quantification of Relative Mitochondrial DNA Copy Number. *Sci Rep* **2018**, *8*, 15347, doi:10.1038/s41598-018-33684-5.
40. Schmittgen, T.D.; Livak, K.J. Analyzing Real-Time PCR Data by the Comparative C(T) Method. *Nat Protoc* **2008**, *3*, 1101–1108, doi:10.1038/NPROT.2008.73.
41. Mancini, A.; Vito, L.; Marcelli, E.; Piangerelli, M.; De Leone, R.; Pucciarelli, S.; Merelli, E. Machine Learning Models Predicting Multidrug Resistant Urinary Tract Infections Using "DsaaS." *BMC Bioinformatics* **2020**, *21*, doi:10.1186/S12859-020-03566-7.
42. Velasquez, M.T.; Ramezani, A.; Manal, A.; Raj, D.S. Trimethylamine N-Oxide: The Good, the Bad and the Unknown. *Toxins (Basel)* **2016**, *8*, doi:10.3390/TOXINS8110326.
43. Silva, F.; Simoes, R.; Couto, R.; Oliveira, P. Targeting Mitochondria in Cardiovascular Diseases. *Curr Pharm Des* **2016**, *22*, 5698–5717, doi:10.2174/1381612822666160822150243.
44. Koller, A.; Fazzini, F.; Lamina, C.; Rantner, B.; Kollerits, B.; Stadler, M.; Klein-Weigel, P.; Fraedrich, G.; Kronenberg, F. Mitochondrial DNA Copy Number Is Associated with All-Cause Mortality and Cardiovascular Events in Patients with Peripheral Arterial Disease. *J Intern Med* **2020**, *287*, 569–579, doi:10.1111/JOIM.13027.
45. Mengel-From, J.; Thinggaard, M.; Dalgård, C.; Kyvik, K.O.; Christensen, K.; Christiansen, L. Mitochondrial DNA Copy Number in Peripheral Blood Cells Declines with Age and Is Associated with General Health among Elderly. *Hum Genet* **2014**, *133*, 1149–1159, doi:10.1007/S00439-014-1458-9.
46. Guo, Y.; Cui, L.; Ye, P.; Li, J.; Wu, S.; Luo, Y. Change of Kidney Function Is Associated with All-Cause Mortality and Cardiovascular Diseases: Results from the Kailuan Study. *J Am Heart Assoc* **2018**, *7*, doi:10.1161/JAHA.118.010596.
47. Manjunath, G.; Tighiouart, H.; Coresh, J.; MacLeod, B.; Salem, D.N.; Griffith, J.L.; Levey, A.S.; Sarnak, M.J. Level of Kidney Function as a Risk Factor for Cardiovascular Outcomes in the Elderly. *Kidney Int* **2003**, *63*, 1121–1129, doi:10.1046/J.1523-1755.2003.00838.X.
48. Flint, A.C.; Conell, C.; Ren, X.; Banki, N.M.; Chan, S.L.; Rao, V.A.; Melles, R.B.; Bhatt, D.L. Effect of Systolic and Diastolic Blood Pressure on Cardiovascular Outcomes. *N Engl J Med* **2019**, *381*, 243–251, doi:10.1056/NEJMOA1803180.
49. Prestes, P.R.; Charchar, F.J. Is There a Link between Mitochondrial DNA and Blood Pressure? *J Hum Hypertens* **2017**, *31*, 761–762, doi:10.1038/JHH.2017.73.
50. Dikalov, S.I.; Ungvari, Z. Role of Mitochondrial Oxidative Stress in Hypertension. *Am J Physiol Heart Circ Physiol* **2013**, *305*, doi:10.1152/AJPHEART.00089.2013.
51. Lei, L.; Guo, J.; Shi, X.; Zhang, G.; Kang, H.; Sun, C.; Huang, J.; Wang, T. Mitochondrial DNA Copy Number in Peripheral Blood Cell and Hypertension Risk among Mining Workers: A Case-Control Study in Chinese Coal Miners. *J Hum Hypertens* **2017**, *31*, 585–590, doi:10.1038/JHH.2017.30.
52. Eirin, A.; Saad, A.; Tang, H.; Herrmann, S.M.; Woollard, J.R.; Lerman, A.; Textor, S.C.; Lerman, L.O. Urinary Mitochondrial DNA Copy Number Identifies Chronic Renal Injury in Hypertensive Patients. *Hypertension* **2016**, *68*, 401–410, doi:10.1161/HYPERTENSIONAHA.116.07849.
53. Eirin, A.; Saad, A.; Woollard, J.R.; Juncos, L.A.; Calhoun, D.A.; Tang, H.; Lerman, A.; Textor, S.C.; Lerman, L.O. Glomerular Hyperfiltration in Obese African American Hypertensive Patients Is Associated With Elevated Urinary Mitochondrial-DNA Copy Number. *Am J Hypertens* **2017**, *30*, 1112–1119, doi:10.1093/AJH/HPX103.
54. Whitaker, R.M.; Stallons, L.J.; Kneff, J.E.; Alge, J.L.; Harmon, J.L.; Rahn, J.J.; Arthur, J.M.; Beeson, C.C.; Chan, S.L.; Schnellmann, R.G. Urinary Mitochondrial DNA Is a Biomarker of Mitochondrial Disruption and Renal Dysfunction in Acute Kidney Injury. *Kidney Int* **2015**, *88*, 1336, doi:10.1038/KI.2015.240.
55. Tin, A.; Grams, M.E.; Ashar, F.N.; Lane, J.A.; Rosenberg, A.Z.; Grove, M.L.; Boerwinkle, E.; Selvin, E.; Coresh, J.; Pankratz, N.; et al. Association between Mitochondrial DNA Copy Number in Peripheral Blood and Incident CKD in the Atherosclerosis Risk in Communities Study. *J Am Soc Nephrol* **2016**, *27*, 2467–2473, doi:10.1681/ASN.2015060661.
56. Zhan, M.; Brooks, C.; Liu, F.; Sun, L.; Dong, Z. Mitochondrial Dynamics: Regulatory Mechanisms and Emerging Role in Renal Pathophysiology. *Kidney Int* **2013**, *83*, 568, doi:10.1038/KI.2012.441.

57. Small, D.M.; Coombes, J.S.; Bennett, N.; Johnson, D.W.; Gobe, G.C. Oxidative Stress, Anti-Oxidant Therapies and Chronic Kidney Disease. *Nephrology (Carlton)* **2012**, *17*, 311–321, doi:10.1111/J.1440-1797.2012.01572.X.
58. Che, R.; Yuan, Y.; Huang, S.; Zhang, A. Mitochondrial Dysfunction in the Pathophysiology of Renal Diseases. *Am J Physiol Renal Physiol* **2014**, *306*, doi:10.1152/AJPRENAL.00571.2013.
59. Navarro-González, J.F.; Mora-Fernández, C.; De Fuentes, M.M.; García-Pérez, J. Inflammatory Molecules and Pathways in the Pathogenesis of Diabetic Nephropathy. *Nat Rev Nephrol* **2011**, *7*, 327–340, doi:10.1038/NRNEPH.2011.51.
60. Wong, J.; McLennan, S. V.; Molyneaux, L.; Min, D.; Twigg, S.M.; Yue, D.K. Mitochondrial DNA Content in Peripheral Blood Monocytes: Relationship with Age of Diabetes Onsetand Diabetic Complications. *Diabetologia* **2009**, *52*, 1953–1961, doi:10.1007/S00125-009-1424-6.
61. Xu, F.X.; Zhou, X.; Shen, F.; Pang, R.; Liu, S.M. Decreased Peripheral Blood Mitochondrial DNA Content Is Related to HbA1c, Fasting Plasma Glucose Level and Age of Onset in Type 2 Diabetes Mellitus. *Diabet Med* **2012**, *29*, doi:10.1111/J.1464-5491.2011.03565.X.
62. Fazzini, F.; Lamina, C.; Raftopoulou, A.; Koller, A.; Fuchsberger, C.; Pattaro, C.; Del Greco, F.M.; Döttelmayer, P.; Fendt, L.; Fritz, J.; et al. Association of Mitochondrial DNA Copy Number with Metabolic Syndrome and Type 2 Diabetes in 14 176 Individuals. *J Intern Med* **2021**, *290*, 190–202, doi:10.1111/JOIM.13242.
63. Wei, R.; Ni, Y.; Bazeley, P.; Grandhi, S.; Wang, J.; Li, S.T.; Hazen, S.L.; Wilson Tang, W.H.; LaFramboise, T. Mitochondrial DNA Content Is Linked to Cardiovascular Disease Patient Phenotypes. *J Am Heart Assoc* **2021**, *10*, 1–720, doi:10.1161/JAHA.120.018776.
64. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kaplon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins (Basel)* **2019**, *11*, doi:10.3390/TOXINS11090490.
65. Jaworska, K.; Bielinska, K.; Gawrys-Kopczynska, M.; Ufnal, M. TMA (Trimethylamine), but Not Its Oxide TMAO (Trimethylamine-Oxide), Exerts Haemodynamic Effects: Implications for Interpretation of Cardiovascular Actions of Gut Microbiome. *Cardiovasc Res* **2019**, *115*, 1948–1949, doi:10.1093/CVR/CVZ231.
66. Restini, C.B.A.; Fink, G.D.; Watts, S.W. Vascular Reactivity Stimulated by TMA and TMAO: Are Perivascular Adipose Tissue and Endothelium Involved? *Pharmacol Res* **2021**, *163*, doi:10.1016/J.PHRS.2020.105273.
67. Era, S.; Kazuo, K.; Imai, H.; Nakamura, K.; Hayashi, T.; Sogami, M. Age-Related Change in Redox State of Human Serum Albumin. *Biochim Biophys Acta* **1995**, *1247*, 12–16, doi:10.1016/0167-4838(94)00166-E.
68. Bito, R.; Hino, S.; Baba, A.; Tanaka, M.; Watabe, H.; Kawabata, H. Degradation of Oxidative Stress-Induced Denatured Albumin in Rat Liver Endothelial Cells. *Am J Physiol Cell Physiol* **2005**, *289*, doi:10.1152/AJPCELL.00431.2004.
69. Rahmani-Kukia, N.; Abbasi, A.; Pakravan, N.; Hassan, Z.M. Measurement of Oxidized Albumin: An Opportunity for Diagnoses or Treatment of COVID-19. *Bioorg Chem* **2020**, *105*, 104429, doi:10.1016/J.BIOORG.2020.104429.
70. Lee, H.-C.; Wei, Y.-H. Mitochondrial Role in Life and Death of the Cell. *J Biomed Sci* **2000**, *7*, 2–15, doi:10.1007/BF02255913.
71. Cayci, T.; Kurt, Y.G.; Akgul, E.O.; Kurt, B. Does MtDNA Copy Number Mean Mitochondrial Abundance? *J Assist Reprod Genet* **2012**, *29*, 855, doi:10.1007/S10815-012-9803-1.
72. Qiu, C.; Hevner, K.; Abetew, D.; Sedensky, M.; Philip, M.; Enquobahrie, D.A.; Williams, M.A. Mitochondrial DNA Copy Number and Oxidative DNA Damage in Placental Tissues from Gestational Diabetes and Control Pregnancies: A Pilot Study. *Clin Lab* **2013**, *59*, 655–660, doi:10.7754/CLIN.LAB.2012.120227.
73. Liu, C.S.; Tsai, C.S.; Kuo, C.L.; Chen, H.W.; Lii, C.K.; Ma, Y.S.; Wei, Y.H. Oxidative Stress-Related Alteration of the Copy Number of Mitochondrial DNA in Human Leukocytes. *Free Radic Res* **2003**, *37*, 1307–1317, doi:10.1080/10715760310001621342.
74. Lin, C.S.; Wang, L.S.; Tsaig, C.M.; Wei, Y.H. Low Copy Number and Low Oxidative Damage of Mitochondrial DNA Are Associated with Tumor Progression in Lung Cancer Tissues after Neoadjuvant Chemotherapy. *Interact Cardiovasc Thorac Surg* **2008**, *7*, 954–958, doi:10.1510/ICVTS.2008.177006.
75. Mercer, J.R.; Cheng, K.K.; Figg, N.; Gorenne, I.; Mahmoudi, M.; Griffin, J.; Vidal-Puig, A.; Logan, A.; Murphy, M.P.; Bennett, M. DNA Damage Links Mitochondrial Dysfunction to Atherosclerosis and the Metabolic Syndrome. *Circ Res* **2010**, *107*, 1021–1031, doi:10.1161/CIRCRESAHA.110.218966.
76. Yu, E.; Calvert, P.A.; Mercer, J.R.; Harrison, J.; Baker, L.; Figg, N.L.; Kumar, S.; Wang, J.C.; Hurst, L.A.; Obaid, D.R.; et al. Mitochondrial DNA Damage Can Promote Atherosclerosis Independently of Reactive Oxygen Species through Effects on Smooth Muscle Cells and Monocytes and Correlates with Higher-Risk Plaques in Humans. *Circulation* **2013**, *128*, 702–712, doi:10.1161/CIRCULATIONAHA.113.002271.
77. Picard, M.; Turnbull, D.M. Linking the Metabolic State and Mitochondrial Dna in Chronic Disease, Health, and Aging. *Diabetes* **2013**, *62*, 672–678, doi:10.2337/DB12-1203/-/DC1.
78. Révész, D.; Verhoeven, J.E.; Picard, M.; Lin, J.; Sidney, S.; Epel, E.S.; Penninx, B.W.J.H.; Puterman, E. Associations Between Cellular Aging Markers and Metabolic Syndrome: Findings From the CARDIA Study. *J Clin Endocrinol Metab* **2018**, *103*, 148–157, doi:10.1210/JC.2017-01625.
79. Huang, C.H.; Su, S.L.; Hsieh, M.C.; Cheng, W.L.; Chang, C.C.; Wu, H.L.; Kuo, C.L.; Lin, T.T.; Liu, C.S. Depleted Leukocyte Mitochondrial DNA Copy Number in Metabolic Syndrome. *J Atheroscler Thromb* **2011**, *18*, 867–873, doi:10.5551/JAT.8698.
80. Hang, D.; Nan, H.; Kværner, A.S.; De Vivo, I.; Chan, A.T.; Hu, Z.; Shen, H.; Giovannucci, E.; Song, M. Longitudinal Associations of Lifetime Adiposity with Leukocyte Telomere Length and Mitochondrial DNA Copy Number. *Eur J Epidemiol* **2018**, *33*, 485–495, doi:10.1007/S10654-018-0382-Z.
81. Carugno, M.; Pesatori, A.C.; Dioni, L.; Hoxha, M.; Bollati, V.; Albetti, B.; Byun, H.M.; Bonzini, M.; Fustinoni, S.; Cocco, P.; et al. Increased Mitochondrial DNA Copy Number in Occupations Associated with Low-Dose Benzene Exposure. *Environ Health Perspect* **2012**, *120*, 210, doi:10.1289/EHP.1103979.
82. Hou, L.; Zhang, X.; Dioni, L.; Barretta, F.; Dou, C.; Zheng, Y.; Hoxha, M.; Bertazzi, P.A.; Schwartz, J.; Wu, S.; et al. Inhalable Particulate Matter and Mitochondrial DNA Copy Number in Highly Exposed Individuals in Beijing, China: A Repeated-Measure Study. *Part Fibre Toxicol* **2013**, *10*, doi:10.1186/1743-8977-10-17.
83. Hou, L.; Zhu, Z.Z.; Zhang, X.; Nordio, F.; Bonzini, M.; Schwartz, J.; Hoxha, M.; Dioni, L.; Marinelli, B.; Pegoraro, V.; et al. Airborne Particulate Matter and Mitochondrial Damage: A Cross-Sectional Study. *Environ Health* **2010**, *9*, 1–9, doi:10.1186/1476-069X-9-48/TABLES/3.
84. Cioffi, F.; Senese, R.; Lasala, P.; Ziello, A.; Mazzoli, A.; Crescenzo, R.; Liverini, G.; Lanni, A.; Goglia, F.; Iossa, S. Fructose-Rich Diet Affects Mitochondrial DNA Damage and Repair in Rats. *Nutrients* **2017**, *9*, doi:10.3390/NU9040323.
85. Bai, Y.; Carrillo, J.A.; Li, Y.; He, Y.; Song, J. Diet Induced the Change of MtDNA Copy Number and Metabolism in Angus Cattle. *J Anim Sci Biotechnol* **2020**, *11*, 1–13, doi:10.1186/S40104-020-00482-X/FIGURES/6.

86. Hernández-Ríos, R.; Hernández-Estrada, S.; Cruz-Robles, D.; Hernández-Lobato, S.; Villalobos-Martín, M.; Johnson, R.J.; Rodríguez-Castellanos, F.; Salazar, J.; García-Arroyo, F.; Sánchez-Lozada, L.G.; et al. Low Fructose and Low Salt Diets Increase Mitochondrial DNA in White Blood Cells of Overweight Subjects. *Exp Clin Endocrinol Diabetes* **2013**, *121*, 535–538, doi:10.1055/S-0033-1349144.

5.7 SUPPORTING INFORMATION

Table S1. Population descriptive statistics about blood lipids.

	Controls (N=151)				CAD (N=389)				
	Min	Max	Mean	SD	Min	Max	Mean	SD	p
TC (mg/dL)	104	351	209.01	46.35	77	353	171.95	47.19	<0.001
TG (mg/dL)	22	505	129.43	74.90	10	463	125.14	66.64	0.827
HDL (mg/dL)	27	107	55.62	16.11	11	126	50.77	16.46	<0.001
LDL (mg/dL)	38	248	127.53	42.83	12	246	95.67	41.46	<0.001
TC/HDL	1.73	8.48	4.02	1.31	1.25	19.64	3.69	1.57	0.001
TG/HDL	0.31	17.41	2.78	2.54	0.25	12.86	2.88	2.10	0.217

Table S2. Descriptive statistics for the population divided for CAD status.

	Controls (N=151)				CAD (N=389)			
	Min	Max	Mean	SD	Min	Max	Mean	SD
Age	44	85	64.34	8.12	27	91	66.43	11.64
BMI (kg/m^2)	19	41	27.81	4.08	18	45	28.77	4.50
TMAO (µM)	0.01	32.59	5.50	4.45	0.27	37.5	5.31	4.53
TMA (µM)	0.34	0.95	0.62	0.13	0.36	1.15	0.60	0.11
TMAO/TMA	0.01	64.87	9.39	8.61	0.42	63.58	8.66	6.81
mtDNAcn (dCt)	24.45	371.63	95.41	58.21	10.09	235.02	56.33	27.29
GFR (ml/min/1.73m^2)	45.85	289.96	92.17	31.23	9.71	211.11	86.62	34.60

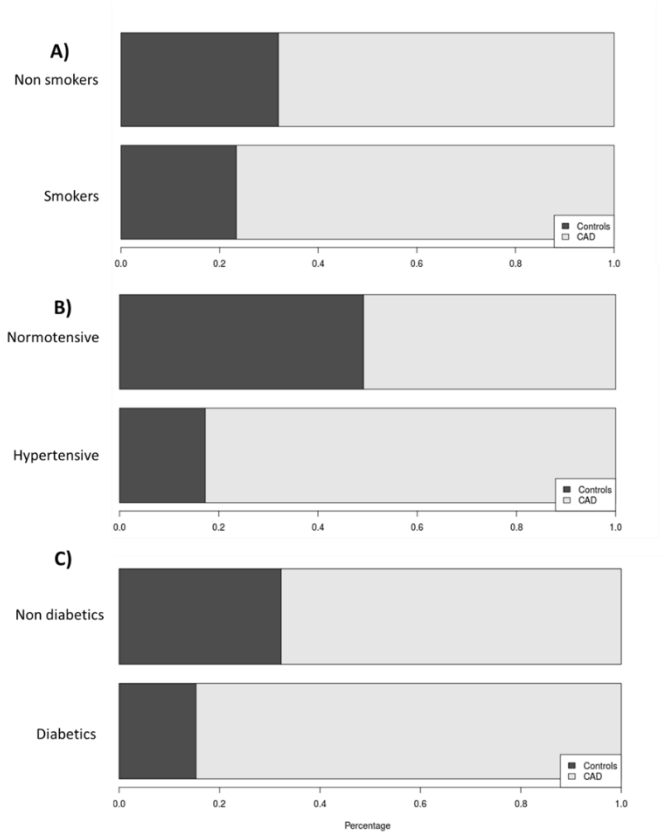


Figure S1. Smoking (A), hypertension (B) and diabetes (C) are confirmed as relevant risk factors for CAD in the analyzed population. A) pearson chi-quare. P=0.028; Odds Ratio [95%CI] = 1.535455 [1.047332 , 2.251073] B) pearson chi-quare. P=6.3*10-15 Odds Ratio [95%CI] = 4.634116 [3.107077 , 6.911653] C) pearson chi-quare. P=1.3*10-4 Odds Ratio [95%CI] = 2.630385 [1.580211 , 4.378483].

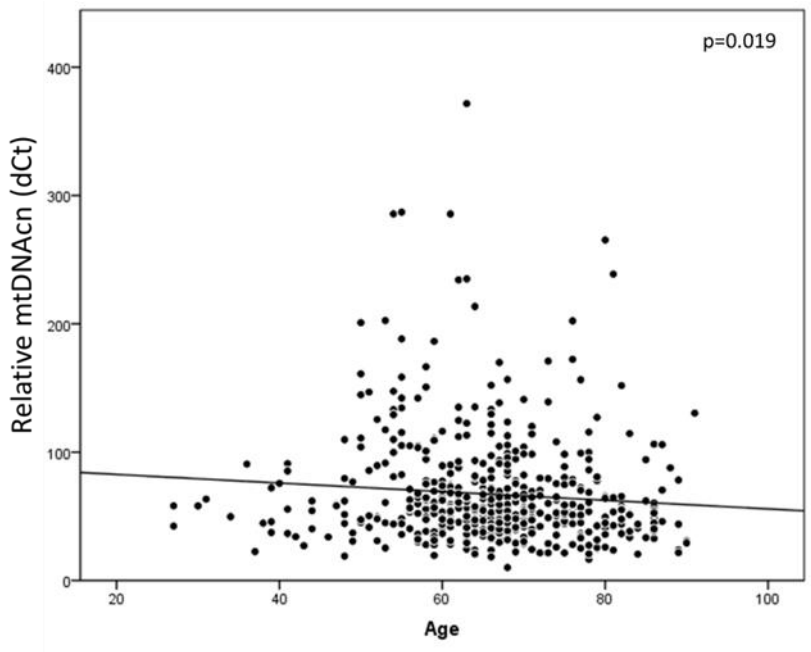


Figure S2. Correlation between mtDNAcn and age. MtDNAcn decreases with age (Spearman's rho=-0.101 ; p=0.019).

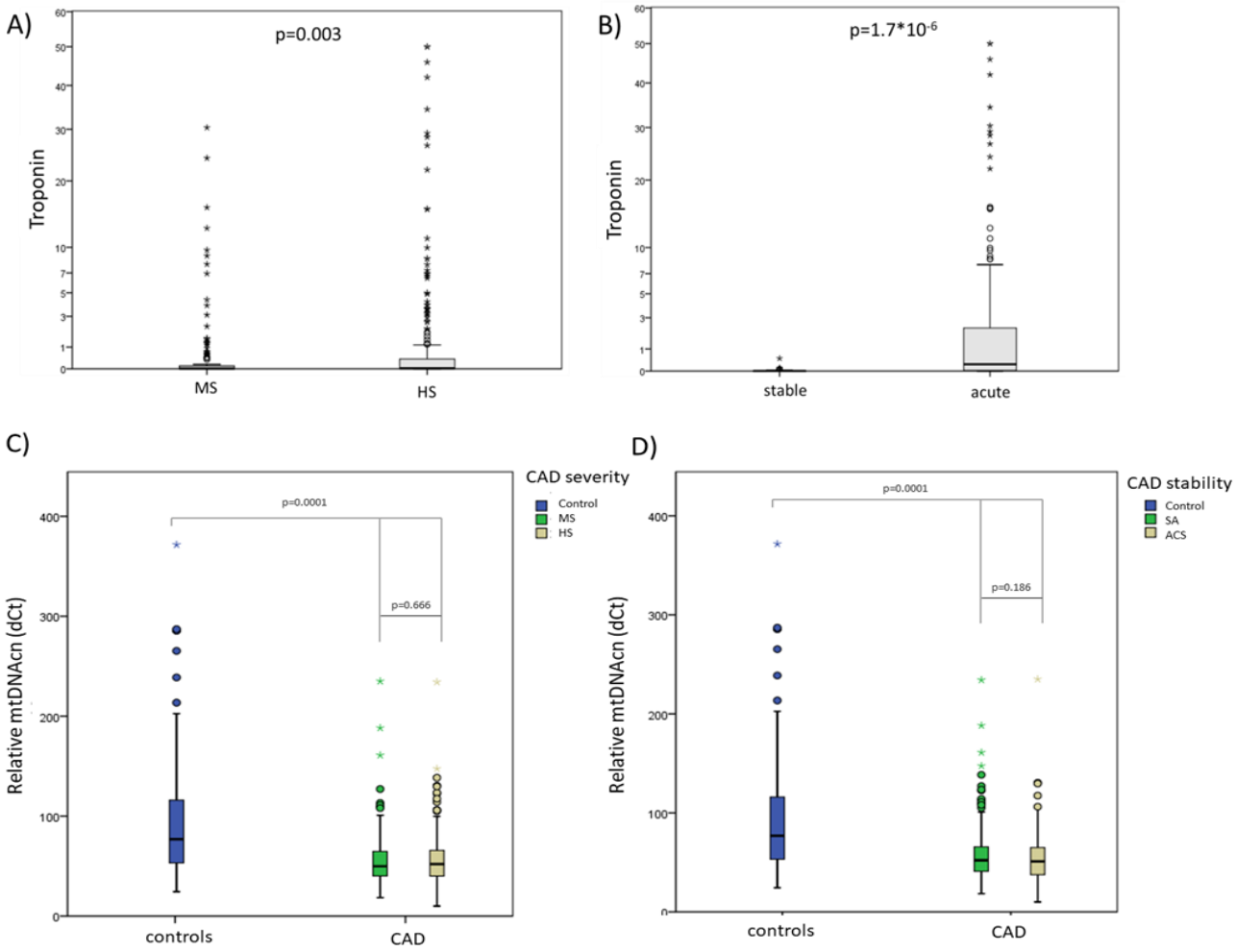


Figure S3. Mean levels of troponin (A,C) or mtDNAcn (B,D) in the population divided for disease severity (A,C) or stability (B,D). SA=stable CAD; ACS=acute coronary syndrome.

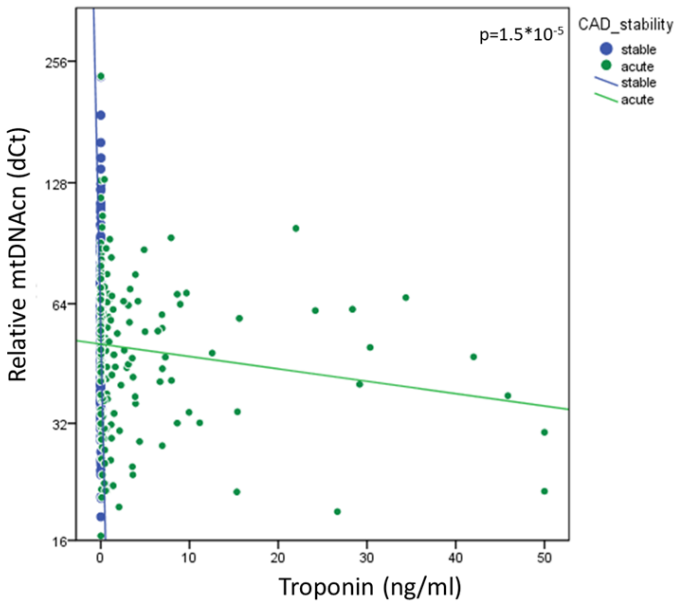


Figure S4. Correlation between mtDNAcn and troponin levels in stable or acute CAD patients.

Chapter 6

Gut-derived trimethylamine (TMA) may promote vascular inflammation disturbing the mitochondrial dynamics in circulating macrophages

6.1 STATE OF ART

Heart disease is a leading cause of mortality worldwide, each year [1]. Traditional predictors of cardiovascular disease (CVD) risk are high blood pressure, elevated glucose and lipids levels, obesity, physical inactivity, stress, smoking, as well as dietary patterns [2]. Indeed, unhealthy Western diet (characterized by increased intake of fat, red meat, and carbohydrates and minimal consumption of fruits and green leafy vegetables) has been associated with the severity of coronary artery lesions [3]. In particular, recent studies have revealed that trimethylamine-N-oxide (TMAO), a compound that derives from diets rich in animals' products, as Western diet is [4,5], could be used as a biomarker of CVD. Indeed, there is evidence supporting the association between circulating levels of TMAO and increased risks of CVDs [6], such as atherosclerosis, hypertension, heart failure, arrhythmia, and coronary artery disease (CAD) [7,8] but also with CVD-related chronic diseases, such as diabetes [9] and chronic kidney disease [10]. TMAO derives from the oxidation of trimethylamine (TMA), which is produced by the intestinal bacteria from dietary precursors of animal origin (i.e., choline, betaine, and carnitine). Once produced, TMA enters the portal circulation, and in the liver, it is further oxidized to TMAO, which has a high turnover rate. Indeed, while more than 90% TMAO is excreted in the urine, a small amount is excreted by respiration and feces, and the remaining is reduced back to TMA and re-enters the circulation. Several hypotheses have been proposed to explain the involvement of TMAO in the development of atherosclerosis and CVD risk. Among them, there is the induction of inflammatory responses, with increased levels of pro-inflammatory monocytes, enhanced cholesterol accumulation, foam cell formation, and endothelial dysfunction. Nevertheless, contrasting evidence is emerging on TMAO role in CVD promotion [11,12]. Indeed, a beneficial and protective role of TMAO has been hypothesized. Remarkably, fish is a major dietary source of TMAO (where it acts as an important osmolyte) but fish consumption is highly recommended to reduce CVD risk. Therefore, additional research is needed to determine the role of TMAO in CVDs.

Interestingly, plasma TMA is recently gaining attention in cardiovascular health studies, and rightly so considering that its toxicity has been known since late 19th century (for review [13]). Studies in rats showed that TMA increased arterial blood pressure and exerted a toxic effect on vascular smooth muscle cells [14]. However, despite the measurements of plasma TMA levels, very few studies have evaluated the effect of this compound on cells and organelles [13,15].

Thus, a clear mechanistic explanation of how TMA and TMAO may promote inflammation is still missing. Recent evidence suggests that the perturbation of mitochondrial homeostasis could be one of the underlying causes [15]. Indeed, mitochondrial dynamics are influenced by both intrinsic and extrinsic factors, including diet, and they may either have an active role in inflammation or be a target. For this reason, mitochondrial DNA copy number (mtDNAcn) [16] has been recently

proposed as a biomarker for CVD prediction. Indeed, mtDNAcn changes turn into altered expression of mitochondrial proteins and altered ATP production and have been rightly suggested as indicators of mitochondrial damage and dysfunction [17].

6.2 AIM OF THE STUDY

The role of circulating TMA and TMAO in CVD is still highly debated and not fully understood. Considering that vascular damage has been proposed as a mechanism by which these diet-derived metabolites may exert their noxious effect and contribute to CVD, the present study aims to better elucidate the potential pro-inflammatory action of TMA and TMAO on circulating macrophages, using THP-1 monocytes as an *in vitro* model. We investigated whether plasma TMA and TMAO may trigger the pro-inflammatory cascade and, considering the prominent role that mitochondria have in inflammation, we also investigated the impact of TMA and TMAO on mitochondrial dynamics, focusing on perturbation of ATP production and cellular membrane potential, as well as on the markers of mitochondrial damage (i.e., mtDNAcn).

6.3 MATERIALS AND METHODS

6.3.1 Cell culture

To study the effect of TMA and TMAO on macrophages, THP-1 cells, a human monocytic leukaemia cell line (ATCC, Rockville, MD), were used.

THP-1 cells were cultured in RPMI 1640 medium 1X with L-glutamine (Corning, Turin, Italy) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Euroclone, Milano, Italy), 1% penicillin/streptomycin (Euroclone, Milano, Italy) and 50 µM β-Mercaptoethanol (Euroclone, Milano, Italy). The cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 2 days and cells were passaged at 80% confluence.

6.3.2 Viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate the cytotoxic effect of TMA and TMAO on THP-1 cells. Briefly, THP-1 cells were seeded in 96-well plates ($1x10^4$ cell/well) in RPMI 1640 1X with L-glutamine, cultured for 24h and treated with several concentrations of TMA (50 µM, 25 µM, 10 µM, 1 µM, 0.5 µM, 0.1 µM) and TMAO (400 µM, 100 µM, 50 µM, 25 µM, 5 µM, 1 µM). At the end of the incubation period, 5 mg/ml MTT solution was added to the cells and incubated for 4h. The insoluble formazan salt product was solubilized by adding dimethyl sulfoxide (DMSO) and its amount was determined by measuring the optical

density at 540 nm using a microplate reader. Cell viability was calculated according to the equation (T/C) × 100%, where T and C represent respectively the mean optical density of the treated group and the control group.

6.3.3 Cell treatments

The TMA and TMAO concentrations that were tested in this study were selected from TMA and TMAO levels previously measured in plasma of coronary artery disease (CAD) patients [18]. Given the broad variation of plasma TMA and TMAO concentrations measured in CAD patients, we only selected two concentrations for the present study. Specifically, the highest concentration and the mean concentration of either TMA or TMAO measured in CAD patients were chosen as the highest and lowest dose tested on THP-1 cells, respectively. First, THP-1 cells were differentiated for 24h with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) [19] to induce differentiation of monocytes into the M0 phenotype macrophages. Then the differentiated macrophages were treated with 0.6 µM TMA, 1.2 µM TMA, 5 µM TMAO and 40 µM TMAO for 24h. Negative controls (differentiated macrophages with vehicle only) were also added. Experiments were run in duplicate. After 24h of incubation, cells were collected. Cells were mechanically detached and centrifuged at 300xg for 10 min; the supernatant was removed, and cell pellets were immediately frozen in liquid nitrogen and stored at -80°C until further use.

6.3.4 mtDNA quantification

Total DNA was extracted using the Genomic DNA Isolation Kit (Cat. 24700, Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions and used to quantify mtDNAcn in the samples. DNA amount and purity were checked by spectrophotometric quantification with Nanodrop (Thermo Fisher Scientific, Italy). Relative quantification by quantitative PCR (Biorad CFX96) was chosen to quantify mtDNAcn, considering nuclear DNA as a normalizer, as previously reported [20]. The mitochondrial primers have been previously validated for their specificity (unique amplification of mtDNA) and the absence of coamplified nuclear insertions of mitochondrial origin (NUMTs). For relative quantification of mtDNAcn, *mtDNA-tRNALeu* (fw: CACCCAAGAACAGGGTTTGT; rv: TGGCCATGGGTATGTTGT), and *Beta-2-Microglobulin* (*B2M*) (fw: TGCTGTCTCCATGTTTGATGTATCT; rv: TCTCTGCTCCCCACCTCTAAGT) primers were chosen to amplify mtDNA and nDNA, respectively. The amplification conditions were: 30 seconds at 95°C followed by 5 seconds at 95°C and 30 seconds at 60°C, these latter repeated for 40 cycles. To check the specificity of each amplification, a melting curve was also performed. Relative mtDNAcn was

determined using the $2^{-\Delta\Delta Ct}$ method. Each analysis was run in duplicate. An inter-run calibrator sample was applied to adjust the results obtained from different amplification plates.

6.3.5 Gene expression analysis

Total RNA was extracted from treated THP-1 cells with the Total RNA Purification Plus Kit (Cat. 48300, Norgen Biotek, Thorold, ON, Canada), according to the manufacturer's instructions. Concentration of RNA and purity were assessed by UV spectrophotometer (NanoDrop, Thermo Fisher Scientific, Italy). Then 1µg RNA was retrotranscribed to cDNA using the PrimeScript RT-PCR Kit (Cat. RR037A, Takara Bio, Göteborg, Sweden) according to the manufacturer's instructions. Gene expression analyses were carried out by quantitative real-time PCR (Biorad CFX96), using the TB Green® Premix Ex Taq™ (Cat. RR420A, Takara Bio, Göteborg, Sweden). The amplification conditions were: 30 seconds at 95°C followed by 5 seconds at 95°C and 30 seconds at 60°C, these latter repeated for 40 cycles. To check the specificity of each amplification, a melting curve was also performed. The expression levels of the target genes were normalized relative to β-actin, using the $2^{-\Delta\Delta Ct}$ method.

The target genes analysed were the inflammation-related *IL-6, IL-8,* and *IL-10* and the mitochondrial *ND6*, *CYTB*, *CO1*, *ATP6*. The sequences of the primers used in the study are listed below: *IL-6* (fw: TGCAATAACCACCCCTGACC; rv: GTGCCCATGCTACATTTGCC), IL-8 (fw: GGACAAGAGCCAGGAAGAAA; rv: CCTACAACAGACCCACACAATA), *IL-10* (fw: AGGCATTCTTCACCTGCTCC; rv: AAGACCCAGACATCAAGGCG), *ND6* (fw: GCTTTGTATGATTATGGGCGT; rv: CACCAACAAACAATGTTCAACC), *CYTB* (fw: ATCACTCGAGACGTAAATTATGGCT; rv: TGAACTAGGTCTGTCCCAATGTATG), *CO1* (fw: GACGTAGACACACGAGCATATTTCA; rv: AGGACATAGTGGAAGTGAGCTACAAC), *ATP6* (fw: TAGCCATACACAACACTAAAGGACGA; rv: GGGCATTTTTAATCTTAGAGCGAAA).

6.3.6 ATP quantification

The ATP content from treated THP-1 cells was quantified using the ATP Colorimetric Assay Kit (Cat. MAK190, Sigma-Aldrich, Germany) according to the manufacturer's instructions. Briefly, pellets from treated THP-1 cells (0.6 µM and 1.2 µM TMA, 5 µM and 40 µM TMAO) were lysed, and the ATP content was determined by phosphorylating glycerol. Negative controls were also included. The absorbance was read at 570 nm and data an ATP calibration curve was used for accurate ATP quantification. All analyses were run in triplicate.

6.3.7 Mitochondrial membrane potential

For the assessment of the mitochondria membrane potential (ΔΨm) in treated THP-1 cells, the Mitochondrial Membrane Potential Kit (Cat. MAK159, Sigma-Aldrich, Germany) was used according to manufacturer's instructions. This assay uses cationic, lipophilic dye JC-10 that can discriminate between living cells and apoptotic cells through differences in their ΔΨm. Briefly, $8x10^4$ cells were seeded into a 96-well plate. Different concentrations of TMA (0.01 µM, 0.1 µM, 0.6 µM, 1.2 µM, 10 µM, 25 µM, 50 µM) and TMAO (0.1 µM, 1 µM, 5 µM, 25 µM, 40 µM, 100 µM, 200 µM, 400 µM) were added to the cells. A positive control (100 ng/mL LPS) and a negative control (vehicle only) were also included. Treated cells were incubated 24h at 37°C. Afterword, treated cells were incubated with the JC-10 dye at 37°C for 60 minutes and the fluorescence was red at λ_{ex}=490/λ_{em}=525 nm and at λ_{ex}=540/λ_{em}=590 nm) through a fluorometer. Each experimental condition was set up in duplicate.

6.3.8 Statistics analysis

Statistical analysis was performed by using SPSS (IBM SPSS Statistics for Windows, Version 24.0, USA) and R version 3.5.3 (R Core Team, Vienna, Austria). The ANOVA test was used to compare the difference between group means. A *p*-value < 0.05 was considered significant throughout the study.

6.4 RESULTS

6.4.1 The Effect of TMA and TMAO on Macrophages Viability (MTT Assay)

Neither TMA not TMAO at the tested concentrations exerted toxic effects on THP-1 cell, as shown by the unchanged cell viability compared to controls (Figure 1).

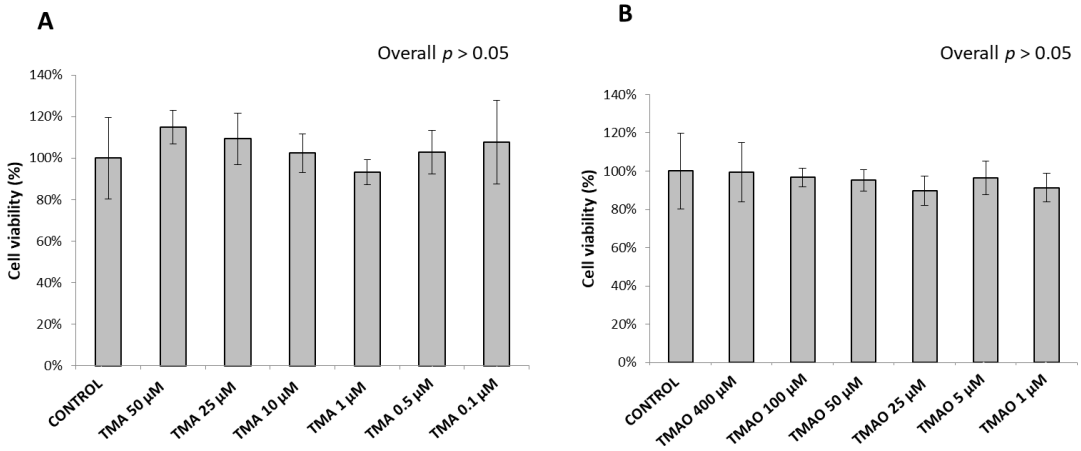


Figure 1. Effect of TMA and TMAO on Macrophages Viability (MTT Assay).

6.4.2 The Effect of TMA and TMAO on inflammation-related genes

The expression levels of the proinflammatory cytokines IL-6 and IL-8, and the anti-inflammatory cytokines IL-10 were investigated in THP-1 cells upon treatments with 0.6 µM and 1.2 µM TMA, and with 5 µM and 40 µM TMAO.

Relative to controls, a significant increase of the expression levels of the pro-inflammatory IL-8 cytokine was observed at all tested TMA concentrations (0.6 µM TMA, $p < 0.05$; 1.2 µM TMA, $p < 0.05$) and at 40 µM TMAO ($p < 0.05$) (Figure 2B). Instead, no changes were observed for *IL-6* gene expression (overall $p > 0.05$) (Figure 2A). Interestingly, we also found that the expression of the anti-inflammatory *IL-10* was upregulated by TMA and TMAO, at the highest doses. (1.2 µM TMA, $p < 0.05$; 40 µM TMAO, $p < 0.05$) (Figure 2C).

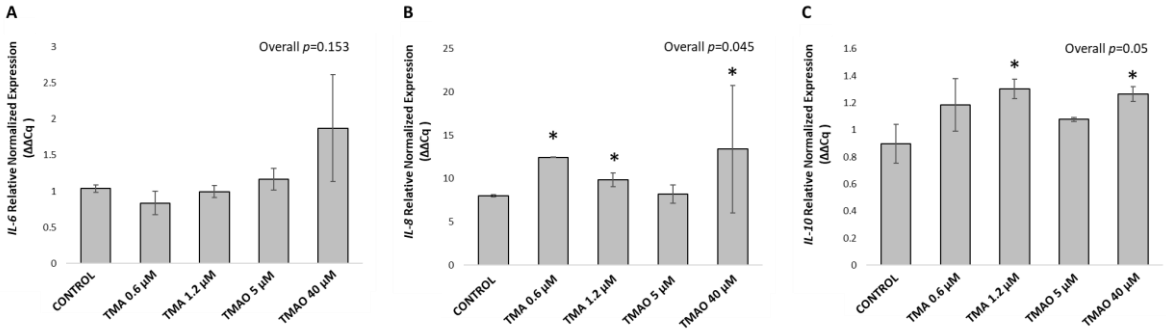


Figure 2. Expression levels of *IL-6* (A), *IL-8* (B) and I*L-10* (C) on THP-1 cells.

6.4.3 The Effect of TMA and TMAO on mtDNAcn

When investigating the mtDNAcn in treated cells, the overall ANOVA showed no significant differences among groups (overall $p > 0.05$). (Figure 3).

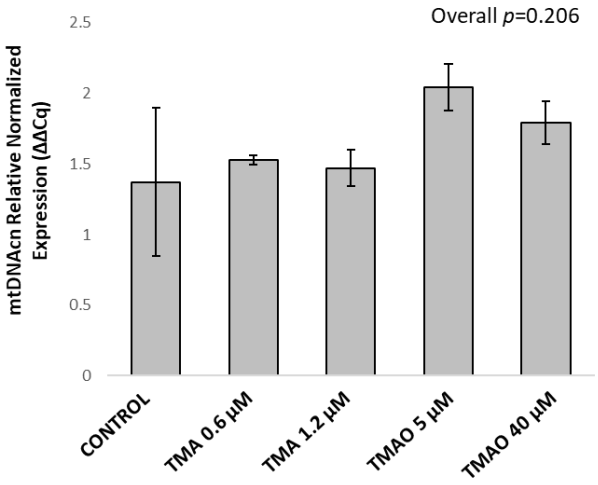


Figure 3. MtDNAcn in THP-1 cells.

6.4.4 The Effect of TMA and TMAO on mitochondrial genes expression

In this study, the capacity of TMA and TMAO to modulate the expression of the mitochondrial genes correlated to ATP formation was investigated in THP-1 cells. Our results showed that both TMA and TMAO at all tested concentrations were able to induce a significant increase in the expression levels of all the analysed mitochondrial genes (*MT-ATP6*, overall $p < 0.001$; *MT-CO1*, overall $p < 0.01$; *MT-CYB*, overall $p < 0.01$; *MT-ND6*, overall $p < 0.01$) (Figure 4). Interestingly, the increase in the expression levels of mitochondrial genes was directly proportional to TMA concentration (*MT-ATP6*, $p < 0.001$; *MT-CO1,* $p < 0.05$; *MT-CYB,* $p < 0.05$; *MT-ND6,* $p = 0.05$). Instead, the increase in the expression levels of mitochondrial genes was inversely proportional to TMAO concentration (*MT-ATP6*, $p < 0.05$; *MT-CO1,* $p < 0.05$; *MT-ND6,* $p < 0.05$).

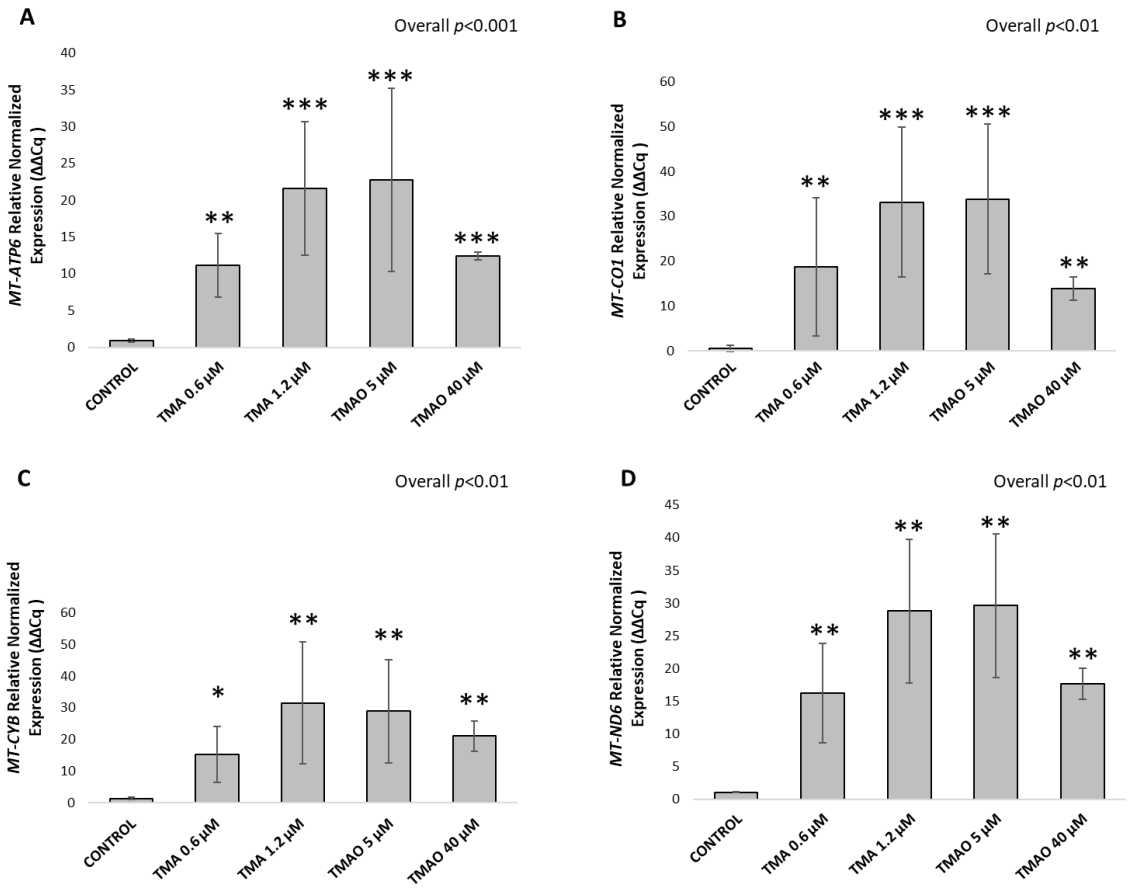


Figure 4. Expression levels of mitochondrial genes *MT-ATP6 (A)*, *MT-CO1 (B), MT-CYB (C),* and *MT-ND6 (D)* in THP-1 cells.

6.4.5 The Effect of TMA and TMAO on ATP production

To determine the effect of TMA and TMAO on intracellular ATP levels, we cultured THP-1 cells in the presence of different concentrations of TMA (0.6 µM and 1.2 µM TMA) and TMAO (5 µM and 40 µM TMAO) for 24h and intracellular ATP was quantified on lysed cells. In the present study, ATP contents is expressed as nmoles ATP per 1 million cells. As ATP content reflects the metabolic activity of cells, our results showed that only TMA slows down the cellular metabolism in THP-1 cells. Indeed, after 24h exposure of TMA, a decrease in ATP content was observed for all the tested TMA concentrations (0.6 µM TMA, $p < 0.05$; 1.2 µM TMA, $p < 0.05$) relative to the control group (Figure 5). Interestingly, we detected an inverse proportionality between intracellular ATP content and TMA dosage.

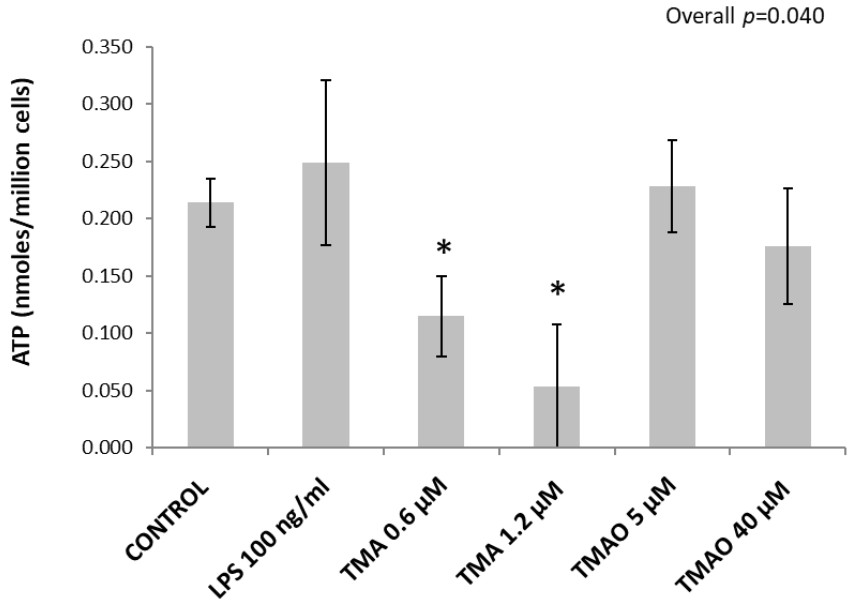


Figure 5. Intracellular ATP levels in THP-1 cells.

6.4.6 The Effect of TMA and TMAO on Mitochondrial Membrane Potential

Mitochondrial membrane potential (ΔΨm), which is defined as the capacity of mitochondria to maintain an electrochemical gradient required to produce ATP, is not only a key indicator of mitochondrial activity, because it reflects ATP production, but also of good cellular health. In this study, the effects of several doses of TMA and TMAO on mitochondrial membrane potential were investigated. After 24h treatment with various TMA and TMAO concentrations, and LPS 100 ng/mL, significant increase of the ΔΨm was induced by LPS and TMA treatments compared to the control group (overall p = 0.001). The treatment 0.1 µM TMA did not induce any significant difference in

the ΔΨm but considering that it is the only exception among all the tested TMA doses, this result might be due to technical issues. Instead TMAO treatment did not induce any significant changes in the ΔΨm in THP-1 cells, at the tested concentrations (Figure 6).

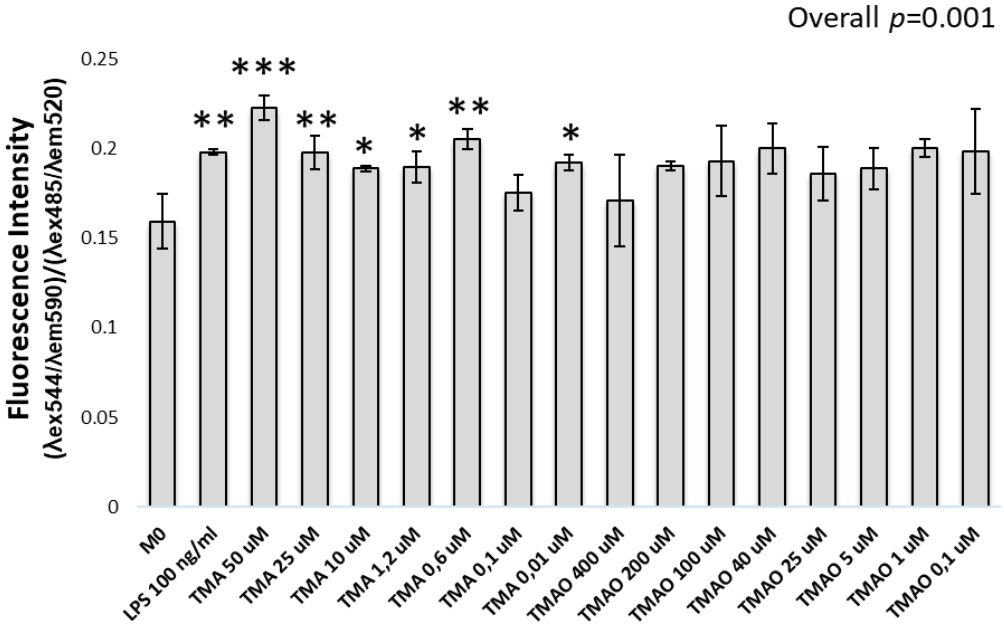


Figure 6. Mitochondrial membrane potential (ΔΨm) of THP-1 cells after TMA and TMAO treatments.

6.5 DISCUSSION AND CONCLUSIONS

In humans nearly 95% of produced TMA is oxidized to TMAO, which for the most part is eliminated by the renal clearance, but it may also accumulate in tissues or be excreted through sweat, faeces, and exhaled air. For this reason, circulating TMA and TMAO levels are usually low, however they show substantial inter- and intra-individual variations [21]. Indeed, several factors determine TMA and TMAO production, and thus their plasmatic concentration, including diet, intestinal microbiota composition, liver FMOs activity, kidneys' clearance capacity, and age.

A positive correlation between elevated plasma TMAO levels and a higher risk for major adverse cardiovascular events and death has been reported [22,23]. However, the results are not always consistent, since not all studies reported a difference in the measured plasma TMAO concentration between healthy subjects and CVD patients [24]. Despite most attention has been channelled towards circulating TMAO levels, some doubts about the harmful potential of TMAO are arising [25] and preliminary evidence suggests that TMA and not TMAO might have a role in CVD *per se* [13,14,26], by virtue of its well-known toxicity. However, very few studies have addressed

circulating TMA levels so far, and results are conflicting, with TMA levels being either elevated [27], or decreased [24] in CVD patients compared to healthy individuals.

Several hypotheses have been proposed to explain the involvement of TMA and TMAO in the development of atherosclerosis and CVD risk, even though a mechanistic explanation is still missing. TMAO is thought to induce inflammatory responses, triggering pro-inflammatory monocytes, and resulting in cholesterol accumulation, foam cell formation, and endothelial dysfunction. TMA seems to contribute to CVD by increasing arterial blood pressure and exerting toxic effects on vascular smooth muscle cells.

The present work investigated the effects that plasma TMA and TMAO may potentially exert on circulating macrophages, using human THP-1 cells as an *in vitro* model, and focusing on inflammatory processes and mitochondrial dynamics. Cells were tested with 2 different concentrations of TMA and 2 different concentrations of TMAO for short exposure (24h). The concentrations of TMA and TMAO used for this study were based on those previously measured by Bordoni *et al*. in plasma from CAD patients [24] and control subjects, and they were chosen to resemble the concentration of the two metabolites in physiological (lower TMA and TMAO doses) and pathological (higher TMA and TMAO doses) conditions.

Our results show that TMA (at all concentrations tested) and TMAO (at the highest dose only) are able to activate macrophages and trigger an inflammatory response, as proven by the increased expression of the pro-inflammatory *IL-8*, that is one of the major mediators of the inflammatory response. Thus, these results support the hypothesis that only high plasma TMAO concentrations may be harmful, while TMA may be noxious at all concentrations and have a role *per se* in CVD onset, by promoting vascular inflammation. Indeed, the role of IL-8 in CVD is widely accepted. Prior research on patients and cell culture models has shown that IL-8 participates in all stages of atherosclerosis and is involved in the pathogenesis of cardiovascular disorders including coronary heart disease, and myocardial infarction [28–30]. However, although the atherogenic effect and association of IL-8 overexpression with the development of cardiovascular disorders are undeniable, the role of IL-8 is still debated [31,32] since the anti-atherogenic effect of IL-8 has been also documented. Interestingly, we found that the anti-inflammatory *IL-10* was upregulated by the highest concentrations of TMA and TMAO, most likely as a compensatory mechanism to counteract the acute inflammatory response induced by *IL-8* overexpression. Indeed, inhibitory role of IL-10 for human and mouse immune responses are known [33,34] and IL-10 has been reported to specifically inhibit IL-8 production in LPS-activated mononuclear cells [34]. In light of our findings, TMA and TMAO are potentially able to activate a complex cytokine environment where proinflammatory cytokines, such as IL-8, are counteracted by inhibitory ones, such as IL-10.

Since the perturbation of mitochondrial homeostasis has been suggested as one possible cause of TMA and TMAO-induced inflammation, in the present study mitochondrial dynamics have been

addressed too. In the tested conditions, neither TMA nor TMAO induced significant changes in the mtDNA content of THP-1 cells, even though Bordoni *et al*. previously detected a strong direct correlation between mtDNAcn and TMA levels measured in plasma from CAD patients [18]. Nonetheless, 24h treatment with either TMA or TMAO boosted mitochondrial respiration, inducing the upregulation of the mitochondrial genes coding for complexes I, III, IV and V of the respiratory chain. Interestingly, the expression of all genes was affected in a dose-dependent manner. Their expression levels were directly proportional to TMA concentration, and inversely proportional to TMAO concentration (except for *MT-CYB* whose expression was not affected by the concentration of TMAO). Surprisingly, we measured a reduction of the intracellular ATP content after 24h treatment with TMA despite the genes coding for the complexes of the respiratory chain were upregulated, most likely because of the activation of compensatory pathways to counteract the depletion of the ATP reservoir. A decreased intracellular ATP content has already been reported by Jalandra et *al.* adenocarcinoma cell lines after 24h, 48h, and 72h treatment with TMA and it was time and dose-dependent [15]. On the contrary, in our study TMAO did not induce changes in ATP production, even though a previous work on cardiomyocytes reported that TMAO inhibited the activity of pyruvate dehydrogenase and weakened the oxidation of pyruvate in mitochondria, impairing the production of ATP [35]. However, in our work, that fact that TMAO did not affect ATP production is coherent with its milder effect (compared to TMA) on the expression of the mitochondrial genes that we previously discussed.

Knowing that the production of ATP is strictly dependent on the electron transfer chain, we investigated the effect of TMA and TMAO on the ΔΨm. Our results showed that the ΔΨm was affected by TMA, but not TMAO. Namely, the ΔΨm was increased by TMA treatment at the tested concentrations, which was not unexpected considering that the increase of ΔΨm normally results in ATP production. Thus, the enhanced ΔΨm that we measured after TMA treatments, most likely results from the boosted respiratory process (as testified by the upregulated mitochondrial genes), in an attempt to compensate for the TMA-induced depletion of ATP reservoirs.

In conclusion, based on what seen on our cellular model, our study reveals that both TMA and TMAO, are able to trigger inflammatory pathways in macrophages. However, TMA, more than TMAO, may disturb cellular homeostasis by altering mitochondrial dynamics. Indeed, in inflamed macrophages the compromised cellular function may lead to the depletion of the intracellular ATP storage. In an attempt to compensate for the TMA-induced depletion of ATP reservoirs and avoid irreversible cellular damage, mitochondria may respond with the upregulation of some respiratory chain components in order to restore the energy balance. Altogether, our findings contribute to reinforce the hypothesis that TMA might be more detrimental than its oxidized product (TMAO) in the context of vascular inflammation. TMA, more that TMAO, may have a contributing role in CVD, even though the exact mechanisms remain to be fully elucidated. Thus, further efforts are

necessary to clarify the mechanistic pathways of TMA-induced vascular inflammation, also in view of CVD prevention, risk stratification, and personalized therapies.

6.6 REFERENCES

1. Roth, G.A.; Johnson, C.; Abajobir, A.; Abd-Allah, F.; Abera, S.F.; Abyu, G.; Ahmed, M.; Aksut, B.; Alam, T.; Alam, K.; et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *J Am Coll Cardiol* **2017**, *70*, 1–25, doi:10.1016/J.JACC.2017.04.052.
2. Roy, S. Atherosclerotic Cardiovascular Disease Risk and Evidence-Based Management of Cholesterol. *N Am J Med Sci* **2014**, *6*, 191–198, doi:10.4103/1947-2714.132916.
3. Oikonomou, E.; Psaltopoulou, T.; Georgiopoulos, G.; Siasos, G.; Kokkou, E.; Antonopoulos, A.; Vogiatzi, G.; Tsalamandris, S.; Gennimata, V.; Papanikolaou, A.; et al. Western Dietary Pattern Is Associated With Severe Coronary Artery Disease. *Angiology* **2018**, *69*, 339–346, doi:10.1177/0003319717721603.
4. Zeisel, S.H.; Mar, M.H.; Howe, J.C.; Holden, J.M. Concentrations of Choline-Containing Compounds and Betaine in Common Foods. *J Nutr* **2003**, *133*, 1302–1307, doi:10.1093/JN/133.5.1302.
5. Obeid, R.; Awwad, H.M.; Rabagny, Y.; Graeber, S.; Herrmann, W.; Geisel, J. Plasma Trimethylamine N-Oxide Concentration Is Associated with Choline, Phospholipids, and Methyl Metabolism. *Am J Clin Nutr* **2016**, *103*, 703–711, doi:10.3945/ajcn.115.121269.
6. Mamic, P.; Chaikijurajai, T.; Tang, W.H.W. Gut Microbiome - A Potential Mediator of Pathogenesis in Heart Failure and Its Comorbidities: State-of-the-Art Review. *J Mol Cell Cardiol* **2021**, *152*, 105, doi:10.1016/J.YJMCC.2020.12.001.
7. Randrianarisoa, E.; Lehn-Stefan, A.; Wang, X.; Hoene, M.; Peter, A.; Heinzmann, S.S.; Zhao, X.; Königsrainer, I.; Königsrainer, A.; Balletshofer, B.; et al. Relationship of Serum Trimethylamine N-Oxide (TMAO) Levels with Early Atherosclerosis in Humans. *Sci Rep* **2016**, *6*, doi:10.1038/SREP26745.
8. Wang, Z.; Klipfell, E.; Bennett, B.J.; Koeth, R.; Levison, B.S.; Dugar, B.; Feldstein, A.E.; Britt, E.B.; Fu, X.; Chung, Y.M.; et al. Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease. *Nature* **2011**, *472*, 57–63, doi:10.1038/NATURE09922.
9. Dambrova, M.; Latkovskis, G.; Kuka, J.; Strele, I.; Konrade, I.; Grinberga, S.; Hartmane, D.; Pugovics, O.; Erglis, A.; Liepinsh, E. Diabetes Is Associated with Higher Trimethylamine N-Oxide Plasma Levels. *Exp Clin Endocrinol Diabetes* **2016**, *124*, 251–256, doi:10.1055/S-0035-1569330.
10. Tang, W.H.W.; Wang, Z.; Levison, B.S.; Koeth, R.A.; Britt, E.B.; Fu, X.; Wu, Y.; Hazen, S.L. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. *N Engl J Med* **2013**, *368*, 1575–1584, doi:10.1056/NEJMOA1109400.
11. Meyer, K.A.; Benton, T.Z.; Bennett, B.J.; Jacobs, D.R.; Lloyd-Jones, D.M.; Gross, M.D.; Carr, J.J.; Gordon-Larsen, P.; Zeisel, S.H. Microbiota-Dependent Metabolite Trimethylamine N-Oxide and Coronary Artery Calcium in the Coronary Artery Risk Development in Young Adults Study (CARDIA). *Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease* **2016**, *5*, doi:10.1161/JAHA.116.003970.
12. Huc, T.; Drapala, A.; Gawrys, M.; Konop, M.; Bielinska, K.; Zaorska, E.; Samborowska, E.; Wyczalkowska-Tomasik, A.; Pączek, L.; Dadlez, M.; et al. Chronic, Low-Dose TMAO Treatment Reduces Diastolic Dysfunction and Heart Fibrosis in Hypertensive Rats. *Am J Physiol Heart Circ Physiol* **2018**, *315*, H1805–H1820, doi:10.1152/AJPHEART.00536.2018.
13. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kaplon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins (Basel)* **2019**, *11*, doi:10.3390/TOXINS11090490.
14. Jaworska, K.; Bielinska, K.; Gawrys-Kopczynska, M.; Ufnal, M. TMA (Trimethylamine), but Not Its Oxide TMAO (Trimethylamine-Oxide), Exerts Haemodynamic Effects: Implications for Interpretation of Cardiovascular Actions of Gut Microbiome. *Cardiovasc Res* **2019**, *115*, 1948–1949, doi:10.1093/cvr/cvz231.
15. Jalandra, R.; Makharia, G.K.; Sharma, M.; Kumar, A. Inflammatory and Deleterious Role of Gut Microbiota-Derived Trimethylamine on Colon Cells. *Front Immunol* **2023**, *13*, 8161, doi:10.3389/FIMMU.2022.1101429/BIBTEX.
16. Ashar, F.N.; Zhang, Y.; Longchamps, R.J.; Lane, J.; Moes, A.; Grove, M.L.; Mychaleckyj, J.C.; Taylor, K.D.; Coresh, J.; Rotter, J.I.; et al. Association of Mitochondrial DNA Copy Number With Cardiovascular Disease. *JAMA Cardiol* **2017**, *2*, 1247–1255, doi:10.1001/JAMACARDIO.2017.3683.
17. Malik, A.N.; Czajka, A. Is Mitochondrial DNA Content a Potential Biomarker of Mitochondrial Dysfunction? *Mitochondrion* **2013**, *13*, 481–492, doi:10.1016/J.MITO.2012.10.011.
18. Bordoni, L.; Petracci, I.; Pelikant-Malecka, I.; Radulska, A.; Piangerelli, M.; Samulak, J.J.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Mitochondrial DNA Copy Number and Trimethylamine Levels in the Blood: New Insights on Cardiovascular Disease Biomarkers. *FASEB J* **2021**, *35*, e21694, doi:10.1096/fj.202100056R.
19. Matacchione, G.; Perugini, J.; Di Mercurio, E.; Sabbatinelli, J.; Prattichizzo, F.; Senzacqua, M.; Storci, G.; Dani, C.; Lezoche, G.; Guerrieri, M.; et al. Senescent Macrophages in the Human Adipose Tissue as a Source of Inflammaging. *Geroscience* **2022**, *44*, 1941–1960, doi:10.1007/S11357-022-00536-0.
20. Fazzini, F.; Schöpf, B.; Blatzer, M.; Coassin, S.; Hicks, A.A.; Kronenberg, F.; Fendt, L. Plasmid-Normalized Quantification of Relative Mitochondrial DNA Copy Number. *Scientific Reports 2018 8:1* **2018**, *8*, 1–11, doi:10.1038/s41598-018-33684-5.
21. Kühn, T.; Rohrmann, S.; Sookthai, D.; Johnson, T.; Katzke, V.; Kaaks, R.; Von Eckardstein, A.; Müller, D. Intra-Individual Variation of Plasma Trimethylamine-N-Oxide (TMAO), Betaine and Choline over 1 Year. *Clin Chem Lab Med* **2017**, *55*, 261–268, doi:10.1515/CCLM-2016-0374.
22. Tang, W.H.W.; Wang, Z.; Fan, Y.; Levison, B.; Hazen, J.E.; Donahue, L.M.; Wu, Y.; Hazen, S.L. Prognostic Value of Elevated Levels of Intestinal Microbe-Generated Metabolite Trimethylamine-N-Oxide in Patients with Heart Failure: Refining the Gut Hypothesis. *J Am Coll Cardiol* **2014**, *64*, 1908–1914, doi:10.1016/J.JACC.2014.02.617.
23. Ge, X.; Zheng, L.; Zhuang, R.; Yu, P.; Xu, Z.; Liu, G.; Xi, X.; Zhou, X.; Fan, H. The Gut Microbial Metabolite Trimethylamine N-Oxide and Hypertension Risk: A Systematic Review and Dose–Response Meta-Analysis. *Advances in Nutrition* **2020**, *11*, 66, doi:10.1093/ADVANCES/NMZ064.
24. Bordoni, L.; Samulak, J.J.; Sawicka, A.K.; Pelikant-Malecka, I.; Radulska, A.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Trimethylamine N-Oxide and the Reverse Cholesterol Transport in Cardiovascular Disease: A Cross-Sectional Study. *Sci Rep* **2020**, *10*, 18675, doi:10.1038/s41598-020-75633-1.
25. Ufnal, M.; Zadlo, A.; Ostaszewski, R. TMAO: A Small Molecule of Great Expectations. *Nutrition* **2015**, *31*, 1317–1323, doi:10.1016/J.NUT.2015.05.006.
26. Restini, C.B.A.; Fink, G.D.; Watts, S.W. Vascular Reactivity Stimulated by TMA and TMAO: Are Perivascular Adipose Tissue and Endothelium Involved? *Pharmacol Res* **2021**, *163*, 105273, doi:10.1016/J.PHRS.2020.105273.

27. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kapłon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins (Basel)* **2019**, *11*, doi:10.3390/toxins11090490.
28. Cavusoglu, E.; Marmur, J.D.; Yanamadala, S.; Chopra, V.; Hegde, S.; Nazli, A.; Singh, K.P.; Zhang, M.; Eng, C. Elevated Baseline Plasma IL-8 Levels Are an Independent Predictor of Long-Term All-Cause Mortality in Patients with Acute Coronary Syndrome. *Atherosclerosis* **2015**, *242*, 589–594, doi:10.1016/J.ATHEROSCLEROSIS.2015.08.022.
29. Velásquez, I.M.; Frumento, P.; Johansson, K.; Berglund, A.; De Faire, U.; Leander, K.; Gigante, B. Association of Interleukin 8 with Myocardial Infarction: Results from the Stockholm Heart Epidemiology Program. *Int J Cardiol* **2014**, *172*, 173–178, doi:10.1016/J.IJCARD.2013.12.170.
30. Boekholdt, S.M.; Peters, R.J.G.; Hack, C.E.; Day, N.E.; Luben, R.; Bingham, S.A.; Wareham, N.J.; Reitsma, P.H.; Khaw, K.T. IL-8 Plasma Concentrations and the Risk of Future Coronary Artery Disease in Apparently Healthy Men and Women: The EPIC-Norfolk Prospective Population Study. *Arterioscler Thromb Vasc Biol* **2004**, *24*, 1503–1508, doi:10.1161/01.ATV.0000134294.54422.2E.
31. Zhao, X.; Zhang, W.; Xing, D.; Li, P.; Fu, J.; Gong, K.; Hage, F.G.; Oparil, S.; Chen, Y.F. Endothelial Cells Overexpressing IL-8 Receptor Reduce Cardiac Remodeling and Dysfunction Following Myocardial Infarction. *Am J Physiol Heart Circ Physiol* **2013**, *305*, doi:10.1152/AJPHEART.00571.2012.
32. Frangogiannis, N.G. Chemokines in the Ischemic Myocardium: From Inflammation to Fibrosis. *Inflamm Res* **2004**, *53*, 585–595, doi:10.1007/S00011-004-1298-5.
33. Waal Malefyt, R. De; Haanen, J.; Spits, H.; Koncarolo, M.G.; Te Velde, A.; Figdor, C.; Johnson, K.; Kastelein, R.; Yssel, H.; De Vries, J.E. Interleukin 10 (IL-10) and Viral IL-10 Strongly Reduce Antigen-Specific Human T Cell Proliferation by Diminishing the Antigen-Presenting Capacity of Monocytes via Downregulation of Class II Major Histocompatibility Complex Expression. *J Exp Med* **1991**, *174*, 915–924, doi:10.1084/JEM.174.4.915.
34. Koppelman, B.; Neefjes, J.J.; De Vries, J.E.; De Waal Malefyt, R. Interleukin-10 down-Regulates MHC Class II Alphabeta Peptide Complexes at the Plasma Membrane of Monocytes by Affecting Arrival and Recycling. *Immunity* **1997**, *7*, 861–871, doi:10.1016/S1074-7613(00)80404-5.
35. Makrecka-Kuka, M.; Volska, K.; Antone, U.; Vilskersts, R.; Grinberga, S.; Bandere, D.; Liepinsh, E.; Dambrova, M. Trimethylamine N-Oxide Impairs Pyruvate and Fatty Acid Oxidation in Cardiac Mitochondria. *Toxicol Lett* **2017**, *267*, 32–38, doi:10.1016/J.TOXLET.2016.12.017.

General Conclusion

TMA and TMAO are among the diet-derived metabolites that have been labelled as potentially harmful for human health. Considering that their dietary precursors are highly abundant in animal-based food, westernized dietary patterns are important sources of these two metabolites. Moreover, the existence of a causal link between the Western diet and the increased prevalence of non-communicable chronic diseases is undiscussed and TMA and TMAO have been suggested to have a role in this association. It has been hypothesized that the impact of TMA and TMAO on human health might result from their effect on mitochondrial dynamics. And this work perfectly fits into this context, with the goal to shed new light on this interesting yet complex topic. In agreement with the evidence gathered so far, which suggests that low TMA levels and high TMAO/TMA ratio may constitute a risk factor for complex multifactorial pathologies, the western dietary pattern was associated with lower circulating levels of TMA, and a higher TMAO/TMA ratio in our study. This result also emphasizes how the measurement of the TMAO/TMA ratio may be more informative than of TMAO alone when looking for risk factors for complex pathologies. Moreover, our study revealed that the link between diet and mitochondrial dynamics might be mediated by TMAO metabolism. Indeed, the TMAO/TMA ratio, which was increased by a western dietary pattern, was also directly associated with mtDNAcn, which can be regarded as a compensatory mechanism to the impairment of energy metabolism by high circulating TMAO levels. Moreover, despite the existence and the role of mtDNA methylation still being controversial topics, our study described for the first time an inverse correlation between the TMAO/TMA ratio and mitochondrial D-Loop methylation, thus confirming that changes in mitochondrial D-loop methylation may occur in response to dietary habits and might mediate the effect of diet on health and disease.

When considering the boundaries between health and disease, also aging has a crucial role, since it is a driving factor of various age-related conditions, including neurodegenerative diseases, cardiovascular disease, and cancer. Diet (quality, abundance of certain nutrients and by-products), together with other environmental exposures (such as physical activity and smoking) can accelerate or decelerate the aging process through changes in the DNA methylation pattern. Interestingly, from our study it emerged that even when following healthy dietary habits, elevated TMA levels in blood (but not elevated TMAO) may increase the epigenetic age, which is a proxy for biological age and reflects the decline in biological functions that accompany age-related diseases. At this point of our research, the idea that TMA might be more awkward than TMAO in relation to human health, started taking shape. Indeed, TMA is a well-known uremic toxin and recent evidence suggests that it may exert its deleterious effects by boosting and exacerbating inflammation. On this regard, since colonic epithelial cells are the first target of TMA's effects (TMA is mainly produced in the colon), the colon adenocarcinoma Caco-2 cell line was used to elucidate the impact of excessive intestinal TMA production on intestinal epithelial cells, focusing on inflammatory signals, mitochondrial dynamics, and epigenetic alterations. Results confirmed the hypothesis that TMA is far from being

a harmless metabolite and it may have a contributing role in microbiota-induced intestinal diseases via the activation of pro-inflammatory signals, and the perturbation of epigenetic machinery. Furthermore, high TMA levels may disrupt mitochondrial dynamics in intestinal cells, as shown by the altered D-loop methylation, the reduction of the mtDNAcn, the downregulation of some components of the respiratory chain and the decreased level of intracellular ATP. Moreover, considering that the alteration of mitochondrial dynamics has been identified as a risk factor for cardiovascular disease development, and that TMA (and not TMAO) has been recently suggested to be implicated in CVD due to its cytotoxic effects on cardiomyocytes and vascular smooth muscle cells, we investigated the role TMA and TMAO in people suffering from CVD. Our results revealed that people suffering from CVD may experience alterations in TMAO metabolism, and a reduction of mitochondrial functionality, as indicated by the decrease in mtDNA content. Moreover, the blood TMA levels were directly correlated to mtDNAcn, suggesting that the impairment of mitochondrial activity might be mediated by low levels of circulating TMA, which reinforces the existence of a link between mitochondrial dynamics and TMAO metabolism. Lastly, our study on THP-1 monocytes revealed that both TMA and TMAO were able to trigger inflammatory pathways in macrophages. However, TMA (more than TMAO) disturbed cellular homeostasis by altering mitochondrial dynamics. Thus, based on our findings TMA might be more detrimental than its oxidized product (TMAO) in the context of vascular inflammation and for this reason it might have a contributing role in CVD, although the exact mechanisms remain to be fully elucidated.

List of Publications

1. Petracci, I.; Gabbianelli, R.; Bordoni, L. The Role of Nutri(Epi)Genomics in Achieving the Body's Full Potential in Physical Activity. *Antioxidants (Basel)* **2020**, *9*, 1–33, doi:10.3390/ANTIOX9060498.
2. Bordoni, L.; Petracci, I.; Calleja-Agius, J.; Lalor, J.G.; Gabbianelli, R. NURR1 Alterations in Perinatal Stress: A First Step towards Late-Onset Diseases? A Narrative Review. *Biomedicines* **2020**, *8*, 1–16, doi:10.3390/BIOMEDICINES8120584.
3. Bordoni, L.; Petracci, I.; Zhao, F.; Min, W.; Pierella, E.; Assmann, T.S.; Martinez, J.A.; Gabbianelli, R. Nutrigenomics of Dietary Lipids. *Antioxidants (Basel)* **2021**, *10*, doi:10.3390/ANTIOX10070994.
4. Bordoni, L.; Petracci, I.; Pelikant-Malecka, I.; Radulska, A.; Piangerelli, M.; Samulak, J.J.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Mitochondrial DNA Copy Number and Trimethylamine Levels in the Blood: New Insights on Cardiovascular Disease Biomarkers. *FASEB J* **2021**, *35*, doi:10.1096/FJ.202100056R.
5. Bordoni, L.; Petracci, I.; Mlodzik-Czyzewska, M.; Malinowska, A.M.; Szwengiel, A.; Sadowski, M.; Gabbianelli, R.; Chmurzynska, A. Mitochondrial DNA and Epigenetics: Investigating Interactions with the One-Carbon Metabolism in Obesity. *Oxid Med Cell Longev* **2022**, *2022*, doi:10.1155/2022/9171684.
6. Bordoni, L.; Malinowska, A.M.; Petracci, I.; Szwengiel, A.; Gabbianelli, R.; Chmurzynska, A. Diet, Trimethylamine Metabolism, and Mitochondrial DNA: An Observational Study. *Mol Nutr Food Res* **2022**, *66*, doi:10.1002/MNFR.202200003.
7. Bordoni, L.; Perugini, J.; Petracci, I.; Di Mercurio, E.; Lezoche, G.; Guerrieri, M.; Giordano, A.; Gabbianelli, R. Mitochondrial DNA in Visceral Adipose Tissue in Severe Obesity: From Copy Number to D-Loop Methylation. *Front Biosci (Landmark Ed)* **2022**, *27*, doi:10.31083/J.FBL2706172.
8. Zhao, F.; Liu, C.; Bordoni, L.; Petracci, I.; Wu, D.; Fang, L.; Wang, J.; Wang, X.; Gabbianelli, R.; Min, W. Advances on the Antioxidant Peptides from Nuts: A Narrow Review. *Antioxidants (Basel)* **2022**, *11*, doi:10.3390/ANTIOX11102020.
9. Bordoni, L.; Malinowska, A.M.; Petracci, I.; Chmurzynska, A.; Gabbianelli, R. Biological Age and Diet: Measuring the Impact of Lifestyle on a 6CpG-Epigenetic Clock. *Nutr Healthy Aging* **2022**, *7*, 121–134, doi:10.3233/NHA-220160.
10. Petracci, I.; Fedeli, D.; Gabbianelli, R.; Bordoni, L. MiR-21, MiR-148, Fatty Acid Content, and Antioxidant Properties of Raw Cow's Milk: A Pilot Study. *ACS Food Science and Technology* **2023**, *2023*, 908, doi:10.1021/ACSFOODSCITECH.3C00072/ASSET/IMAGES/LARGE/FS3C00072_0008.JPEG.