Universitat Autònoma de Barcelona

TRIMETHYLAMINE-NOXIDE (TMAO) AND HDL DYSFUNCTION IN PATIENTS AT HIGH CARDIOVASCULAR RISK

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TRIMETHYLAMINE-N-OXIDE (TMAO) AND HDL DYSFUNCTION IN PATIENTS AT HIGH CARDIOVASCULAR RISK

Doctoral Program in Biochemistry, Molecular Biology and Biomedicine
Department of Biochemistry and Molecular Biology
Universitat Autònoma de Barcelona
Institut de Recerca de l'Hospital de la Santa Creu i Sant Pau

Directors

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A la meva padrina,

sé que estaries molt orgullosa de mi

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ABBREVIATIONS

AAA: abdominal aortic aneurysm

ABCA1: ATP binding cassette subfamily A member 1

ABCG1: ATP binding cassette subfamily G member 1

ACS: acute coronary syndrome

ANCOVA: analysis of covariance

ANOVA: analysis of variance

APC-PCI: activated protein C - protein C inhibitor

APO: apolipoprotein

ASCVD: atherosclerotic cardiovascular disease

A1AT: alpha 1-antitrypsin

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CAD: coronary artery disease

CETP: cholesteryl ester transfer protein

CK: creatine kinase

CKD: chronic kidney disease

CRP: C-reactive protein

cTn: cardiac troponin

CVD: cardiovascular disease

DMEM: Dulbecco's modified eagle's medium

ECG: electrocardiogram

eGFR: estimated glomerular filtration rate

FBS: fetal bovine serum

FMO3: flavin-containing monooxygenase-3

FMT: fecal microbiota transplantation

γBB: gamma-butyrobetaine

GRACE: global registry of acute coronary events

HDL: high density lipoprotein

hsCRP: high-sensitivity C-reactive protein

hs-cTnT: high-sensitive cardiac troponin T

IDL: intermediate-density lipoprotein

IFN-γ: interferon gamma

LCAT: lecithin–cholesterol acyltransferase

LC-MS: liquid chromatography mass spectrum

LDL: low density lipoprotein

LDLR: low-density lipoprotein cholesterol receptor

MACE: major adverse cardiovascular events

MCE: macrophage cholesterol efflux

MI: myocardial infarction

MIF: migration inhibitory factor

MMP: matrix metalloproteinase

NSTEMI: non-ST-elevation myocardial infarction

OCT: organic cation transporter

PAPs: plasmin-antiplasmin complexes

PBS: phosphate-buffered saline

PCI: percutaneous coronary intervention

PCSK9: pro-protein convertase subtilisin/kexin type 9

PIIINP: aminoterminal propeptide of type III procollagen

PLTP: phospholipid transfer protein

PON1: paraoxonase 1

P/S: penicillin/streptomycin

RCT: reverse cholesterol transport

ROS: reactive oxygen species

SNP: single nucleotide polymorphism

SR-BI: scavenger receptor class B type I

STEMI: ST segment elevation myocardial infarction

TIMI: Thrombolysis in Myocardial Ischemia

TIMP: tissue inhibitor of metalloproteinase

TG: triglyceride

TMA: trimethylamine

TMAO: trimethylamine N-oxide

TML: trimethyllysine

TNF: tumor necrosis factor

tPA: tissue plasminogen activator

UA: unstable angina

VIVA: Viborg Vascular

VLDL: very low density lipoprotein

VSMC: vascular smooth muscular cell

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ABSTRACT

High density lipoprotein (HDL) cholesterol (HDL-C) levels are usually inversely correlated with cardiovascular disease (CVD). However, treatments that raised HDL-C levels failed to prevent cardiovascular risk and the focus moved to the study of HDL functions rather than HDL-C itself. Their main cardioprotective function is HDL-mediated macrophage cholesterol efflux (MCE). Gut-related metabolites, trimethylamine N-oxide (TMAO), trimethyllysine (TML) and gamma-butyrobetaine (γBB) have been directly linked to CVD.

Our first objective was to evaluate HDL-mediated MCE in abdominal aortic aneurysm (AAA) patients and its capacity to predict AAA progression and time-to-surgery. HDL-mediated MCE was impaired in AAA patients compared with the control group. However, no significant differences in HDL-mediated MCE capacity were observed in 3 different progression subgroups in patients with small/medium size AAA. Moreover, HDL-mediated MCE capacity was not correlated with the progression of AAA.

The second objective was to assess the associations among HDL-mediated MCE and the microbial-derived metabolites TMAO, γBB, and TML at baseline with cardiovascular mortality in patients with an acute ST-elevation myocardial infarction (STEMI), testing whether these gut microbial-derived metabolites modulate the associations between HDL-mediated MCE and mortality.

HDL-mediated MCE decreased in patients with STEMI, and was further impaired in those who died during follow-up. Moreover, the circulating concentrations of TMAO, γBB, and TML were higher in the deceased STEMI patients when compared with the STEMI survivors or unstable angina (UA) patients. However, after statistical adjustment, only the ATP binding cassette subfamily A member 1 and subfamily G member 1 (ABCA1/G1)-mediated macrophage cholesterol efflux remained significantly associated with mortality. Furthermore, neither the

TMAO, γBB, nor TML levels altered the HDL-mediated macrophage cholesterol efflux *in vitro*.

RESUM

Els nivells de colesterol de les lipoproteïnes d'alta densitat (HDL) es correlacionen de manera inversa amb la malaltia cardiovascular. No obstant això, els tractaments que augmenten els nivells de colesterol associat a les HDL no han aconseguit prevenir el risc cardiovascular i el focus d'atenció s'ha traslladat a les funcions de les HDL més que al colesterol de HDL. La principal funció cardioprotectora es l'eflux de colesterol des de macròfags a través de les HDL. Els metabòlits relacionats amb la microbiota, el N-òxid de trimetilamina (TMAO), la trimetilisina (TML) i la gamma-butirobetaina (γBB) s'han relacionat de manera directa amb la malaltia cardiovascular.

El nostre primer objectiu va ser determinar l'eflux de colesterol des de macròfags en pacients amb aneurisma d'aorta abdominal (AAA) i avaluar la seva capacitat per a predir la progressió de l'AAA i la necessitat de cirurgia. L'eflux de colesterol des de macròfags es trobava disminuït en els pacients amb AAA en comparació amb el grup control. No obstant això, no es van observar diferències significatives en l'eflux de colesterol des de macròfags en els 3 subgrups diferents de progressió en els pacients amb AAA de grandària petita o mitjana. A més, l' eflux de colesterol des de macròfags no es va correlacionar amb la progressió del AAA.

El segon objectiu va ser avaluar les associacions entre l'eflux de colesterol des de macròfags i els metabòlits derivats de la microbiota TMAO, γBB i TML amb la mortalitat cardiovascular en pacients amb un infart de miocardi amb elevació del ST (IAMCEST), provant d'esbrinar si aquests metabòlits derivats de la microbiota intestinal modulen les associacions entre l'eflux de colesterol des de macròfags i la mortalitat

L'eflux de colesterol des de macròfags es trobava disminuït en els pacients amb IAMCEST, i encara més disminuït en els IAMCEST que morien durant els 2 anys de seguiment. A més, les concentracions de TMAO, γBB i TML es trobaven augmentades en els pacients amb IAMCEST morts en comparació amb els

supervivents de l'IAMCEST o els pacients amb angina inestable. No obstant això, després de l'ajust estadístic, només l'eflux de colesterol dels macròfags mediat pels transportadors ABC (de l'anglès ATP-binding cassette) membre 1 i subfamília G membre 1 va continuar estant significativament associat a la mortalitat. A més, els nivells de TMAO, γBB o TML no van alterar l'eflux de colesterol des de macròfags mediat per les HDL *in vitro*.

~ INTRODUCTION ~

1. Abdominal aortic aneurysm

1.1. Diagnosis and epidemiology

Aneurysm, from the Ancient Greek word ἀνεύ́ρυσμα, is defined as a progressive dilatation of an artery. The most frequent dilatation occurs in the infrarenal abdominal aorta, known as abdominal aortic aneurysm (AAA). This is an irreversible and permanent dilatation of the infrarenal abdominal aorta diameter greater than 30 mm, corresponding usually, in men, to more than two standard deviations above the mean diameter. AAA is also diagnosed if the maximum diameter is ≥50% greater than the suprarenal diameter [1,2]. Measuring is performed using both abdominal ultrasound and duplex ultrasonography as imaging techniques [3]. Computed tomography angiography is also used, mainly for the diagnosis of rupture and the follow up after repair [4]. Studies showed that the diagnosis using imaging techniques can reach a sensitivity of 67% and a specificity of 97% for predicting the need for AAA repair within 10 years [5].

AAA affects more men than women, to a ratio of 5:1 [6]. Based on population screening and large-scale randomized controlled trials performed in Europe, AAA prevalence ranges from 4 to 8% in men and 0 to 2% in women [7,8].

Different countries have studied the cost effectiveness of screening the population for AAA diagnosis. In Denmark, an important reduction of mortality risk from AAA, peripheral arterial disease, and hypertension concomitant to the screening was observed. Remarkably, this reduction had never been reported in the literature and might be linked primarily to the initiation of pharmacological therapy. The main conclusion was that countries should consider the implementation of a combined screening program if no screening or isolated AAA screening was offered [9]. Conversely, a similar AAA screening program for women was considered unlikely to be cost-effective in the United Kingdom health system. The study concluded that thresholds established for men could not be applied to

women, and that more studies on aortic diameter distribution in women and on potential quality of life gain associated with screening were needed [10].

AAA is generally asymptomatic, but progressive aneurismal dilation is associated with eventual severe consequences of aortic rupture. Symptoms of an intact AAA are unspecific, including pain or tenderness on palpation. These symptoms are usually related to clinical complications. For rupture, the signs include hemodynamic collapse, pallor, and abdominal and/or back pain, among others [11].

1.2. Physiopathology

The physiopathology of AAA is a complex and multifactorial process. Histologically, AAA is characterized by alterations in the connective tissue, mainly due to high proteolytic activity produced by both infiltrating and resident cells, leading to a decreased amount of elastin [12]. A huge range of different cell types have an implication on the pathology, including vascular smooth muscular cells (VSMCs), endothelial cells, neutrophils, monocyte/macrophages, lymphocytes, adipocytes, mast cells and platelets.

Moreover, there are a vast number of molecular components acting as AAA inducers: matrix metalloproteinases (MMPs), angiotensin II, mineralocorticoids, NADPH oxidase, myeloperoxidase, endothelial nitric oxide synthase, O_2^-, H_2O_2 and HOCl. [13].

Among all the cell types, probably the most relevant are VSMCs, since apoptosis of VSMCs and aorta media degeneration are the main features of AAA. This apoptosis is associated with inflammation, reactive oxygen species (ROS) production and endoplasmic reticulum stress [14].

Secondly, due to an imbalance between the amount of active MMPs and endogenous tissue inhibitors of metalloproteinases (TIMPs), the amount of elastin, collagen, and glycosaminoglycans are decreased in AAA respect to normal aortas. MMPs are produced by endothelial cells, VSMCs, and adventitial

fibroblasts. Furthermore, inflammatory cells can also be a source of MMPs in the case of AAA [15].

Increased MMP-1 (collagenase-1) expression has been described in human AAA, with a concomitant decrease of the MMP-1 inhibitor levels [16]. MMP-13 (collagenase-3) expression is also higher in human AAA, especially in those with symptoms and at high risk of rupture [17]. MMP-3 (stromelysin-1) expression is elevated in AAAs, mainly due to an increased production by macrophages [18]. Polymorphisms in *MMP2*, *MMP3* and *MMP-13* genes are an independent risk factor for AAA formation [19]. MMP-12 (metalloesterase), secreted and produced mainly by macrophages, may not be directly implicated in the AAA pathogenesis, but can facilitate the actions of other MMPs in extracellular matrix degradation [20]. *MMP-9* (gelatinase B) gene expression is associated with expression of genes involved in inflammation and cholesterol metabolism [21] and is produced by fibroblasts, VSMCs and by infiltrating adventitial macrophages during AAA formation [22]. MMP-14 is also associated with AAA in human and murine models [23].

Another molecule implicated in the pathology of AAA is angiotensin II. However, its contribution is less clear. Increased angiotensin II might induce AAA to a minor extent and, therefore, other factors such as hypercholesterolemia would be needed [24].

Inflammation is a key step in AAA formation, and this involves neutrophils, macrophages, B and T cells. Neutrophil infiltration is one of the first processes in AAA formation. Neutrophil extracellular traps could be a crucial component of continued and progressive inflammation in AAA [25]. Macrophages have a dual role in AAA, inducing the pathology through macrophage infiltration and production of MMPs, cytokines and chemokines, but also forming part of the repair response [26]. Both T cells and B lymphocytes are abundant in aneurysmal tissues. B-cell depletion was associated with protection against AAA formation, but the different B-cell subtypes (B1 and B2) might play different and even opposing roles in the

disease [27,28]. Also, a T-cell depletion attenuates AAA formation, and different subtypes of T cells such as regulatory T cells, CD4+ Th1 and Th2 contribute to AAA pathogenesis [29,30].

ROS play a key role in AAA development by activating MMPs and inducing proinflammatory genes and apoptosis. NADPH oxidase expression, a major source of ROS, is increased in AAA [31]. However, there are other sources of ROS such as neutrophils [32].

1.3. AAA progression and biomarkers

Mechanisms implicated in AAA progression mainly act through extracellular matrix turnover and inflammation. Aortic lumen shows intraluminal thrombus formation and inflammatory cell adhesion and infiltration, which promote apoptosis of VSMCs, elastin degradation and collagen deposition, thus changing the vessel wall structure.

Several different definitions of AAA progression have been used, including expansion rate, rupture, and symptoms. The parameter most widely used to study AAA progression is the expansion rate or growth rate, since the aortic diameter is determinant in deciding whether a patient will be treated with surgery or not. In men, the threshold for considering elective abdominal aortic aneurysm repair is ≥5.5 cm diameter [33]. Ultrasonography is recommended for small aneurysm follow-up; every three years for aneurysms between 3 and 3.9 cm, annually for aneurysms between 4.0 and 4.9 cm, and every 3 - 6 months for aneurysms greater than 5 cm [34].

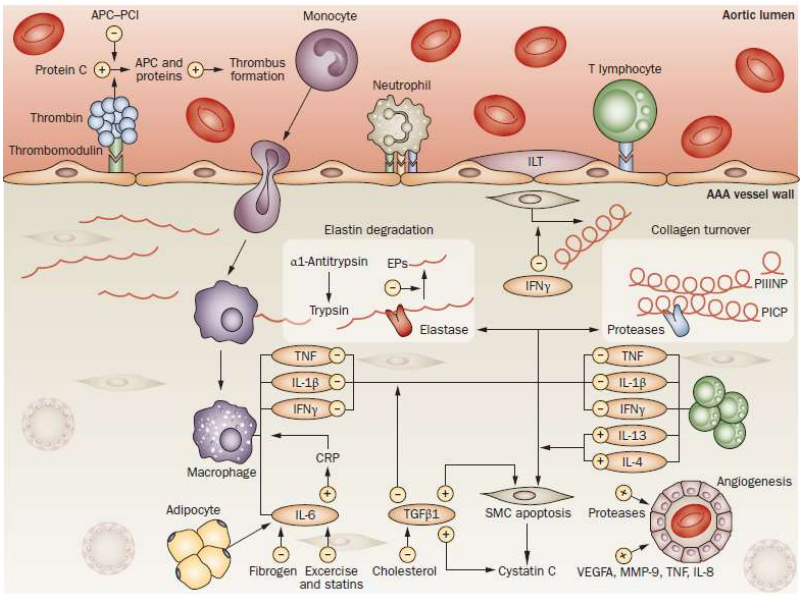

Figure 1. Mechanisms implicated in abdominal aortic aneurysm progression: extracellular matrix turnover and inflammation. Extracted from Hellenthal FA, Buurman WA, Wodzig WK, Schurink GW. Biomarkers of abdominal aortic aneurysm progression. Part 2: inflammation. Nat Rev Cardiol. 2009 Aug;6(8):543-52. doi: 10.1038/nrcardio.2009.102. Epub 2009 Jun 23. PMID: 19546866.

Mortality associated with ruptured AAA is approximately 90% [35]. Identification of patients at high risk of rapid AAA expansion and rupture is thus essential for providing targeted therapy. In that sense, AAA size has been considered the gold standard for clinical prediction of rupture risk. However, a huge effort is being made to find a circulating biomarkers that could be used as an AAA progression predictor [36].

An eligible biomarker should have a causal relationship with the disease, be involved in the pathophysiological pathways, echo the severity and progression of the disease, and should be measurable in a clinical practice context. When referring to AAA, two special subtypes of biomarkers should be specially considered: constituents and active enzymes of the extracellular matrix and circulating biomarkers of inflammation.

1.3.1. Extracellular matrix biomarker

Elastin degradation is strongly implicated in AAA progression. A positive correlation between serological elastin peptides and AAA progression has been reported by some studies [37–39]. Elastase is the main enzyme implicated in elastin degradation. Trypsin is an elastase inhibitor and α1-antitrypsin (A1AT) is a trypsin inhibitor. Some investigators have studied the role of A1AT in predicting the AAA progression [40]; however, the same group reported both a positive association and no relationship with risk of AAA in two different publications [41].

Concomitantly, increased collagen degradation combined with inadequate collagen deposition was also associated with AAA progression and rupture. Accordingly, products of collagen catabolism are potential biomarkers of AAA progression. Increased aminoterminal propeptide of type III procollagen (PIIINP) levels have been found in AAA patients in parallel with an increased AAA diameter [42]. A positive correlation between PIIINP and expansion rate has also been reported [39].

As mentioned above, an imbalance between MMPs and TIMPs causes an aggravation in the dilatation of human abdominal aorta [16]. However, among all the MMPs and TIMPs, only MMP-9 plasma levels were found to be associated with AAA size and expansion [37]. Other authors did not find any correlation between plasma levels of MMP-9 and annual AAA expansion [43].

Cystatin C is one of the most important extracellular inhibitors of cysteine proteases, such as cathepsin K, cathepsin S and caspases. Cathepsin K has elastase and collagenolytic activities, whereas cathepsin S acts similarly to MMPs. There is only one study of cystatin C as a biomarker of AAA progression and the main finding was a poor-to-moderate negative correlation between cystatin C levels and annual AAA expansion rate [44].

Serine proteases and the fibrinolytic system are also implicated in AAA pathogenesis, hence a positive correlation between circulating levels of tissue plasminogen activator (tPA) and AAA has been reported [45]. Both plasmin-antiplasmin complexes (PAPs) and tPA seem to have a role in AAA progression. Plasma levels of PAPs, combined with initial AAA size, are able to predict the expansion of small AAA to operable sizes, and correlate with AAA expansion rate [38].

Activated protein C and its inhibitor, protein C inhibitor, form the complex APC-PCI, which is a sensitive indicator of thrombin formation and a representative measure of coagulation activation. It has been reported that patients with AAA have a three-fold higher median APC-PCI level than control patients. AAA patients with low levels of APC-PCI do not have a thrombus-lined aneurysm [46]. APC-PCI levels have been associated with AAA diameter but not with the growth rate [47].

1.3.2. Circulating biomarkers of inflammation

A variety of cytokines, including tumor necrosis factor (TNF), interferon γ (IFN-γ) and transforming growth factor 1β have been proposed as progression biomarkers. However, only IFN-γ levels at the time of AAA diagnosis correlate with an increased aneurysm expansion rate. The results of the study seemed to be influenced by a wide range of initial maximal aortic diameter, a short follow up, and a high average expansion rate [48].

Migration inhibitory factor (MIF) is a pleiotropic cytokine released from macrophages, T cells and the pituitary gland during inflammatory responses. In addition to its well-known involvement in inflammation, new MIF biological functions have emerged. Circulating levels of MIF positively correlate with initial AAA size; furthermore, a moderate positive correlation between MIF and annual AAA expansion rate was established [49].

Osteoprotegerin is a member of the TNF receptor superfamily and also acts as an osteoclastogenesis inhibitory factor. There is a positive correlation between serum osteoprotegerin levels and AAA expansion rate [50].

C-reactive protein (CRP) was associated with symptomatic and ruptured AAAs, while asymptomatic AAA was not associated with an increase in CRP levels in a large case-control study [51]. However, another study found that patients with other inflammatory conditions and asymptomatic AAA also presented high CRP levels [52]. AAA size was associated with log-transformed serum levels of high-sensitivity CRP (hsCRP) [53]. However, no significant correlation was observed between hsCRP and AAA expansion rate. Therefore, inflammation might be a response to expansion rather than the primary cause of the disease [54].

Cotinine is a nicotine metabolite, which was found in abundance in the plasma of cigarette smokers [55]. A 3-year study of 43 patients found that the growth rate of small aneurysms was faster in smokers than in nonsmokers [56]. However, a poor correlation was found between serum levels of cotinine and AAA progression [41]. Homocysteine, a sulfur-containing amino acid formed during metabolism of the essential amino acid methionine, has been linked to AAA pathophysiology in *in vitro* studies. These studies indicated that homocysteine induced electrolysis in the arterial media during aneurysm formation through MMP-2 activation [57]. A weak but significant linear correlation was found between aortic diameter and homocysteine levels [45]. Other studies, however, did not find any correlation between AAA progression and homocysteine [58].

Osteopontin, produced by endothelial cells, VSMC, macrophages and other cell types was associated with AAA symptoms and annual growth rate but not with AAA size [59].

1.4. Risk factors and treatment

Age, male sex, family history, smoking status, hypertension and coronary and peripheral arterial disease are the strongest risk factors associated with AAA [60]. Therefore, factors such as age and gender, for example, are considered in AAA follow up, on top of aorta diameter [61].

The incidence of AAA in the general population increases with age. Deaths by aneurysm rupture rarely occur in patients below the age of 65. The risk increases by 40% every five years [62]. This risk is higher in men than in women, in whom the disease appears on average ten years later. Hormonal and genetic factors, added to exposure to different risk factors, have been proposed as a possible explanation for the gender gap [63].

AAA would appear to have a genetic component, since an increase in its prevalence can be observed in family members of AAA patients. A positive first-degree family history of AAA increases a person's risk by up to 10 times, and at younger ages [64]. Smoking increases AAA relative risk 7.6 times, and in smokers of more than 25 cigarettes/day, this risk raises to 15 times compared to the risk of non-smokers [65]. The number of years a person has smoked is more important than the number or cigarettes, and every year of smoking enhances AAA relative risk by 4% [66].

Surprisingly, diabetes mellitus seems to exert a protective role against AAA development [67]. The mechanisms are unknown but it has been speculated that an increase in the synthesis of extracellular matrix and the proliferation of VSMCs with a concomitant suppression of MMPs activity may be, at least in part, the reason [68].

Current treatment is based on the correct control of risk factors through the use of antiplatelet drugs and statins. Recent studies have shown that the use of statins and low-dose aspirin were associated with lower AAA growth rates and decreased progression [69,70]. However, major randomized double-blind trials are still scarce,

and no official recommendation regarding medical treatment exists in current clinical guidelines [11,71]. Different antihypertensive drugs have been used in clinical trials and observation studies, including beta blockers, angiotensin converter enzyme inhibitors and calcium channels blockers, but no conclusive results have been obtained; therefore, they are not used in everyday clinical practice [60].

2. Acute coronary syndrome

2.1. Diagnosis and epidemiology

Acute coronary syndrome (ACS) describes a compendium of conditions associated with a sudden reduction in blood flow to the heart. Commonly, this is a consequence of progressive vascular disease, affecting one or more major branches of the coronary arteries [72]. ACS encompasses acute myocardial infarction and unstable angina (UA). According to the 4th universal definition of myocardial infarction (MI), the clinical definition of MI denotes the presence of acute myocardial injury detected by abnormal quite specific cardiac biomarkers in the setting of an adequate clinical context. Cardiac troponins (cTn) value above the 99th percentile is then defined as myocardial injury and if there is a rise and/or fall of cTn values [73].

The leading symptom that triggers diagnostic and therapeutic procedures in patients with suspected ACS is usually chest pain. Taking into account the electrocardiogram (ECG) profile, two groups of patients can be defined [74]:

1) Patients with acute chest pain and persistent (>20 min) ST-segment elevation: this condition is termed ST-elevation ACS and generally reflects an acute total coronary occlusion. Most patients will ultimately develop an ST-elevation myocardial infarction (STEMI).
2) Patients with acute chest pain but no persistent ST-segment elevation. ECG changes may include transient ST-segment elevation, persistent or transient ST-segment depression, T-wave inversion, flat T waves or pseudo-normalization of T waves or a normal ECG.

Patients with no persistent ST-segment can be divided into non-ST-elevation myocardial infarction (NSTEMI) and UA patients (Figure 2). UA patients do not have myocardial necrosis, unlike NSTEMI patients, who have elevated cardiac troponin values.

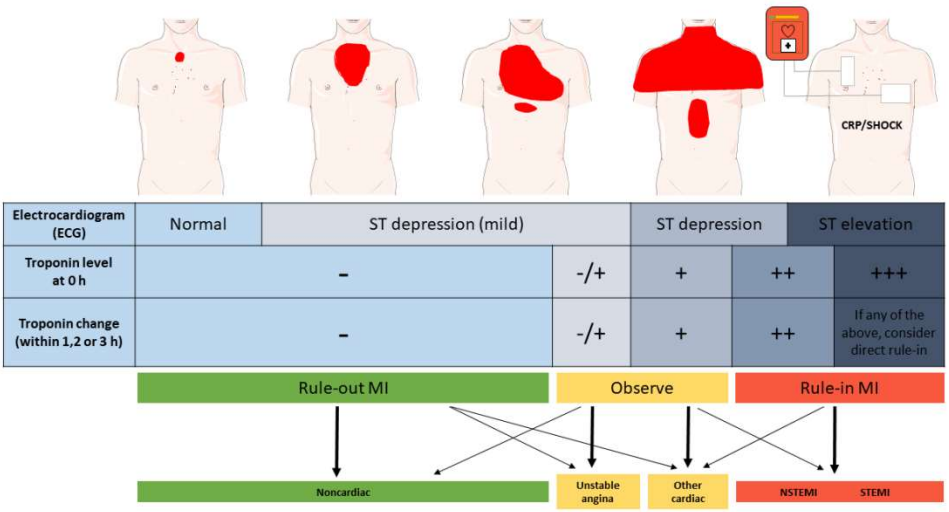

Figure 2. Diagnostic algorithm and triage in acute coronary syndrome. Adapted from Collet JP, Thiele H, Barbato E, Barthélémy O, Bauersachs J, Bhatt DL, Dendale P, Dorobantu M, Edvardsen T, Folliguet T, Gale CP, Gilard M, Jobs A, Jüni P, Lambrinou E, Lewis BS, Mehilli J, Meliga E, Merkely B, Mueller C, Roffi M, Rutten FH, Sibbing D, Siontis GCM; ESC Scientific Document Group. 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. 2021 Apr 7;42(14):1289-1367. doi: 10.1093/eurheartj/ehaa575.

ACS is, in turn, a type of coronary artery disease (CAD), and it is always symptomatic. Cardiovascular diseases (CVDs), including CAD, are the leading cause of death globally. It has been estimated that 17.9 million people died from CVDs in 2019, representing 32% of all global deaths, according to the World Health Organization.

The total prevalence of CAD is 6.7% in adults older than 20 years of age; 7.4% for males and 6.2% for females. In the same age range, the overall prevalence for MI is 3%, and a gender difference can also be observed: there is a prevalence of 4% in males and 2.3% in females. For angina, the prevalence is 3.6% for adults older than twenty years of age [75].

The relative incidence of STEMI has decreased in recent years, from 133 per 100 000 in 1999 to 50 per 100 000 in 2008, while the incidence of NSTEMI has risen [76].

2.2. Physiopathology

The first stage of ACS physiopathology is the formation of a fibrolipid plaque in coronary arteries, also known as atherosclerosis. A healthy vessel is made up of three layers: intima, media and adventitia. The main cells in the intima layer are endothelial cells, forming a monolayer; the media layer is made up of VSMCs; the adventitia layer is the most external and consists of connective tissue and collagen [77].

Endothelial dysfunction is the first sign of arterial disease, an eventuality of atherosclerotic plaque formation and is a consequence of ROS formation, leucocyte migration and adhesion, increasing endothelial permeability and lipid infiltration.

Subsequently, plaque with a lipid core containing cholesterol and cholesterol esters, some in crystalline form, surrounded by two layers, is formed. The first layer is composed of numerous macrophages derived from monocytes of the arterial lumen, as well as medial VSMCs, rich in intracytoplasmic droplets of cholesterol, which are known as foam cells. These foam cells are highly active and produce inflammatory cell mediations, interleukins, and procoagulant tissue factor, among others. The second, external layer is composed of connective tissue, mainly collagen produced by VSMCs [78].

Once the plaque is formed, the endothelium enhances replication and focal areas of endothelial denudation over the plaque appear, exposing the underlying connective tissue matrix and allowing a monolayer of platelets to adhere at the site.

Thrombosis is usually the trigger for myocardial damage, and this occurs through two different processes: endothelial erosion and plaque disruption. Endothelial erosion is caused by an expansion of the endothelial denudation, which exposes the subendothelial connective tissue. Thrombus is then formed and adheres to the plaque. In plaque disruption, the plaque cap tears to expose the lipid core to blood in the arterial lumen. Both processes reflect an increase in inflammatory activity within the plaque, and this causes a reduction in collagen synthesis by VSMCs and also an increased production of MMPs, which can degrade all the components of the connective tissue matrix [79].

Plaque disruption is the main cause of major coronary thrombi in white males with high levels of low density lipoprotein (LDL) cholesterol (LDL-C) and low levels of high density lipoprotein (HDL) cholesterol (HDL-C). In contrast, in women, endothelial erosion causes around 50% of major thrombi [80,81]. There are some characteristics which may indicate that a plaque is vulnerable and an episode of thrombosis may happen:

- A large lipid core involving at least 50% of the overall plaque volume with apoptotic content
- A thin plaque cap in which the collagen structure is disorganized
- A high density of macrophages and inflammatory cells
- A low density of smooth muscle cells within the cap, with calcifications
- A high tissue factor content with proliferation of blood vessels (neoangiogenesis) [82]

2.3. Risk factors and biomarkers

2.3.1. Risk factors

ACS is commonly associated with certain risk factors, including modifiable and non-modifiable ones. Smoking, high blood pressure, high blood cholesterol, diabetes mellitus, physical inactivity, and being overweight or obese are among the

modifiable risk factors. The non-modifiable risk factors include a family history of chest pain, heart disease or stroke, male gender, age and ethnicity [83].

For a long time, only mutations in the low-density lipoprotein cholesterol receptor (LDLR) or pro-protein convertase subtilisin/kexin type 9 (PCSK9) were considered as causal of CAD [84,85]. In 2007, with the introduction of arrays that enabled genome-wide genotyping of an increasing number of genomic variants and the formation of large international consortia, the number of genes implicated in CAD increased considerably. One of the first discoveries in modern CAD genetics research was the chromosome 9p21 locus, which together with the *LPA* locus, represents the locus with the strongest effect on CAD risk [86]. Since then, more than 200 loci, such as the one at chromosome 9p21 locus, have been reported to be associated with CAD. Genes implicated in CAD were classified into genes implicated in initiation of plaque formation, genes implicated in plaque progression and genes with unknown mechanisms [87]. Some of these CAD-associated genes have been used as therapeutic targets, like apolipoprotein (apo) C-3 gene, *APOC3* [88], or angiopoietin-like protein 3/4 gene, *ANGPTL3/4* [89].

2.3.2. Biomarkers

2.3.2.1. Biomarkers of vulnerable plaque

Imaging techniques are the gold standard for identifying vulnerable plaques. However, these techniques are expensive, invasive, and highly qualified staff are needed to interpret the results, so their use in clinical practice is limited. The biomarkers proposed as vulnerable plaque predictors are inflammatory and pro-thrombotic, since these processes are related to clinical stages of ACS.

CRP, measured with highly sensitive methods, is one of the inflammatory biomarkers that has been most studied. High CRP levels are associated with risk factors such as hypertension, smoking, metabolic syndrome and obesity, among others. CRP levels have a high predictive value for coronary disease in healthy populations [90]. They can be used as a complement to cardiovascular risk

stratification in healthy populations with medium cardiovascular risk, but their use in the general population is generally not recommended. However, CRP in combination with troponin does add prognostic value; a higher mortality is expected if high CRP levels are present in a troponin-negative case of ACS [91,92].

Lipoprotein-associated phospholipase A2, interleukin 6, soluble adhesion molecules, and pregnancy-associated plasma protein A are other biomarkers that have been studied but, in general, show less evidence of vulnerable plaque prediction. Their use is not recommended in clinical practice, since measuring them with the available assays did not improve the early diagnosis of acute MI [93].

2.3.2.2. Biomarkers of cardiac damage

In certain situations of cardiac necrosis, cardiac cells can release molecules into the blood stream. The amount of these molecules reflects the amount of cardiac injury, even if the injury is in the early stages. Among the molecules used as biomarkers we can find creatine kinase (CK), partially cardiospecific CK-MB, myoglobin, and cardiac troponins.

CK is a non-cardiospecific enzyme present in all tissues that catalyze the phosphorylation of creatinine. It is mainly located in stretched musculature and its levels are influenced by sex, age and ethnicity. CK-MB is a more cardiospecific isoenzyme, although other pathologies can also increase levels of it. Myoglobin is a protein that rapidly reaches the circulation but its use as a biomarker is limited as, similarly to the previous commented biomarkers, it is also expressed in skeletal muscle [94,95].

The use of cTn as biomarkers was developed at the beginning of the 21st century and marked a dramatic change in the management of ACS patients. Their increase (cardiac troponins are mainly detected by specific antibodies) reflects myocardial necrosis, although it does not indicate the underlying mechanism. Cardiac troponins form a complex with actin filaments in the cardiac myofibrils. This complex is made up of three major troponins: T, I and C. Some isoforms of T

and I troponins are completely cardiospecific. When there is a cardiac injury, troponins are quickly released into circulation [96]. Thus, cardiac troponins have a key role in the diagnosis and prognosis of NSTEMI patients. Initial cardiac troponin levels add prognostic information about short and long-term mortality to clinical and ECG variables [97].

The gold standard for measuring cTn levels in clinical practice is using highly sensitive techniques that allow detection of even small variations in either T or I cTn concentration, making it possible to recognize serial cTn changes typical of myocardial ischaemia earlier than with the so called contemporary methods.

2.3.3. Risk scores

One of the first risk scoring systems was proposed in 1988 and was performed on UA patients, taking into account features like angina course, angina frequency and ST changes. The first large study was the GUSTO-I trial, in which the mortality at 30 days of about 41,000 patients with myocardial infarction was studied. Among all of the features studied, age, location of the MI, and physiological characteristics representing myocardial functions are the most valid prognostic factors. The Thrombolysis in Myocardial Ischemia (TIMI) registry and the following TIMI 11B and ESSENCE provided a huge dataset for risk stratification, but it was restricted only to patients with non-ST elevation ACS. In addition, the scoring systems indicated a risk of mortality or MI only at 1 year and 14 days respectively [98,99].

The Global Registry of Acute Coronary Events (GRACE) study was designed to reflect an unbiased population of patients with ACS. It was carried out over 10 years in 94 hospitals of 14 countries. The risk score is based on nine variables: ST segment deviation, age, heart rate, systolic blood pressure, creatinine, Killip class, cardiac arrest at admission, and elevated biomarkers of necrosis. The first study was performed and also validated on more than 22,000 patients. The score was estimated in hospital risk of death, or the combination of death or MI, and the

same outcomes up to 6 months post discharge. The latest version (GRACE risk score 2.0) provides a 3-year follow-up period [100].

2.4. Management

Management of ACS in patients requires antiplatelet treatment, injectable anticoagulants, modification of cardiac demand, and statins. With STEMI patients, immediate revascularization with primary percutaneous coronary intervention (PCI) is essential. NSTEMI patients should have angiography within 72 hours, ideally sooner.

Antithrombotic treatment is essential in non-ST elevation ACS patients with and without invasive management. The decision to apply antithrombotic treatment should be made according to the patient's characteristics, clinical presentation, comorbidities, co-medication, and procedural aspects. Both antiplatelet and anticoagulant treatments are used during pre-, peri- and post-interventional treatments. Pre-interventional treatment consists of the administration of an antiplatelet drug, usually a $P2Y_{12}$ receptor inhibitor, just before invasive management. Unfractionated heparin is primarily recommended as an anticoagulant in the pre-intervention stage, combined with GP IIb/IIa inhibitors. Post-interventional and maintenance treatment consists of a $P2Y_{12}$ receptor inhibitor, in addition to aspirin, during approximately 12 months. An early invasive strategy within 24 hours is strongly recommended in patients with diagnosis of NSTEMI or with transient ST-segment elevation or with a GRACE risk score >140 [97].

In STEMI patients, primary PCI is the preferred reperfusion strategy. It should be performed within 12 hours of symptom onset. A time of 120 minutes from STEMI diagnosis to PCI-mediated reperfusion is established. The recommended treatment in patients undergoing primary PCI intervention is also antiplatelet and anticoagulant therapy. Moreover, a potent $P2Y_{12}$ inhibitor is recommended before

PCI and maintained over 12 months, aspirin as soon as possible, and anticoagulation treatment based on unfractionated heparin.

If the anticipated time from STEMI diagnosis to PCI-mediated reperfusion is >120 min, then immediate fibrinolysis is indicated. The patient must be transferred to a PCI-capable center following fibrinolysis. Antiplatelet treatment, using clopidogrel in addition to aspirin, and anticoagulation treatment with, preferably, enoxaparin are used as co-therapy with fibrinolysis [101].

3. Gut microbiota metabolites

The human gastrointestinal tract contains more than one hundred trillion microorganisms, giving the gut a high diversity. These microorganisms are organized into communities. The part of the gastrointestinal tract with the highest abundance of microorganisms is the colon, with a density of bacterial cells of 10^{11} to 10^{12} approximately. The gut genome is a thousand times more complex than the human genome, with more than 3 million genes producing thousands of metabolites, while the human genome has only about 23,000 genes [102,103]. The most common technique for determining and/or quantifying microorganisms is the extraction of DNA and the amplification of the 16S ribosomal RNA gene directly extracted from stools [104].

Among all the metabolites produced by the gut, one of the most studied in recent years, because of its relationship with various cardiovascular diseases, is trimethylamine (TMA) and its oxidative product, Trimethylamine-N-oxide (TMAO).

3.1. Microbial pathways leading to TMA production in the gut

Various gut microorganisms can form TMA from dietary precursors. There are various microbial pathways for TMA production, depending on the precursor. In the gut microbiota, several families of bacteria, such as Firmicutes, Proteobacteria and Actinobacteria phyla, isolated from commensal bacteria in the human intestine, have been identified as choline and carnitine consumers and, therefore, potential TMA producers. Bacteroidetes, however, appear to be unable to produce TMA [105]. The choline utilization (*cut*) gene cluster, which is responsible for anaerobic choline degradation, has been shown to contain a gene coding for specific glycyl radical enzyme choline TMA-lyase (*cutC*) and its corresponding radical S-adenosyl-L-methionine activating protein (cutD), which uses choline as a substrate [106].

Another microbial metabolic pathway via which TMA is generated is a two-component CntA/CntB oxygenase/reductase system, which uses carnitine as a substrate [107]. Another bacterial lyase, based on *yeaW/X* gene products (YeaW/X TMA lyase), is responsible for TMA production, using choline, betaine, L-carnitine and ɣ-butyrobetaine (ɣBB) as a substrate [108].

Interventions on the intestinal lumen are a possible way of reducing TMA and TMAO production, in order to prevent the synthesis of these metabolites. This is the case of the structural analogs of choline, which specifically inhibit TMA lyase. Precisely, 3,3-dimethyl-1-butanol inhibits both *in vitro* and *ex vivo* TMA lyase without presenting lethal effects on targeted bacteria; thus it has been proposed as a plausible treatment for atherosclerosis and thrombosis [109,110].

Modification of the microbiota composition is another intervention strategy proposed for targeting the TMA/TMAO pathway. This modification can be done in different ways: with probiotics, fecal microbiota transplantation (FMT) or with bioactive food ingredients. An extended range of probiotics has been studied, but none of them seemed to significantly alter TMAO levels [111,112]. The results of FMT are controversial: while it has been successfully used to prove the transmissibility of atherosclerosis susceptibility [113], FMT failed to reduce TMAO levels or parameters related to vascular inflammation [114].

Inter-individual variability in the composition of the gut microbiota can be influenced by dietary patterns. A greater increase in post-prandial plasma TMAO was observed after consuming both eggs and beef, compared to fruit, which was used as a control. High TMAO producers, found in the groups that had eaten eggs and beef, had more Firmicutes than Bacteroidetes and a less diverse gut microbiome. More specifically, *Clostridiaceae*, *Lachnospiraceae* and *Veillonellaceae,* Clostridiales of the Firmicutes phylum, were the most abundant genre. Conversely, low TMAO producers, found in the fruit control group, had more

Bacteroidales of the Bacteroidetes phylum, of which *Bacteroidaceae* and *Prevotellaceae* were predominant [115].

3.2. Dietary precursors of TMA and TMAO

TMAO can be produced directly from food. It is found in fish and marine invertebrates like mussels or other seafood. Concentrations in fresh seafood can vary considerably between species, habitat, depth, and season. The average TMAO content is between 8 and 789mg per 100g of ray-finned fish and between 262 and 789mg per 100g of cartilaginous fish. Compared to other dietary sources, fish has the biggest impact on TMAO concentrations. The consumption of fish yielded 50 times higher postprandial circulating TMAO concentrations than eating other foods that are rich in choline or carnitine [116].

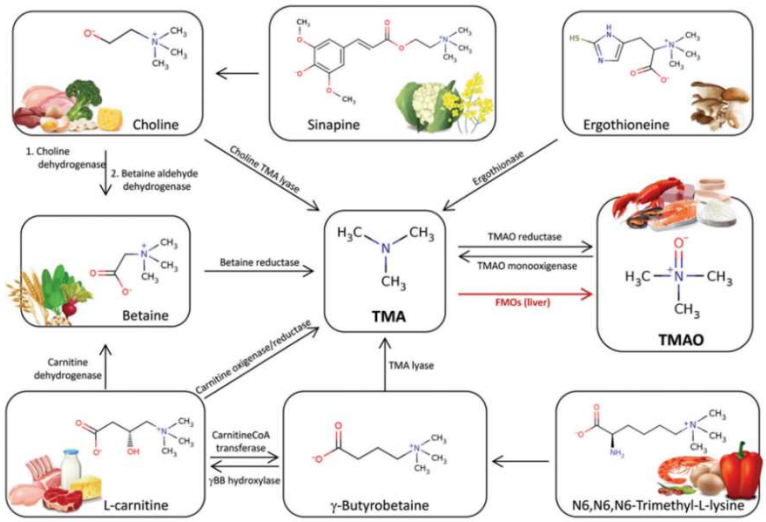

Figure 3. Synthesis and metabolism of gut microbiota metabolites. Extracted from Simó C, García-Cañas V. Dietary bioactive ingredients to modulate the gut microbiota-derived metabolite TMAO. New opportunities for functional food development. Food Funct. 2020 Aug 1;11(8):6745-6776. doi: 10.1039/d0fo01237h. Epub 2020 Jul 20. PMID: 32686802.

Furthermore, TMAO can be produced by oxidation of TMA generated by the gut, using different dietary precursors: L-carnitine, choline, N6,N6,N6-trimethyl-L-lysine (trimethyllysine, TML), betaine and ergothioneine.

L-carnitine is synthetized endogenously from methionine and lysine in the liver, kidneys, heart, testes, and brain. In certain conditions, an exogenous source may be needed to supplement endogenous synthesis. Foods of animal origin (especially meat and dairy products) are rich in L-carnitine. It can also be found in grains and vegetables, but in lower concentrations. The amount of carnitine in dairy products ranges from 3 to 42mg per 100g whereas in red meat it can vary between 45 and 65mg per 100g [117]. The intermediate γBB, also called precarnitine, is an immediate biosynthetic precursor of carnitine. As explained previously, it is also formed in the conversion of L-carnitine to TMAO [118].

Choline can also be considered a conditionally essential nutrient. As the endogenous production by the liver may not be enough for human requirements it needs to be obtained from the diet. Choline is present in foods in different forms, mainly in water-soluble forms that include free choline, phosphocholine and glycerophosphocholine, and lipid-soluble forms that include phosphatidylcholine and sphingomyelin. The main source of choline are eggs and liver, followed by fish, whole grain cereals, vegetables, fruit, milk, fat, and oils [119].

TML is a non-protein amino acid with an important role as a precursor of L-carnitine. TML is present as a free amino acid in legumes, grains, leafy and solanaceae vegetables, and fruit [120]. In vegetables, the highest concentration is found in sweet peppers, but the amount of TML is higher in eggs (13.7mg per 100g) and in shrimps (12.2mg per 100g) [121].

3.3. TMAO metabolism

TMAO is a small organic compound whose chemical formula is $(CH_3)_3NO$ and is classified as an amine oxide. In the tissues of marine organisms, it exerts a protective role against the adverse effects of temperature, salinity, high urea, and hydrostatic pressure. TMAO stabilizes the folded state of proteins, but the mechanism by which TMAO interacts with proteins remains unknown [122]. Once the gut microbiota has produced TMA from dietary precursors, it crosses the

intestinal lumen. TMA can only be produced and absorbed by the small intestine, and is then transported via portal circulation to the liver [123]. TMA is afterwards converted into TMAO by Flavin monooxygenase (FMO) in the liver of the host. Among the five members of the FMO family, only FMO1 and FMO3 have the ability to oxidize TMA to TMAO. FMO3 is the main isoform expressed in the human liver [124].

Certain rare deleterious mutations of the *FMO3* gene are known to cause reduced or absent TMAO formation, which in turn causes an accumulation of TMA. This autosomal recessive condition is called trimethylaminuria or "fish malodor syndrome" (OMIM 602079). Early reports of this condition date back to 1970 and they described affected individuals as experiencing urine, sweat, and breath that smelled like rotting fish [125]. Interestingly, a sexually dimorphic expression pattern has been observed both in mice and humans, with females showing higher expression than males [126]. This gender difference may be explained by hormonal regulation, as testosterone is responsible for the lower hepatic FMO3 expression in males and estrogens induce *FMO3* gene expression in females [127]. Studies on humans have produced divergent results on gender-related differences in plasma TMAO concentrations. Several studies investigating TMAO expression in humans did not find significant differences between the sexes in plasma TMAO levels [128–130]; others, however, reported higher levels either in females [124,131] or in males [132,133].

Modulating the gene expression of *FMO3* would be another target of action for regulating plasma TMA/TMAO levels in the liver. Various studies in mice have shown a key role of FMO3 in lipid metabolism [134,135], insulin resistance [136], and thrombosis [137]. Recently, it has been proposed that FMO3 inhibition could be a suitable strategy in controlling the production of TMAO. FMO3 inhibition by dietary 3,3'-diindolylmethane or indole-3-carbinol supplementation reduced hepatic TMAO in insulin-resistant mice, with a decrease in activated protein kinase R-like endoplasmic reticulum kinase (PERK) and forkhead box protein O1

(FoxO1), and improved glucose tolerance. Partial inhibition of FMO3 activity can have beneficial TMAO-lowering effects without causing odor (trimthylaminuria) [138].

Urinary excretion is the major elimination pathway of TMAO from systemic circulation. TMAO is almost exclusively eliminated by the kidneys in an unmodified form via filtration and active secretion mechanisms. A kinetic study in 40 healthy men with d9-TMAO revealed that TMAO can be detected in plasma only 15 minutes after oral ingestion. The elimination of TMAO is greatest during the first 6 hours (showing an enrichment of 67%) after consumption of the tracer. An estimated 96% of the administrated d9-TMAO was excreted unmodified in urine after 24 hours, leaving only 4% retained in the body, with none detected in feces [139].

Active secretion mechanisms, which are implicated in TMAO excretion, occur through different transporters. The most important transporters are the organic cation transporters (OCT). Specifically, OCT1 to 3 are a TMAO transporters, and OCT1 and OCT2 are decisive in TMAO kinetics in mice. TMAO can be absorbed by OCT2 in humans, but its contribution to tubular secretion is not clearly observed under normal conditions [140]. OCT1/2 knockout mice present higher levels of TMAO than wild-type mice. OCT1 is mainly located in the liver and OCT2 in the kidney. The increase in TMAO levels is a consequence of low renal excretion; the liver/plasma ratio of TMAO is not altered but the kidney/plasma ratio of TMAO is decreased. Moreover, OCT2 is physiologically relevant to TMAO renal tubular uptake across all the concentrations observed in humans [141].

Different studies performed in human cohorts have demonstrated the association of TMAO with kidney markers. The estimated glomerular filtration rate (eGFR) correlates negatively with TMAO values, whereas blood urea nitrogen, cystatin C, and serum creatinine levels have a positive association with TMAO levels [142,143]. TMAO can directly contribute to renal insufficiency. Long-term TMAO ingestion

in mice models resulted in cystatin C elevation and increased tubulin, accompanied by increases in renal fibrosis [144].

TMAO levels measured at baseline are a prognostic marker for kidney function, since patients with chronic kidney disease have higher TMAO levels. Advanced chronic kidney disease (CKD) populations have elevated TMAO levels compared to the healthy population associated in turn with increased cardiovascular events [145]. CKD patients who are on hemodialysis also have increased TMAO levels [132] . Use of loop diuretics, furosemide, for example, is associated with high TMAO levels. The proposed mechanism is an abolishment in tubular TMAO secretion, causing an accumulation in plasma and the kidney [146].

3.4. Gut microbiota metabolites and cardiovascular disease

In recent years, the concept of pathological variation in the gut microbiota as the cause of several diseases has emerged, mostly because of studies in rodents and humans. TMAO, and some metabolites, have been associated with an increased risk of atherosclerosis, thrombosis, stroke, heart disease, type 2 diabetes and obesity [147].

The first publication linking TMAO and cardiovascular disease was in 2011. It was an untargeted metabolomics study which identified three candidate molecules which correlated significantly - after adjusting for traditional cardiac risk factors and medication usage - with CVD. These three molecules were TMAO, choline, and phosphatidylcholine [148]. Since then, a large number of studies have been performed to validate this finding. One of the most important studies proposed that a concentration of plasmatic TMAO levels above 4.6 μM could predict an increased CVD risk [149].

A recent systematic review and meta-analysis of 19 prospective studies concluded that elevated TMAO concentrations were associated with a 62% increased risk for all-cause mortality [150]. Another meta-analysis of 11 prospective studies with more than 10,000 patients found that elevated circulating TMAO was associated with a

23% higher risk of cardiovascular events and 55% higher risk of all-cause mortality [151].

The mechanisms by which TMAO can cause increased risk of CVD are not clearly elucidated. The main mechanism implicated would appear to be the ability of TMAO to enhance atherosclerosis. A diet rich in choline, TMAO and/or betaine could up-regulate multiple macrophage scavenger receptors, which are linked to atherosclerosis. Further, reverse cholesterol transport significantly decreases in mice fed with carnitine, choline or TMAO supplemented diets. Inhibition of microbiota-TMA generation through the inhibition of TMA lyases may reduce atherosclerotic plaque due to a reduction in foam cell accumulation [109,148,149]. However, a recent study cast doubts on this concept as dietary supplementation with choline, betaine or TMAO did not induced atherosclerosis in two different mice models, despite resulting in increased TMAO concentrations [152].

Another proposed mechanism is the ability of TMAO to promote thrombosis. It has been shown in animal models that TMAO enhances platelet responsiveness to multiple agonists by stimulating Ca^{2+} release from intracellular stores, heightening thrombosis potential [153]. A recent *in vitro* study in human coronary endothelial cells also revealed that TMAO promotes thrombosis through increasing tissue factor expression and activity [154]. Plasma TMAO levels have also been associated with a higher thrombotic event risk in human subjects [153,155].

Lastly, *in vivo* studies in mice and *in vitro* studies in cultured human aortic endothelial and vascular smooth muscle cells have shown that physiological levels of TMAO induce expression of inflammatory cytokines and adhesion molecules. This activation was mediated, in part, by the NF-κB signaling pathway [156].

4. High density lipoprotein

4.1. HDL composition

The most important plasmatic lipids are cholesterol and triglycerides; both have a key role in the global lipid metabolism. Cholesterol and triglycerides are found in circulation as lipoproteins, particles containing both lipids and proteins. Lipoproteins are classified according to their density: HDL, LDL, intermediate-density lipoprotein (IDL), and very low-density lipoprotein (VLDL). The density of HDLs ranges from 1,063 to 1,210 g/mL in the ultracentrifuge; it is the smallest and the densest of all serum lipoproteins [157].

HDL particles owe their high density to their high protein content, which accounts for about 35 - 65% of their weight [158]. The current official count of the HDL proteome is 251 proteins, as defined in three different reports from more than three independent laboratories (from 45 studies surveyed). Among all of these proteins, only five apolipoproteins (apoA-I, A-II, C-II, C-III, and E) were reported in all the studies [159]. Apolipoproteins and enzymes are considered the functional component of HDL particles. ApoA-I represents approximately 70% of the total protein moiety of HDL. It is mainly synthesized in the liver and in the small intestine. The main function of apoA-I is to act as a cellular receptor ligand, as an activator of lecithin–cholesterol acyltransferase (LCAT), endowing HDL with multiple anti-atherogenic activities. The second most abundant apolipoprotein is apoA-II, which represents 20% of the total protein content. It is synthesized mainly in the liver, and to a lesser extent in the small intestine, being a structural and functional apolipoprotein [160–162].

The remaining protein components (<10%) include minor apos, enzymes, lipid transfer proteins, acute-phase proteins, complement components, proteinase inhibitors, and other protein components. The most important enzymes are LCAT, cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP). These enzymes have a key role in HDL metabolism, which will be described in the following section.

HDL is an extremely heterogeneous class of lipoproteins and different subtypes can be isolated depending on the density, size, shape, and lipid and protein composition. HDL has traditionally been divided into two subclasses: the less dense and relatively lipid-rich form, HDL2, and the denser and relatively protein-rich form, HDL3. Approximately 60% of total HDL is in the HDL3 subclass, whereas 40% is HDL2 [163,164]. HDL can also be separated according to surface charge and shape, using an agarose gel electrophoresis. The two types obtained are α-migrating particles, the majority of circulating HDL, and preβ-migrating particles, consisting of nascent discoidal and poorly lapidated HDL [165]. Other isolation/separation techniques can be used to separate HDL subclasses, based on size or protein composition, for example. Recently, several authors have proposed a new uniform nomenclature system for HDL subfractions based on physical properties. The aim was to facilitate future characterization of HDL subfractions and improve the interpretation of findings using different approaches, but also to assess the clinical effects of different agents that modulate HDL structure or function [166].

A classification based on the HDL proteome has also recently been proposed. Furtado et al. identified and characterized the proteomes of 15 novel protein-defined HDL subspecies. Each subspecies comprised 1% to 18% of total HDL, and their concentrations were stable within an individual over 1 to 2 years [167].

The lipid content may vary depending on the HDL subtype, but is estimated to be approximately 35 - 65% of the total weight. Lipids are in part on the HDL surface, forming a monolayer of phospholipids that encloses the particle. Phosphatidylcholine is the principal plasma phospholipid and accounts for 35 - 50% of total lipids in HDL. This monolayer also contains minor sphingolipids, like ceramide and sphingomyelin (which represent 5 - 7% of HDL total lipid mass), along with free cholesterol and other sterols (which account for an additional 5 - 10%). The core of HDL is composed of cholesteryl esters (35 - 40%

of HDL lipids), with few triglycerides (TG) (which represent 2 - 3% of HDL lipids) [161,162,168].

4.2. HDL metabolism and biological activities

HDL biosynthesis begins in the liver (70%) and small intestine (30%) with the secretion of lipid-free apoA-I, the main HDL protein, into the blood stream. ApoA-I is then rapidly lipidized and converted into nascent HDL (termed preβ-HDL). Both apoA-I and preβ-HDL have the ability to interact with transmembrane ATP-binding cassette subfamily A member 1 (ABCA1), inducing a free cholesterol and phospholipid transference translocation from the cell membrane [169]. As a result, LCAT, activated by apoA-I, esterifies the free cholesterol of these nascent discoidal particles. The esterified cholesterol is then internalized in the core particle, starting its transformation from discoidal to spherical HDL or α-HDL [170].

Mature HDL is also a LCAT substrate, allowing further removal of free cholesterol from peripheral tissues, while becoming HDL with a cholesterol ester-enriched core. Furthermore, mature HDL can accept phospholipids, free cholesterol, and apos from other lipoproteins. PLTP promotes the transfer of excess phospholipids and free cholesterol from triglyceride-rich lipoproteins into HDL, inducing HDL particle fusion, but also a remodeling process where preβ-HDL particles can also be generated. HDL cholesterol esters can be transferred to VLDL or LDL by CETP, delivering TG in return, contributing to HDL remodeling by lipases and the generation of smaller HDL [162,171].

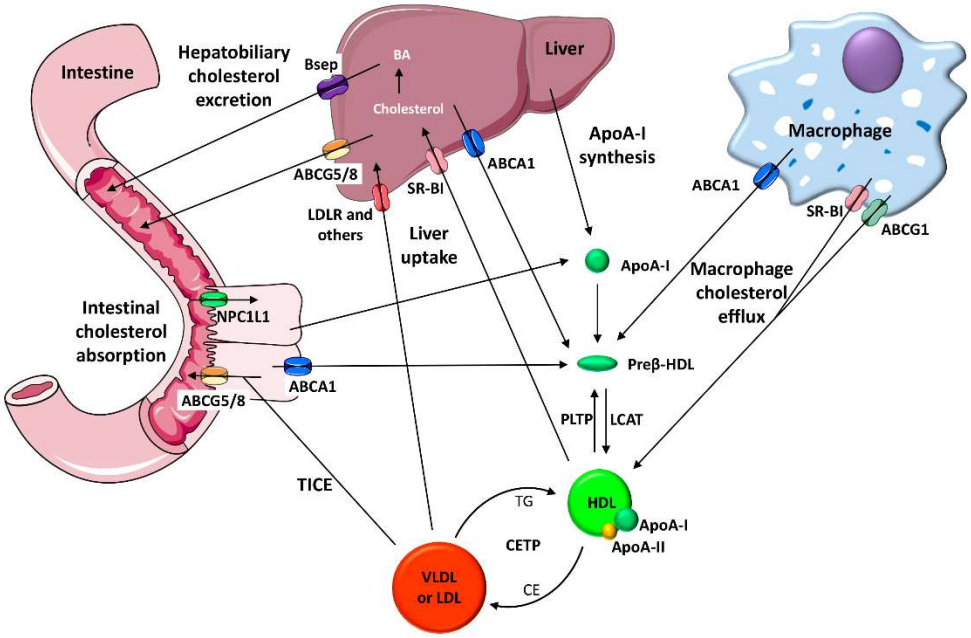

Figure 4. A schematic diagram of HDL metabolism and reverse cholesterol transport. Extracted from Canyelles M, Tondo M, Cedó L, Farràs M, Escolà-Gil JC, Blanco-Vaca F. Trimethylamine N-Oxide: A Link among Diet, Gut Microbiota, Gene Regulation of Liver and Intestine Cholesterol Homeostasis and HDL Function. Int J Mol Sci. 2018 Oct 19;19(10):3228. Doi: 10.3390/ijms19103228. PMID: 30347638; PMCID: PMC6214130.

Cholesterol can then be taken up selectively from HDL after binding to the scavenger receptor class B type I (SR-BI) in the liver, or by being captured together with the whole HDL particle by other receptors in the liver or the kidneys. VLDL and LDL are cleared by the liver through LDL receptor and other receptor-dependent pathways; part of the cholesterol contained in these particles has been transferred from HDL by the CETP. From there, cholesterol will be partly transformed into bile acids and removed together with the unesterified cholesterol through the biliary pathway. Cholesterol and bile acids may then be finally excreted from the body by the feces. In addition to the main hepatobiliary pathway of cholesterol elimination, an alternative nonbiliary transintestinal route for cholesterol elimination has also been reported. The transintestinal cholesterol

excretion route facilitates the transfer of cholesterol from the circulating plasma directly into the intestinal lumen through enterocytes [172].

The concept that HDL has multiple atheroprotective activities has been proved in a large number of *in vitro* and *in vivo* studies. These pleiotropic effects include the capacity to protect vascular endothelium via cholesterol efflux from macrophage foam cells, decreased cellular death, lower vascular constriction, reduced inflammatory response, protection from pathological oxidation, combating bacterial infection, decreased platelet activation, and improved glucose metabolism [173].

The most relevant effect is probably the HDL-mediated ability to remove cholesterol from macrophages in atherosclerotic plaques, the initial step in reverse cholesterol transport. This mechanism will be further explained in section 4.4.

HDL-mediated anti-inflammatory effects include its ability to reduce the production of cytokines by macrophages and a decrease in the expression of intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin in vascular endothelial cells. This last effect is mediated by SR-BI receptor signaling and precludes the penetration of monocytes and neutrophils into the arterial wall [174]. Both HDL lipidome and proteome contribute to the anti-inflammatory activity of HDL. Therefore, modifications such as glycation or oxidation can not only impair HDL anti-inflammatory capacity but generate HDL with pro-inflammatory activity [175,176]. HDL and apoA-I can attenuate oxidative stress by preventing LDL oxidation by free radicals, which has pro-atherogenic effects [177].

HDL has also a role in endothelial function, by inducing vasodilatation and preventing apoptosis. The vasodilatation is due to an increase in the production of nitric oxide and prostacyclin [178]. ApoA-I and HDL-associated lysosphingolipids are the major components responsible for the inhibition of endothelial cell apoptosis [179]. The antithrombotic effect of HDL is due to its ability to inhibit:

platelet activity, and aggregation, the expression of endothelial tissue factor induced by thrombin, and the Von Willebrand factor [177].

4.3. HDL hypothesis and the role of HDL in CVD

HDL is considered the main antiatherogenic lipoprotein, while VLDL, IDL and, particularly, LDL are proatherogenic lipoproteins. The widely held concept of HDL-C as the good cholesterol and the idea that HDL-C can protect against coronary heart disease was originally based on epidemiological studies in which people with low levels of HDL-C presented an increased risk of major adverse CVD events. The Framingham Heart Study was the first to demonstrate that cardiovascular morbidity and mortality were more than eight times higher in people with HDL-C levels below 35 mg/dL compared to those with HDL-C levels above 65 mg/dL. An increase in HDL-C levels entailed a reduction in CVD risk, independently of sex and LDL-C levels [180,181]. These results were also validated in subsequent analyses of the Framingham cohort and confirmed by other studies such as the PROCAM study, in which the inverse association of HDL-C with CAD was highly significant, even after adjustment for several other risk factors. A meta-analysis of four prospective studies revealed that for every 1 mg/dL increase in plasma levels of HDL-C, there was a reduction in cardiovascular risk of 2% in men and 3% in women, independently of other risk factors, including plasma LDL-C [182].

These epidemiological findings were supported by a series of animal studies: HDL infusions in rabbits showed an inhibition of atherosclerosis. Mice overexpressing apoA-I were protected from atherosclerosis and, more importantly, induced-overexpression of apoA-I was able to regress pre-existing atherosclerosis disease. Overall, the HDL hypothesis established that HDL has a causal role in atherosclerotic development and also in CVD, so strategies focused on increasing HDL-C levels would reduce atherosclerosis and the associated cardiovascular risk [183].

However, after decades of study, the results are much more conflicting. Most therapeutic strategies that have succeeded in increasing circulating HDL-C levels have failed to provide clinical benefits with regards to CVD. The main strategy for increasing HDL-C levels was the use of CETP enzyme inhibitors. The interest in CETP inhibitors as a drug is based on their role in HDL metabolism: species that lack CETP activity are naturally resistant to the development of atherosclerosis; also, human genotypes associated with low CETP activity have shown a reduction in cardiovascular risk [184].

Up to now, four CETP inhibitors have been tested in large clinical trials: torcetrapib, dalcetrapib, evacetrapib, and anacetrapib. Despite a 30 to 70% increase of HDL-C, none of them were able to reduce CVD or mortality significantly. In fact, the clinical trial of the first drug studied, torcetrapib, had to be stopped prematurely due to an increase in the incidence of death and CVD in the torcetrapib group [185,186]. One possible explanation is that HDL composition and function were altered in a neutral, or even harmful, way.

On the other hand, mendelian randomization studies have also shown that polymorphisms that increase HDL-C did not change cardiovascular risk. Mutations in ABCA1, which cause a 30% reduction in HDL-C, did not adversely affect cardiovascular risk. In conclusion, the current data do not support the evidence for a direct causal relationship between molecularly defined HDL-C changes and CVD [187,188].

These results have called into question the causality of the relationship between HDL and CVD. Indeed, it has become clear that changes in HDL-C levels cannot be used as a biomarker of cardiovascular risk. HDL encompasses a heterogeneous group of particles that differ in size, shape, density, electrophoretic mobility, and apolipoprotein content. As a consequence, HDL cardioprotective functions rather than the quantity of HDL-C have become the current focus of interest [189,190].

4.4. HDL-mediated cholesterol efflux

Many HDL studies focused on its role as an antioxidant, an anti-inflammatory agent and, above all, as an acceptor of macrophage cholesterol efflux (MCE). MCE is the first step in reverse cholesterol transport (RCT) and represents a cell type that is important, in the pathogenesis of atherosclerosis. Other cell types are also important in RCT.

RCT involves the transport of cholesterol from the cell membrane of peripheral tissues to the liver, where it will be distributed to other tissues mainly through VLDL secretion, or removed from the body through feces.

Whereas cholesterol efflux to HDL occurs in all tissues, the fraction that originates from the macrophage foam cells located in the arterial wall is considered the most critical RCT component, which is directly related to atherosclerosis [191]. MCE occurs through passive aqueous diffusion and also through protein transmembrane transporters. At least three cholesterol transporters are involved in the HDL-mediated macrophage cholesterol efflux: ABCA1, ATP binding cassette subfamily G member 1 (ABCG1) and SR-B1. Efflux via ABCA1 and ABCG1 are active whereas SR-BI-mediated efflux is a passive process.

In this passive aqueous diffusion, molecules of free cholesterol are desorbed from the cell membrane and incorporated into an acceptor such as HDL. This gradient enables HDL to accept additional free cholesterol from cell membranes [192].

ABCA1 promotes the unidirectional transport of free cholesterol and phospholipids to the lipid-free apoA-I, preβ-HDL, and small HDL. Tangier disease is an inherited autosomal recessive disorder caused by mutations in the *ABCA1* gene. Patients with Tangier disease present a defective ABCA1 transporter function with significantly reduced levels of HDL-C and reduced efflux in the ABCA1-mediated pathway. Conversely, ABCG1 and SR-BI facilitate the efflux to mature α-migrating HDL [172].

The relative contribution of each pathway to the net efflux is not entirely understood and depends on the cholesterol content and distribution in cells and tissues. In lipid-laden foam cells of the atherosclerotic plaque, cholesterol efflux is mainly mediated by aqueous diffusion pathways, ABCA1 and ABCG1, with a minimal contribution from the SR-BI transporter. In the absence of cholesterol enrichment, the pathway used for cholesterol efflux to human serum is mainly aqueous diffusion, followed by the SR-BI pathway [192].

The first publication studying the link between HDL-mediated MCE and CVD was published in 2011. The conclusion was that MCE, a key metric of HDL function, cannot be explained simply by circulating levels of HDL-C. Moreover, MCE was strongly and inversely associated with both subclinical atherosclerosis and obstructive coronary artery disease [193]. However, the cross-sectional design of the study did not allow a direct relationship between this HDL function and CVD to be established. Three years later, another publication demonstrated that cholesterol efflux capacity was inversely associated with CVD in a general population-based cohort that was CVD-free at baseline and was followed up for 9 years. This association remained after adjustment for traditional cardiovascular risk factors, HDL-C levels, and HDL particle concentration [194].

It should also be noted that cholesterol efflux assays only quantify the first step of the atheroprotective RCT pathway, without addressing the efficiency of the remaining RCT steps, or other already mentioned HDL atheroprotective properties, such as their antioxidant, anti-inflammatory, and antithrombotic potential. An assay that evaluates the transfer of radiolabeled cholesterol from macrophages to feces has been widely applied to mice to determine the macrophage RCT rate of the entire pathway. This RCT multistep pathway, which is initiated by macrophages, can be modulated in the liver and small intestine. Studies investigating genetically engineered mice and mice treated with different RCT-enhancing therapies indicate that this major HDL antiatherogenic function is a potentially important predictor of atherosclerosis susceptibility [172,195].

~ HYPOTHESIS AND OBJECTIVES ~

Our group reported that AAA patients with a large aortic diameter showed an impaired HDL-mediated MCE. This inverse association was observed after adjusting for age, statin use, and lipid, apoA-I, and lipoprotein levels. Furthermore, HDL-C concentrations predicted the aneurysmal growth rate in another prospective study. As HDL-C does not always reflect HDL cardioprotective functions, we hypothesized that HDL-mediated MCE capacity could predict both AAA growth rate and the need for surgical repair.

On the other hand, MCE capacity is inversely associated with CVD risk and mortality, although there is heterogeneity among studies and evidence of publication bias. There is also a potential link among gut microbial-derived metabolites and HDL function. We expected that MCE and gut-related metabolites (TMAO, γBB, and TML) would have a prognostic value for predicting cardiovascular mortality in patients with STEMI and, further, that gut-related metabolites might modulate the association between MCE and STEMI mortality.

Therefore, in this study, our first objective was to evaluate HDL-mediated MCE in a cohort of AAA patients and controls and evaluate its capacity to predict AAA progression and the need for surgery in patients with small/medium AAA.

The second objective was to evaluate the associations among HDL-mediated MCE and the microbial-derived metabolites TMAO, γBB, and TML at baseline with future cardiovascular mortality in patients with an acute STEMI, testing whether these gut microbial-derived metabolites modulate the associations between HDL-mediated MCE and mortality.

~ MATERIALS AND METHODS ~

1. Study design and patient selection

1.1. Abdominal aortic aneurysm

All samples were obtained from a cohort of the Viborg Vascular (VIVA) trial in Denmark (URL: http://www.clinicaltrials.gov. Unique identifier: NCT00662480). The trial was approved by the regional ethics committee (M20080025) on 28 March 2008 and all the subjects gave their informed consent. The study was carried out in accordance with the ethical principles set forth in the Declaration of Helsinki. This was a randomized controlled trial with the aim of examining the efficacy and cost effectiveness of a screening program for early AAA detection. From October 2008 until October 2010, approximately 50,000 65 to 74-year-old Danish men were chosen at random to receive either an invitation for vascular screening or to be a control. The age range was chosen with the objective of maximizing the possibility of detecting the effect of screening, since this group has a higher prevalence of non-symptomatic cardiovascular disease and yet a long life expectancy. During the screening, an ankle brachial index measurement, ultrasound scan of the aorta and a lifestyle questionnaire were performed by trained project nurses. Specifically, to measure the aorta diameter, an ultrasound 4 Mhz transducer was placed longitudinally above and slightly to the left of the navel. If a dilatation was found, the maximal perpendicular anteroposterior diameter was measured. If a dilatation was not found, the anteroposterior diameter was measured two centimeters above the bifurcation.

We rando mLy selected a representative subsampling of one hundred and fifty-eight patients with different AAA sizes classified into three groups, according to their aortic diameter: a large-size group (aortic diameter >50 mm; n = 39), a small/medium-size group (aortic diameter between 30 and 50 mm; n = 81) and a control group (aortic diameter <30 mm; n = 38). The average concentration of HDL-C and apoA-I in this subcohort of patients was similar to that obtained in the whole trial.

The large-size group was referred for a computed tomography scan and vascular assessment. The small/medium-size group underwent medical monitoring for clinical control to check for diameter expansion. Monitoring consisted of ultrasonographic follow-up of the aortic diameter (a minimum of two follow-ups in a 5-year period) to obtain a linear growth rate per year. Based on this rate, the patients were divided into three subgroups: low progression (growth rate of <1 mm per year; $n = 26$), medium progression (growth rate between 1 and 5 mm per year; $n = 29$) and high progression (growth rate of >5 mm per year; $n = 26$). The patients were assigned to surgery according to increases in the aortic diameter and evaluation of clinical parameters.

1.2. Acute coronary syndrome

Blood samples from patients with STEMI were obtained from a retrospective study that included 253 patients, who were older than 18 years old, and who were admitted to the Hospital de la Santa Creu i Sant Pau in Barcelona between January 2012 and November 2014. All the subjects gave informed consent. The study was carried out in accordance with the ethical principles set forth in the Declaration of Helsinki and approved by the Ethical Comitee of Hospital de la Santa Creu i Sant Pau (protocol code IIBS-TRO-2015-61, 4 December 2015). STEMI diagnosis was carried out according to the principles of the Universal Definition of Myocardial Infarction, defined in the current guidelines [196,197], that is, an increase in ST segment in the electrocardiogram measured in the J point in two adjacent derivations. All patients were followed for a period of two years for cardiovascular adverse events, all-cause death, and hospital readmissions, via telephone interview and/or electronic medical record review. The primary outcome of this study was cardiovascular death defined as death by cardiogenic shock, fatal myocardial infarction or heart failure.

A representative sub-sample of STEMI patients was selected: 35 who died during admission or during follow-up, and 36 who survived at the end of the period when they were being monitored. The GRACE risk score provides an estimate of the

probability of death within 6 months of hospital discharge in patients with ACS and it was calculated (GRACE 2.0 risk score) [100] in all patients.

A subgroup of 33 patients with suspected MI symptoms, but with high-sensitive cardiac troponin T (hs-cTnT) concentrations below the cut-off used to define myocardial damage (14 ng/L), or not reaching an hourly increase >3.0 ng/L during three-hour serial sampling, was diagnosed with UA and was used as a control group. This control group was chosen so they had the same acute background as the STEMI patients but without myocardial damage.

UA and STEMI survivors were matched with the deceased STEMI group for age, sex, body mass index, arterial hypertension, diabetes mellitus and smoking status.

2. Blood samples and clinical biochemical parameters

Whole blood samples from AAA patients were collected in Vacutainer® tubes and fractionated by centrifugation at 1,300 g for 15 minutes at room temperature to obtain plasma. Plasma was aliquoted into 1.5 mL tubes and kept frozen at -80ºC until analysis. Blood samples from ACS patients were collected in EDTA anticoagulated tubes; in the case of UA patients, upon admission, and in STEMI patients, immediately before the angiographic procedure performed at the time of the event. Plasma was obtained via centrifugation at 10,000 g for 10 minutes and kept frozen at -80ºC until analysis.

In the AAA study, total cholesterol and TG concentrations in the plasma were determined enzymatically using commercial kits and apoA-I levels were determined by an immunoturbidimetric assay, all of them in the autoanalyzer COBAS 501c (Roche Diagnostics, Basel, Switzerland). HDL-C levels were measured in serum supernatant obtained after precipitation of apo B-containing lipoproteins with 0.44 mmol/L phosphotungstic acid (Merck, Darmstadt, Germany) and 20 mmol/L magnesium chloride (Sigma-Aldrich, Madrid, Spain). LDL-C levels were calculated using the Friedewald equation because none of the patients had TG levels over 3 mmol/L.

In the ACS study, hs-cTnT was measured using an electrochemiluminescent method (Elecsys Troponin T hs STAT) commercialized by Roche Diagnostics (Basel, Switzeland) and totally automatized to the Cobas e601 platform. TG, LDL-C, and CRP levels were measured enzymatically using commercial kits adapted for a COBAS 6000 autoanalyzer (Roche Diagnostics, Basel, Switzerland). Plasma HDL-C levels were measured after the precipitation of apo B-containing lipoprotein particles with 0.44 mmol/L phosphotungstic acid (Merck, Darmstadt, Germany) and 20 mmol/L magnesium chloride (Sigma-Aldrich, Madrid, Spain). Plasma creatinine, to estimate the eGFR (using the Chronic Kidney Disease Epidemiology Collaboration formula), was analyzed with an Architect c16000 analyzer (Abbott Diagnostic, Abbott Park, IL, US).

3. TMAO, γBB and TML measurements

The plasma determination of TMAO, γBB and TML was carried out at the Omic Sciences center, which is part of the omics technology infrastructure recognized as a Singular Scientific and Technical Infrastructure (ICTS) by the Ministry of Economy and Competitiveness created by the Rovira i Virgili University of Tarragona and Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM).

First, 25 μL of human plasma or serum was mixed and vigorously vortexed for 20 seconds with 300 μL of acetonitrile:methanol:water (5:4:1; v:v:v) containing two internal standards (IS). The internal standards used were d_3-methylcarnitine (d_3-MeCar) to quantify the γBB and TMAO concentrations and $^{13}C_3$-TML to quantify the TML, both at 5 ppm. After 30 minutes of re-equilibration on ice, the samples were centrifuged for 10 minutes at 25,100 g and 4ºC, and the supernatant was transferred to a vial prior to liquid chromatography mass spectrum (LC-MS) analysis.

Matrix-matched calibration curves were prepared using a human plasma pool spiked with internal standards. The concentration ranges of the calibration curves

were 0 - 250 μM, 0 - 25 μM and 0 - 20 μM for the TMAO, γBB and TML, respectively.

The extracts were analyzed using an ultra-high performance LC system coupled with a 6490 triple-quadrupole mass spectrometer (QqQ, Agilent Technologies) with an electrospray ion source (LC-ESI-QqQ) working in positive mode. An ACQUITY UPLC BEH HILIC column (1.7 mm, 2.1 × 150 mm, Waters) and a gradient mobile phase consisting of water with 50 mM of ammonium acetate (phase A) and acetonitrile (phase B) were used for chromatographic separation. The gradient was as follows: isocratic for 30 seconds at 75% B, from 0.5 to 2 minutes decreased to 65% B, from 2 to 2.1 minutes decreased to 45% B, from 2.1 to 3.9 minutes isocratic at 45% B, for 0.1 minute raised to 75% B, and, finally, column equilibrated at 75% B until 5.5 minutes.

The flow used in the method was 0.6 mL/min. Then, 2 μL of plasma extract was injected into the LC system. The mass spectrometer parameters were as follows: drying and sheath gas temperatures of 280°C and 400°C, respectively; source and sheath gas flows of 20 and 12 L/min, respectively; nebulizer flow of 60 psi; capillary voltage of 2500 V; nozzle voltage of 500 V; and iFunnel HRF and LRF of 110 and 80 V, respectively. The QqQ worked in multiple reaction monitoring (MRM) mode using defined transitions. The transitions for TML, $^{13}C_3$-TML (IS), γBB, TMAO, and d_3-MeCar(IS) and the collision energy (V) were: TML 189→84(17), 189→130(30); $^{13}C_3$-TML(IS) 192→84(21), 192→130(13); γBB 146→87(16), 146→60(12); TMAO 76→58(16), 76→59(8); d_3-MeCar (IS) 165→63(16), 165→103(16).

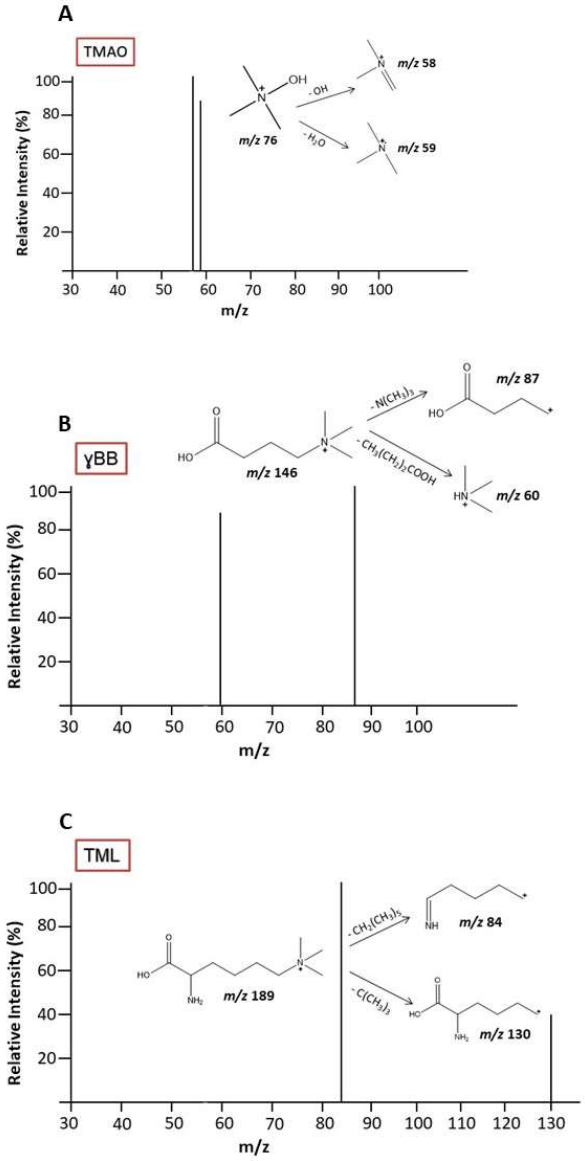

Figure 5. TMAO, γBB and TML transitions used in the LC-MS analysis with the fragments, m/z and relative intensity

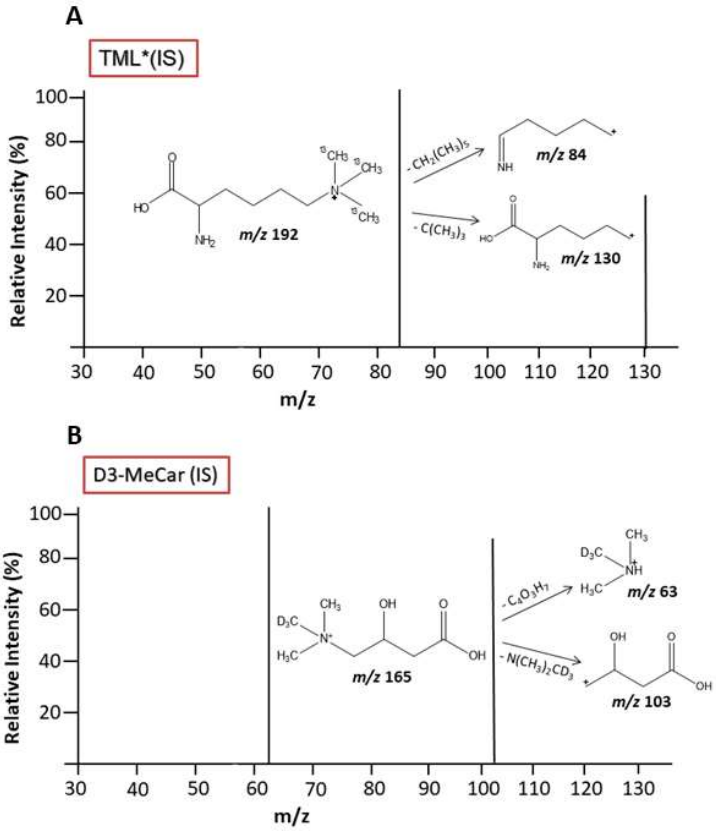

Figure 6. Internal standard transitions used in the LC-MS analysis with the fragments, m/z and relative intensity

4. Macrophage cholesterol efflux

4.1. Cell culture

J774A.1 (ATCC® TIB-67™) murine macrophage cell line was used. The vial containing the cells was stored in liquid nitrogen in vapor phase until the start of the experiments. The cells were seeded and expanded in a 75 cm^2 culture flask with complete growth medium in 37ºC incubators with 5% of CO_2 in air atmosphere.

The complete growth medium was high-glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) with heat-inactivated fetal bovine serum (FBS) to a final concentration of 10% v/v and penicillin/streptomycin (P/S) at 1% v/v of concentration. Cell cultures were observed daily by optic microscopy to detect any microbial contamination and to control the cell density. The medium was renewed every two days, with a previous wash with sterile phosphate-buffered saline (PBS). When the flask reached the confluence (an estimate density of 80 - 90%), cells were dislodged from the flask substrate using a cell scraper in a new, complete growth medium and dispensed into a new flask (the most common subcultivation ratio was 1:3 to 1:5).

4.2. Radioactive macrophage cholesterol efflux

J774A.1 cells were seeded at a density of 200,000 cells per well with DMEM complete growth medium in 6-well plates with 2 mL per well and were allowed to grow for 72 hours. Then, labeling medium was prepared by dispensing 1 μCi of [^{3}H]-cholesterol per mL of DMEM, supplemented with 5% FBS and P/S. The spent medium was removed from the wells and incubated with 1 mL per well of the labelling medium for 48 hours. Next, the labeling medium was poured out and the macrophages washed twice with warm sterile PBS and incubated with serum-free DMEM, supplemented with 0.2% fatty acid-free bovine serum albumin (BSA), for 18 hours, to allow equilibration of the radiolabeled cholesterol with the intracellular cholesterol pools. After equilibration, medium was removed and cells

washed with warm sterile PBS. The macrophages were then incubated for 4 hours with the cholesterol acceptor: 75 µL of apoB-depleted plasma (equivalent to 2.5% of plasma).

At the end of incubation, the medium from each well was collected and centrifuged at 1,000 g for 10 minutes to remove detached cells. Then, an aliquot of 200 µL from the upper fraction of the supernatant of cell-free medium was transferred to a scintillation vial. Four mL of scintillation liquid was added to the vials, vigorously mixed and put into the radioactivity counter. An aliquot was transferred to avoid removing sedimented cells from the bottom of the centrifugation vials. The total counts in the total volume of the medium (1 mL) were calculated by a correction factor.

Thereafter, 1 mL of 0.1 M NaOH were added to the plates and incubated on a shaker at 4ºC for almost 72 hours. Then, all the well content of was transferred to a scintillation vial with 4 mL of scintillation liquid and radioactivity measured. The MCE capacity was expressed as a percentage of total cholesterol using the following formula:

$$\text{cholesterol efflux (\%)} = \frac{\text{medium } [^3H]\text{-radioactivity (cpm)}}{\text{medium } [^3H]\text{-radioactivity (cpm)} + [^3H]\text{-radiactivity from cell extract (cpm)}} \times 100$$

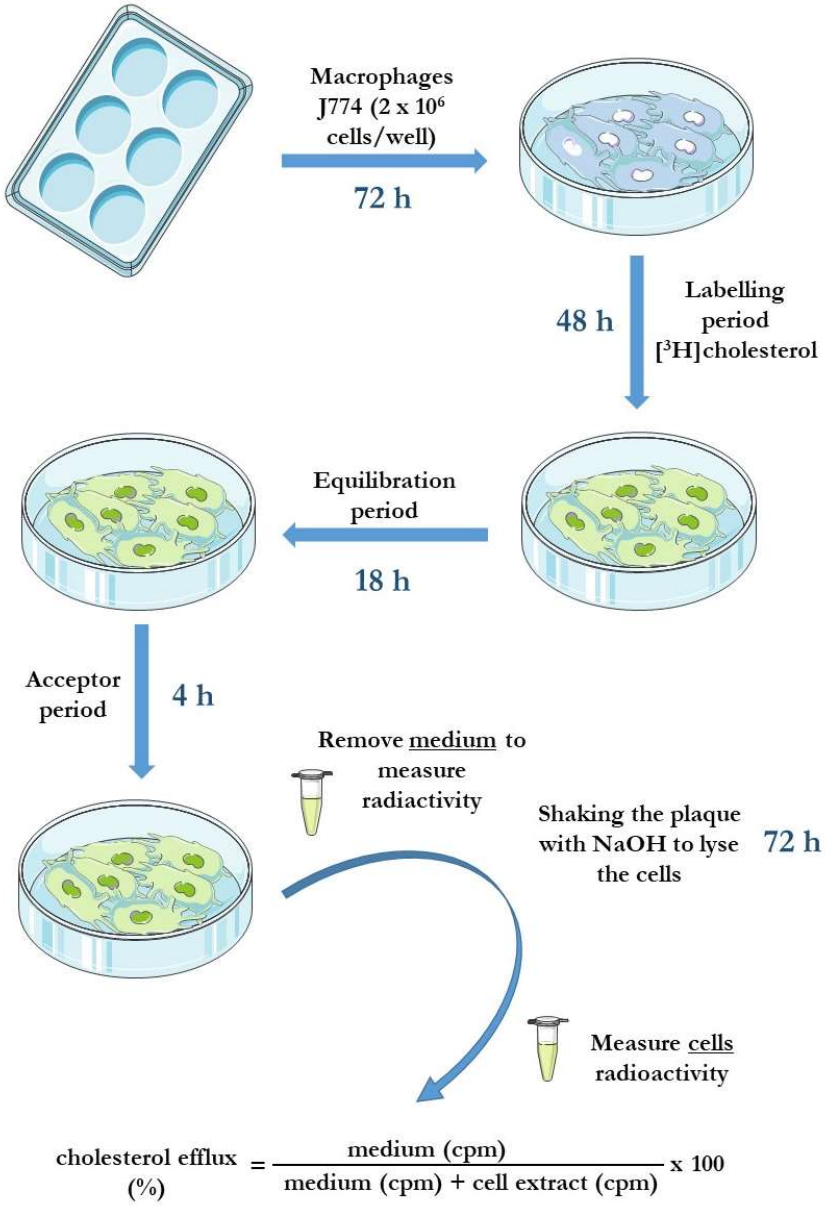

Figure 7. Methodological steps of radioactive cholesterol efflux

4.3. Fluorescent macrophage cholesterol efflux

Murine J774A.1 macrophages were seeded at a density of 75,000 cells per well with DMEM complete growth medium in a 48-well plate. Then, they were allowed to grow for 24 hours.

To label the macrophages, a fluorescent TopFluor-cholesterol probe (Avanti Polar Lipids, Alabaster, AL, US) was used. It consisted of a boron dipyrromethene difluoride (BODIPY) molecule anchored to cholesterol. The labelling medium was prepared with high-glucose DMEM containing 0.125 mM of total cholesterol, where the fluorescent cholesterol accounted for 20% and the rest was unlabeled cholesterol. The labelling medium was prepared the day before labelling. In summary, fluorescent and unlabeled cholesterol were diluted in DMEM medium complexed with 10 mmol/L of methyl-β-cyclodextrin (Sigma-Aldrich, Madrid, Spain). Then, the medium was sonicated for 50 minutes, shaken in a 37ºC bath for 3 hours and finally stored at 4ºC overnight.

After 24 hours, confluent monolayers were labelled for 1 hour. The labelled cells were subsequently equilibrated for 18 hours with DMEM containing 0.2% fatty-acid free BSA (Sigma-Aldrich, Madrid, Spain). Finally, the cells were incubated for 24 hours with 1% APOB-depleted plasma in a Roswell Park Memorial Institute (RPMI) medium. Acyl-CoA cholesterol acyltransferase inhibitor, Sandoz 58-035 (Sigma Aldrich, Madrid, Spain) at a concentration of 5 μmol/L, was present during the whole experimental procedure.

This experiment was performed under baseline conditions and also under experimental settings, mainly stimulating the concerted ABCA1/ABCG1-dependent efflux pathways by pre-treating the cells with 2 μmol/L of the liver X receptor agonist, T0901317 (Cayman Chemicals, Ann Arbor, MI, US), during the equilibration process or, alternatively, under experimental settings that only stimulated the ABCA1-dependent pathway by pre-treating the cells with 0.3 mmol/L of cyclic adenosine monophosphate (cAMP, Sigma-Aldrich, Madrid, Spain).

Fluorescence intensity was measured in the medium using the microplate reader, Synergy HT (BioTek Instruments, Winooski, VT, US), at λEx/Em = 485/530 nm. The cells were solubilized with 1% cholic acid and mixed on a plate shaker for 4 hours at room temperature. Before fluorescence quantification, cholesterol efflux capacity was calculated according to the formula: [media fluorescence / (media fluorescence + cells fluorescence)] × 100. All conditions were run in duplicate. Values were normalized using an APOB-depleted plasma pool within each efflux assay.

The experiment was also performed with TMAO, γBB or TML as possible efflux inductors. The process was the same but the macrophages were incubated with either 0, 5, 20, and 40 μM of TMAO, 0, 0.5, and 2 μM of γBB or 0, 0.5, 1, and 2 μM TML (Sigma-Aldrich, Madrid, Spain) for 24 h with a 1% APOB-depleted plasma pool. The pool of plasma samples from normolipemic patients was obtained from the Clinical Laboratory of the Hospital de la Santa Creu i Sant Pau. The cholesterol efflux capacity was determined as described above and expressed as a percentage after 24 hours.

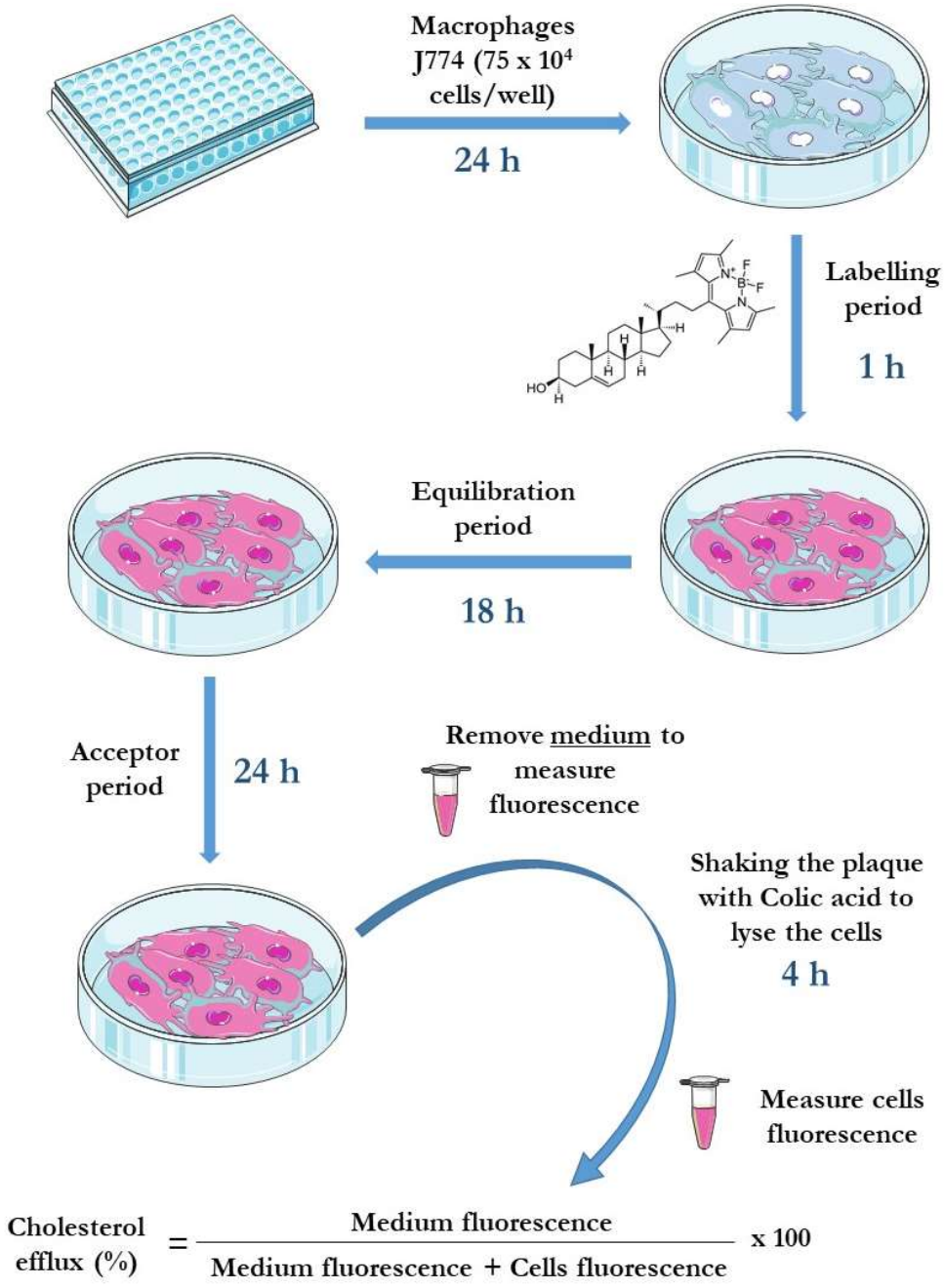

Figure 8. Methodological steps of fluorescent cholesterol efflux

5. Statistical methods

Data are presented as mean ± standard deviation (SD) for continuous variables and as frequencies and percentages for categorical variables. A chi-square test was used to compare the categorical data between groups. The normality of the data was analyzed using the Kolmogorov-Smirnov and D'Agostino and Pearson omnibus test.

A one-way analysis of variance (ANOVA) test was used to compare the continuous variables, and Tukey's post-test was used for comparing differences among groups. A Kruskal-Wallis test was used to compare continuous variables not following a Gaussian distribution, and Dunn's post-test was used for comparing differences among the groups. Correlations between variables were analyzed using Pearson's correlation analysis.

In the AAA study, multivariate lineal regression models were used to explore the association between efflux and the aortic baseline diameter and growth rate, adjusting for potential confounders. A multivariate Cox regression, analyzing tertiles as a categorical variable using the upper tertile as reference, was performed to explore the association between efflux and time to surgery, adjusting for potential confounders. Clinical confounders were chosen based on previous evidence that associated some clinical parameters and statin use with AAA.

In the ACS study, multivariate analysis of covariance (ANCOVA) was used to explore associations with mortality, adjusting for potential confounders. The potential confounders were chosen based on their association with the dependent variable or their association with mortality.

The statistical software R (http://www.r-project.org) and GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) were used to perform all statistical analyses. A p-value < 0.05 was considered as a significant difference.

~ RESULTS ~

Publication 1: Macrophage cholesterol efflux downregulation is not associated with abdominal aortic aneurysm (AAA) progression.

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Macrophage Cholesterol Efflux Downregulation Is Not Associated with Abdominal Aortic Aneurysm (AAA) Progression

Marina Canyelles [1,2], Mireia Tondo [1], Jes S. Lindholt [3], David Santos [4,5], Irati Fernández-Alonso [4], David de Gonzalo-Calvo [4,6,7], Luis Miguel Blanco-Colio [7,8], Joan Carles Escolà-Gil [2,4,5,*,†], José Luís Martín-Ventura [7,8,*,†] and Francisco Blanco-Vaca [1,2,5,*,†]

1 Servei de Bioquímica, Hospital de la Santa Creu i Sant Pau, IIB Sant Pau, 08041 Barcelona, Spain; mcanyelles@santpau.cat (M.C.); mtondo@santpau.cat (M.T.)
2 Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, 08041 Barcelona, Spain
3 Centre of Individualized Medicine in Arterial Disease (CIMA), Department of Cardiology, Odense University Hospital, 5000 Odense, Denmark; Jes.Sanddal.Lindholt@rsyd.dk
4 Institut de Recerca de l'Hospital de la Santa Creu i Sant Pau- IIB Sant Pau, 08041 Barcelona, Spain; dsantos@santpau.cat (D.S.); irati.fernandezalonso@gmail.com (I.F.-A.); david.degonzalo@gmail.com (D.d.G.-C.)
5 CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), 28029 Madrid, Spain
6 Institute of Biomedical Research of Barcelona (IIBB)–Spanish National Research Council (CSIC), 08036 Barcelona, Spain
7 CIBER de Cardiovascular (CIBERCV), Instituto de Salud Carlos III, 28029 Madrid, Spain; lblanco@fjd.es
8 IIS-Fundación Jiménez Díaz, 28040 Madrid, Spain
* Correspondence: jescola@santpau.cat (J.C.E.-G.); JLmartin@fjd.es (J.L.M.-V.); fblancova@santpau.cat (F.B.-V.)
† Senior authors.

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Abstract: Recent studies have raised the possibility of a role for lipoproteins, including high-density lipoprotein cholesterol (HDLc), in abdominal aortic aneurysm (AAA). The study was conducted in plasmas from 39 large size AAA patients (aortic diameter > 50 mm), 81 small/medium size AAA patients (aortic diameter between 30 and 50 mm) and 38 control subjects (aortic diameter < 30 mm). We evaluated the potential of HDL-mediated macrophage cholesterol efflux (MCE) to predict AAA growth and/or the need for surgery. MCE was impaired in the large aortic diameter AAA group as compared with that in the small/medium size AAA group and the control group. However, no significant difference in HDL-mediated MCE capacity was observed in 3 different progression subgroups (classified according to growth rate < 1 mm per year, between 1 and 5 mm per year or >5 mm per year) in patients with small/medium size AAA. Moreover, no correlation was found between MCE capacity and the aneurysm growth rate. A multivariate Cox regression analysis revealed a significant association between lower MCE capacity with the need for surgery in all AAA patients. Nevertheless, the significance was lost when only small/medium size AAA patients were included. Our results suggest that MCE, a major HDL functional activity, is not involved in AAA progression.

Keywords: abdominal aortic aneurysm; cardiovascular disease; cholesterol efflux; HDL; apoA-I; aortic diameter; growth rate; need for surgery; reverse cholesterol transport

1. Introduction

An abdominal aortic aneurysm (AAA) is defined as a permanent dilatation of the abdominal aorta above the threshold of a diameter of 30 mm, as determined using imaging techniques [1]. The prevalence of AAA ranges from 4–8% in men and 0–2% in women, based on population screening and large-scale randomized controlled trials [2,3]. The estimated prevalence of AAA in the US is over one million, with approximately half of AAA cases being women, nonsmokers and aged less than 65 years [4]. AAA is generally asymptomatic, and progressive aneurismal dilation is finally associated with the severe consequences of aortic rupture. To prevent AAA rupture, surgical repair is indicated when the aortic diameter exceeds 55 mm. Other than the AAA diameter, factors such as age, sex, body size and image characteristics should be considered in AAA evaluations [5]. For smaller aneurysms (30–50 mm), follow-up to monitor the growth rate is mandatory to estimate the median growth (mm per year) and/or rupture risk [6]. In these patients, there is no definitive pharmacological treatment. Recent studies showed that the use of statins and low-dose aspirin were associated with lower AAA growth rates and decreased progression [7,8]. However, major randomized double-blind trials are scarce, and no official recommendation regarding medical treatment exists in current guidelines [6,9].

The AAA pathophysiology remains unclear, but it is believed to be associated with alterations in the connective tissue in the aortic wall, mainly due to the high proteolytic activity produced by both infiltrating and resident cells, leading to a decrease in the amount of elastin [10]. A large number of exogenous immune cells, including neutrophils, lymphocytes and macrophages, infiltrate into the aortic tissue, eliciting a significant immune inflammatory response in the AAA wall. These inflammatory cells may enhance smooth muscle cell (SMC)-mediated secretion of matrix metalloproteinases, thereby impairing the stability and mechanical properties of the aortic wall, resulting in destruction of the medial extracellular matrix [11]. The AAA wall is also characterized by the presence of cholesterol crystals, which also induce inflammasome activation [12]. In this context, cholesterol accumulation enhances macrophage differentiation toward a pro-inflammatory state [13].

Among well-established risk factors for AAAs, dyslipidemia has grown in importance since a recent genetic meta-analysis and a Mendelian randomization study demonstrated the potential causal association of lipoproteins with this condition [14,15]. In a prospective study cohort of AAA patients, we found significantly decreased apolipoprotein A-I (apoA-I) (the main protein of high-density lipoprotein (HDL) particles) concentration, and plasma HDL cholesterol (HDLc) concentration predicted the aneurysmal growth rate [16]. However, strong evidence indicates that circulating HDLc levels may only represent a surrogate marker of atherogenesis. The ability of HDL to induce macrophage cholesterol efflux (MCE) is considered one of the main atheroprotective functions of HDL [17]. We recently reported that AAA patients showed impaired HDL-mediated MCE, which could be mechanistically linked to AAA. This inverse association was confirmed after adjusting for age, statin use, plasma lipids, apoA-I and HDLc levels [18]. However, this study included only AAA patients with a large aortic diameter (>50 mm). The association between HDL-mediated MCE and AAA progression has never been evaluated. In this study, we aimed to evaluate HDL-mediated MCE in a cohort of AAA patients with small/medium aortic diameters as a tool to test the potential of MCE to predict AAA growth and/or the need for surgery.

2. Materials and Methods

2.1. Study Design and Participants

All samples were obtained from a Danish cohort derived from the Viborg Vascular (VIVA) trial (URL: http://www.clinicaltrials.gov. Unique identifier: NCT00662480). The trial was approved by the regional ethics committee on Health Research Ethics (M20080025) on 28 March 2008. All the subjects gave informed consent. The study was performed in accordance with the ethical principles set forth in the Declaration of Helsinki. One hundred and fifty-eight male patients aged 64–74 years with different AAA sizes were randomly selected and classified into three groups according to their aortic

diameter, which was measured by abdominal ultrasound: a large size group (aortic diameter > 50 mm; $n = 39$, based in the US Aneurysm Detection and Management study), small/medium size group (aortic diameter between 30 and 50 mm; $n = 81$) and control group (aortic diameter < 30 mm; $n = 38$). This subset was selected from a large collection of plasmas from the VIVA trial [19], and HDLc/apoA-I levels as well as other clinical parameters were similar to the complete collection.

The large size group was referred for a computed tomography scan and vascular assessment. The small/medium size group underwent medical monitoring for clinical control to check for diameter expansion. Monitoring consisted of ultrasonographic follow-up of the aortic diameter (a minimum of two follow-ups in a 5-year period) to obtain a linear growth rate per year. Based on the rate, the patients were divided into three subgroups: low progression (growth rate of < 1 mm per year; $n = 26$), medium progression (growth rate between 1 and 5 mm per year; $n = 29$) and high progression (growth rate of > 5 mm per year; $n = 26$). The patients were assigned to surgery according to increases in the aortic diameter and evaluation of clinical parameters.

2.2. Lipid, Apolipoprotein and Lipoprotein Analyses

Whole blood samples were collected in Vacutainer® tubes and fractionated by centrifugation at 1300× *g* for 15 min at room temperature to obtain plasma. Plasma was aliquoted into 1.5 mL tubes and kept frozen at −80 °C until analysis. Plasma total cholesterol and triglyceride (TG) concentrations were determined enzymatically using commercial kits and a COBAS 501c autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland). ApoA-I levels were determined by an immunoturbidimetric assay (Roche Diagnostics). HDLc levels were measured in plasma obtained after precipitation of apoB-containing lipoprotein particles with phosphotungstic acid and magnesium ions (Roche Diagnostics). Low-density lipoprotein (LDL) cholesterol levels were calculated with the Friedewald equation.

2.3. Macrophage Cholesterol Efflux Assays

The MCE capacity of apoB-depleted plasma samples (equivalent to 5% of plasma containing mature HDL, nascent preβ-HDL particles and HDL regulatory proteins) was determined using J774.A1 [^{3}H]-cholesterol-labeled murine macrophages according to a previously described protocol [18,20]. Briefly, macrophages were seeded and grown for two days in the Roswell Park Memorial Institute (RPMI) growth medium. Macrophages were then incubated for 48 h with a loading medium containing 1 μCi of radiolabeled cholesterol/well. The cells were washed and incubated with a serum-free medium supplemented with fatty acid-free Bovine serum albumin (BSA) for 18 h to allow equilibration of the radiolabeled cholesterol with the intracellular cholesterol pools. After equilibration, the medium was removed, and the cell cultures washed. The macrophages were then incubated for 4 h in the presence of apoB-depleted plasma (equivalent to 5% of plasma), after which cholesterol efflux was determined and expressed as ([^{3}H]-cholesterol medium)/([^{3}H]-cholesterol cells medium) × 100. The samples were assayed in duplicate in five independent batches using six-well plates. To minimize the effects of intraplate variation, both AAA and control samples were included in each experiment.

2.4. Statistical Analysis

Data are presented as mean ± standard deviation (SD) for continuous variables and as frequencies and percentages for categorical variables. A chi-square test was used to compare the categorical data between groups. The normality of the data was analyzed using the Kolmogorov–Smirnov and D'Agostino and Pearson omnibus test. A one-way analysis of variance (ANOVA) test was used to compare the continuous variables, and Tukey's post-test was used for comparing differences among groups. Correlations between variables were analyzed using Pearson's correlation analysis. Multivariate lineal regression models were used to explore the association between efflux and the aortic baseline diameter and growth rate, adjusting for potential confounders. A multivariate Cox regression, analyzing tertiles as a categorical variable using the upper tertile as reference, was performed to explore the association between efflux and time to surgery, adjusting for potential confounders. Clinical

confounders were chosen based on previous evidence that associated some clinical parameters and statin use with AAA. The statistical software R (http://www.r-project.org) and GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) were used to perform all statistical analyses. A p-value < 0.05 was considered to represent a significant difference in all the analyses.

3. Results

The clinical and plasma biochemical characteristics of the patients and controls are shown in Table 1. The body mass index (BMI) and diastolic blood pressure (DBP) of the AAA patients were significantly higher than those of the control group, whereas the ankle-brachial index (ABI) was lower than that of the control group. No differences among the groups were observed in terms of smoking habits, diabetes, arterial hypertension, a history of cardiovascular events, use of statins and use of low-dose aspirin. As previously reported [16,18], the apoA-I concentrations in the AAA patients at presentation were significantly lower than those in the control group. Only patients with large size AAAs presented with significantly lower total cholesterol concentrations as compared with those in the control group, and this was concomitant with lower concentrations of LDL cholesterol. Patients with small/medium size AAAs had higher concentrations of very-low density lipoprotein VLDL cholesterol and TG. There was no significant difference in HDLc concentrations when this parameter was compared among the three groups.

Table 1. Clinical and plasma biochemical parameters of the abdominal aortic aneurysm (AAA) patients and controls.

Parameters	Large Size (n = 38)	Small/Medium Size (n = 81)	Control (n = 39)	ANOVA or Chi-Square p-Value
Age (y)	69.71 ± 2.88	69.65 ± 2.81	68.87 ± 2.69	ns
BMI (%)	28.03 ± 2.42 †	27.41 ± 3.48 †	25.80 ± 2.55	<0.01
Total cholesterol (mmol/L)	4.52 ± 0.71 †	4.87 ± 0.88	5.24 ± 0.77	<0.001
TG (mmol/L)	0.98 ± 0.40 #	1.44 ± 0.66 †	1.10 ± 0.36	<0.001
ApoA-I (g/L)	1.53 ± 0.28 †	1.59 ± 0.32 †	1.80 ± 0.32	<0.001
HDLc (mmol/L)	1.09 ± 0.43	1.09 ± 0.40	1.22 ± 0.42	ns
LDLc (mmol/L)	2.98 ± 0.81 †	3.13 ± 0.90	3.52 ± 0.89	<0.05
VLDLc (mmol/L)	0.45 ± 0.19 #	0.65 ± 0.26 †	0.50 ± 0.16	<0.001
Aortic diameter (mm)	62.52 ± 15.35 †,#	36.35 ± 4.54 †	18.16 ± 2.90	<0.001
DBP (mm Hg)	91.00 ± 13.62 †	88.00 ± 12.30 †	80.97 ± 11.36	<0.01
Lowest ABI	0.99 ± 0.11 †	0.95 ± 0.19 †	1.10 ± 0.09	<0.001
Smoking	8 (2%)	32 (41%)	15 (40%)	ns
Diabetes	3 (8%)	10 (13%)	4 (8%)	ns
Arterial hypertension	15 (40%)	41 (52%)	23 (61%)	ns
Previous CVD	1 (18%)	11 (14%)	7 (3%)	ns
Statin use	16 (42%)	41 (52%)	20 (53%)	ns
Low-dose aspirin	8 (50%)	35 (44%)	19 (22%)	ns

BMI = body mass index; TG = triglycerides; ApoA-I = apolipoprotein A–I; HDLc = High-density lipoprotein cholesterol; LDLc = low-density lipoprotein cholesterol; VLDLc = very low-density lipoprotein cholesterol; DBP = diastolic blood pressure; ABI = ankle brachial index; CVD = cardiovascular disease (acute myocardial infarction, angina or stroke); ANOVA = analysis of variance. Results expressed as mean ± standard deviation (SD). † $p < 0.05$ compared to the control group, # $p < 0.05$ compared to small/medium size, ns = non-significant.

The clinical and plasma biochemical characteristics of the small/medium size AAA groups, classified as subjects with low, medium and high AAA progression, are shown in Supplementary Materials Table S1. No significant differences were found for lipid, apoA-I and lipoprotein levels and almost all of the studied clinical parameters when they were compared among the three AAA progression groups, thereby indicating that these parameters were not related with AAA progression, at least in our sub-cohort of the VIVA trial. The only exception was the lower use of statins and aspirin in the high progression subgroup.

The ability of apoB-depleted plasma to induce MCE was evaluated in all the groups. MCE was impaired in the large size AAA group as compared with that in the small/medium size AAA group and the control group (Figure 1a). However, no significant differences in HDL-mediated MCE capacity were observed when the different AAA progression subgroups were compared (Figure 1b).

A significant linear trend was detected among the indicated groups after a one-way ANOVA analysis was performed (R square = 0.1010; $p < 0.0001$). The association between MCE capacity and AAA remained significant after adjusting for age, BMI and DBP (Supplementary Materials Table S2).

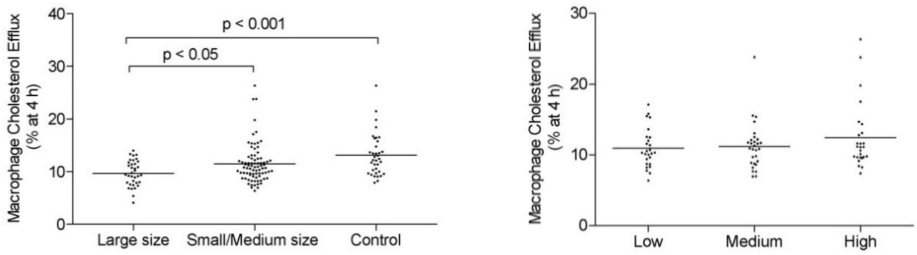

Figure 1. High-density lipoprotein (HDL)-mediated macrophage cholesterol efflux (MCE) capacity. (**a**) Large size abdominal aortic aneurysm (AAA) (n = 38), small/medium size AAA patients (n = 81) and control group (n = 39). (**b**) Small/medium size AAA group based on progression: low (n = 26), medium (n = 29) and high (n = 26) progression. Scatter dot blots are shown, and the line represents the mean. Differences were assessed using Tukey's multiple comparison test.

Univariate Pearson correlation tests revealed that the MCE capacity correlated negatively with the aortic baseline diameter and BMI and positively with apoA-I and HDLc in all subjects (Supplementary Materials Table S3). However, after adjusting for potential confounders, such as age, smoking, BMI, statin use and DBP, the MCE capacity did not correlate with the aortic baseline diameter (Table 2). Furthermore, when Pearson's correlation tests were performed only in the AAA patients, the significant correlation between the MCE capacity and aortic diameter disappeared, whereas BMI and the apoA-I and HDLc concentrations remained significant (Supplementary Materials Table S3).

Table 2. Multivariate linear regression of the aortic baseline diameter and macrophage cholesterol efflux (MCE) capacity in all subjects, adjusted for age, body mass index (BMI), smoking, statin use and diastolic blood pressure (DBP).

Coefficients			
Model	**Standardized Coefficients**	***t***	***p-Value***
	Beta		
Age	0.050	0.634	0.527
BMI	0.145	1.622	0.107
Smoke	0.027	0.325	0.745
Statins	0.103	1.273	0.205
DBP	0.275	3.457	0.001
MCE capacity	−0.115	−1.295	0.198
Dependent variable: aortic baseline diameter			

Moreover, no significant correlation was found between the MCE capacity and AAA growth rate in the small/medium size group (Table 3), even after adjusting for potential confounders (Supplementary Materials Table S4). Importantly, in the small/medium size AAA group, the positive associations

between MCE capacity and apoA-I and HDLc concentrations remained significant, as well as the negative association with BMI.

Table 3. Univariate correlations between high-density lipoprotein (HDL)-mediated macrophage cholesterol efflux (MCE) and abdominal aortic aneurysm (AAA) growth rate, apolipoprotein A-I (apoA-I), high-density lipoprotein cholesterol (HDLc) and body mass index (BMI) in the small/medium size AAA group.

	Growth Rate	ApoA-I	HDLc	BMI
MCE capacity in small/medium size AAA group	0.11 (−0.11–0.32)	0.36 (0.16–0.54)	0.37 (0.16–0.55)	−0.36 (−0.54–(−0.15))
p-value	ns	<0.001	<0.001	<0.01

Results expressed as r Pearson coefficient (95% confidence interval), ns = non-significant.

A multivariate Cox regression analysis was also conducted across HDL-mediated MCE tertiles to evaluate the association of the MCE capacity with the need for surgery, adjusted for potential confounders (smoking, a history of cardiovascular disease, use of low-dose aspirin, statins or angiotensin-converting enzyme inhibitors, DBP, BMI, lowest ABI and initial AAA diameter). When all the AAA patients were included (small/medium and large size groups), the need for surgical repair hazard ratio was significant after adjusting for potential confounders (Figure 2a and Supplementary Materials Table S5). However, this analysis did not reveal significant associations between the MCE capacity and the need for surgical repair when only small/medium size AAA patients were considered (Figure 2b and Supplementary Materials Table S6).

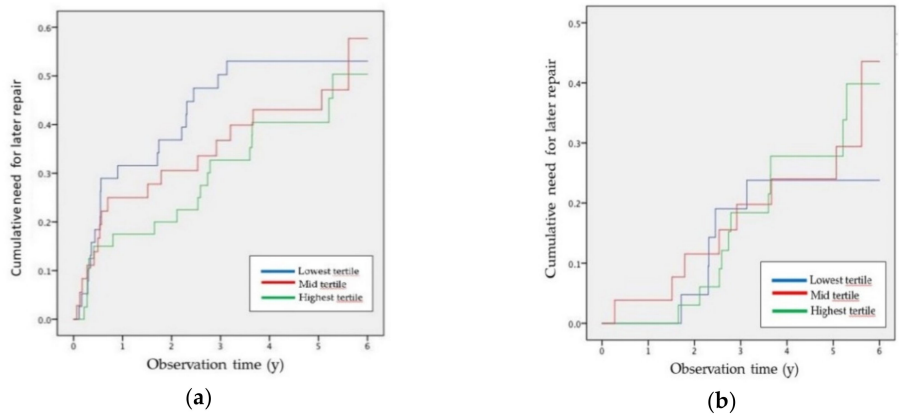

Figure 2. Cox regression of the cumulative need for surgery based on high-density lipoprotein HDL-mediated macrophage cholesterol efflux (MCE) tertiles in all AAA patients (**a**) and in small/medium size AAA patients (**b**). The observation time to surgery is represented in years. The cumulative need for later repair represents the need for surgery over the follow-up. The lowest MCE tertile is shown in blue, the mid tertile is shown in green and the highest tertile is shown in red.

4. Discussion

Recent reports support the concept that lipoproteins play a role in the pathogenesis of AAAs [14,15,21]. In agreement with our previous results in AAA subjects with large AAA diameter, LDLc and apoA-I were downregulated in the late stages of the disease [18]. However, cholesterol transported by HDL did not change and it is not considered a good surrogate marker of the lipoprotein antiatherogenicity. We recently demonstrated that that HDL-facilitated MCE, one of the potential main

surrogate markers of HDL function, was impaired in large size AAA patients [18]. In this study, in a larger cohort of AAA patients, we confirmed that HDL-facilitated MCE was downregulated in large size AAA patients compared with that of controls and small/medium size AAA patients. In a previous study, we also demonstrated oxidative modifications in some apoA-I residues of HDLs isolated from AAA tissue (>50 mm) obtained after surgery [22]. These apoA-I modifications were closely associated with reduced HDL-mediated MCE capacity in vitro and in vivo [22]. Myeloperoxidase-induced modification of apoA-I is mainly responsible for the loss of apoA-I cholesterol acceptor activity in AAAs by affecting the conformational stability of apoA-I and enhancing apoA-I displacement from HDLs and, therefore, catabolism [23]. Thus, oxidative modifications in apoA-I residues could partially explain the downregulation of HDL-mediated MCE capacity, particularly in late AAA stages.

Since we demonstrated that HDL-mediated MCE capacity was impaired in the late stages of AAA, a clinically relevant question is whether HDL-mediated MCE capacity is associated with the progression of the disease in small/medium size AAA patients. In this study, we evaluated the ability of apoB-depleted plasma, which includes mature HDL, nascent preβ-HDL particles and HDL regulatory proteins, to induce MCE in a cohort of AAA patients at different stages of AAA evolution. To our knowledge, our study is the first prospective study to evaluate the association of HDL-mediated MCE capacity with both the AAA growth rate and/or need for surgical repair in small/medium size AAA patients. When we evaluated the MCE capacity in the small/medium size AAA group, there were no differences in AAA progression, at least in terms of aneurysm growth. As the growth rate can be an incomplete reflection of the real progression of aneurysm [24], we also investigated the association of HDL-mediated MCE capacity with the time to surgery. When all the AAA patients were included (small/medium and large size groups), and after adjusting for potential confounders, the need for surgical repair was associated to lower MCE capacity. However, significance was lost when only small/medium size AAA patients were included. This was interpreted as a biased HR estimation caused by the inclusion of large size AAA patients, due to the impaired MCE capacity of these patients, whose surgical repair was already indicated. New cohort analyses with a larger number of events and more uniformity (for example, including only subjects with small AAAs between 30 and 40 mm) would be needed, however, to further confirm this point.

Overall, our results indicate that determining HDL function using MCE as a surrogate is irrelevant in terms of predicting AAA progression and need for surgical repair, as is also the case of HDLc. Indeed, HDLs display a wide variety of pleiotropic effects including antioxidant, anti-inflammatory, anti-protease, anti-thrombotic, anti-infectious, anti-apoptotic and vasodilatory roles that may be involved in AAA development. It should be noted that the injection of reconstituted discoidal HDLs reduced experimental AAA formation [25]. In line with these findings, we previously demonstrated that the injection of an apoA-I mimetic peptide (D4F) and overexpression of the main anti-inflammatory/antioxidant HDL enzyme paraoxonase (PON) 1 inhibited experimental AAA progression [16,26]. Furthermore, the serum activity of PON1 was reduced in a small cohort of AAA patients [26]. These results suggest that other HDL functions beyond MCE, such as their antioxidant and anti-inflammatory properties, may be significant determinants of AAA development and would warrant further study.

The present study has some limitations. We used a single sample collected to measure MCE. It would be interesting to test the variation of MCE in two sequential samples of the same small/medium AAA patients at two time points of their follow-up (at least on those who progress and would not require surgery) to evaluate whether the changes in MCE are correlated to changes in aortic diameter. In addition, it is important to note that to obtain correlation with AAA growth or time to surgery, we previously tested the whole VIVA cohort [27,28], so in this case, lack of power could also account for the obtained negative results. However, considering alpha = 0.05 and power = 80%, it was estimated that a difference of 2% in MCE could be detected by studying a minimum of 20 subjects in each group.

5. Conclusions

HDL-mediated MCE capacity was impaired in large size AAA patients but a lower MCE was not associated with the AAA growth rate and/or the need for surgical repair in small/medium size AAA patients, suggesting that this major HDL functional activity may not be mechanistically involved in AAA progression.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/4/662/s1, Table S1: Clinical and biochemical parameters of small/medium size AAA patients, Table S2: One-way ANCOVA of MCE in all subjects adjusted for age, BMI and DBP, Table S3: Univariate correlations in all subjects between HDL-mediated macrophage cholesterol efflux (MCE) capacity and aortic diameter, apoA-I, HDLc and BMI, Table S4: Multivariate lineal regression of Growth Rate with MCE capacity in small/medium size AAA group adjusted for age, BMI, smoke, statins and DBP, Table S5: Multivariate Cox regression analysis of 5-year predictors of need to surgery in all AAA patients. MCE tertiles were analyzed as a categorical variable using the upper tertile as reference, Table S6: Multivariate Cox regression analysis of 5-years predictors of need to surgery in small/medium AAA patients. MCE tertiles were analyzed as a categorical variable using the upper tertile as reference.

Author Contributions: Conceptualization, F.B.-V., J.L.M.-V. and J.C.E.-G.; experimental procedures, M.C., I.F.-A. and D.S.; formal statistical analysis, M.C., D.d.G.-C. and J.S.L.; writing—original draft preparation, M.C. and M.T.; writing—review and editing, L.M.B.-C., J.C.E.-G., F.B.-V. and J.L.M.-V. All authors have read and agreed to the published version of the manuscript.

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Apart from the published results of the thesis, TMAO and γBB were measured in AAA patients and a control group. No differences were found among patients with large AAA, patients with small/medium AAA and controls for both metabolites. There was only a slightly increase in TMAO levels in the small/medium AAA group compared to the controls. Neither were differences found when the small/medium AAA group was divided into low, medium and high progression.

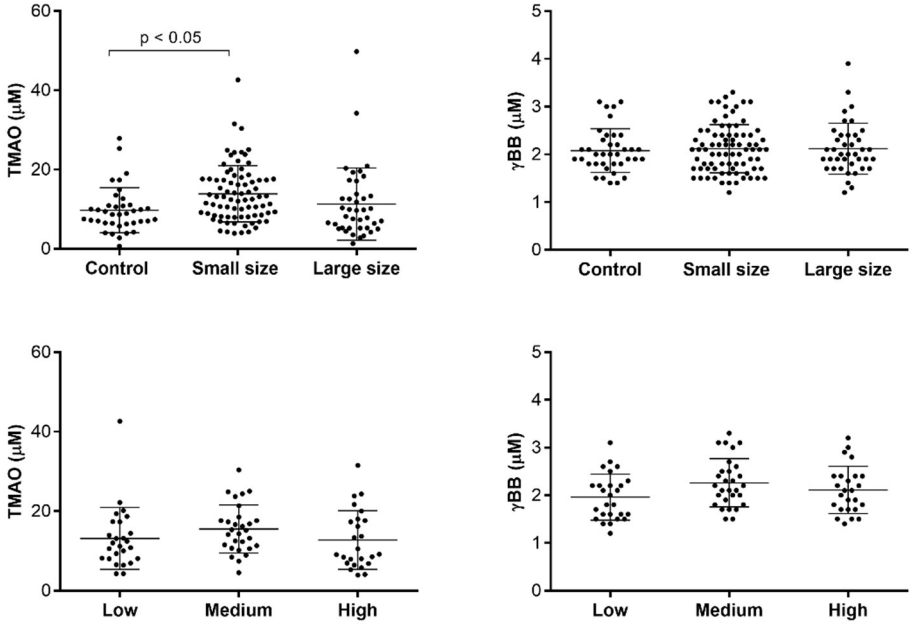

Figure 9. Plasma TMAO and γBB levels of AAA patients. Scatter dot blots are shown, and the line represents the mean. Differences were assessed using Tukey's multiple comparison test.

TMAO was not correlated with the parameters of AAA, aorta diameter and growth rate, nor with MCE.

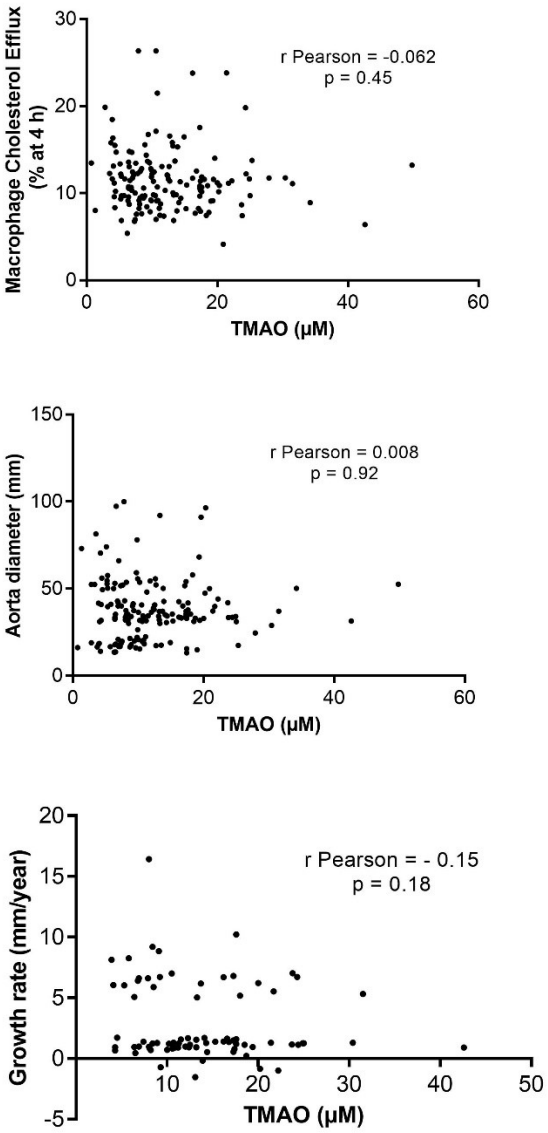

Figure 10. The correlation between TMAO and MCE, aorta diameter and growth rate. Results were presented as r Pearson.

Publication 2: The capacity of APOB-depleted plasma in inducing ATP-binding cassette A1/G1-mediated macrophage cholesterol efflux, but not gut microbial-derived metabolites, is independently associated with mortality in patients with ST-Segment elevation myocardial infarction.

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The Capacity of APOB-Depleted Plasma in Inducing ATP-Binding Cassette A1/G1-Mediated Macrophage Cholesterol Efflux—But Not Gut Microbial-Derived Metabolites—Is Independently Associated with Mortality in Patients with ST-Segment Elevation Myocardial Infarction

Marina Canyelles [1,2,3,4], Álvaro García-Osuna [2], Alexandra Junza [3,5], Oscar Yanes [3,5], Núria Puig [1,4], Jordi Ordóñez-Llanos [2,6], Alessandro Sionis [7,8], Jordi Sans-Roselló [7], Aitor Alquézar-Arbé [9], David Santos [1,3], Noemi Rotllan [1,3], Josep Julve [1,3], Mireia Tondo [2,3,*,†], Joan Carles Escolà-Gil [1,3,*,†] and Francisco Blanco-Vaca [2,3,4,*,†]

1 Institut de Recerca de l'Hospital Santa Creu i Sant Pau, Institut d'Investigacions Biomèdiques, IIB Sant Pau, 08041 Barcelona, Spain; mcanyelles@santpau.cat (M.C.); npuigg@santpau.cat (N.P.); daymer11@hotmail.com (D.S.); NRotllanV@santpau.cat (N.R.); JJulve@santpau.cat (J.J.)
2 Department of Clinical Biochemistry, Hospital de la Santa Creu i Sant Pau, IIB Sant Pau, 08041 Barcelona, Spain; agarciao@santpau.cat (Á.G.-O.); jordonez1952@gmail.com (J.O.-L.)
3 CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), 28029 Madrid, Spain; alexandra.junza@urv.cat (A.J.); oscar.yanes@urv.cat (O.Y.)
4 Department de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, 08041 Barcelona, Spain
5 Metabolomics Platform, Department of Electronic Engineering, Universitat Rovira i Virgili, 43204 Reus, Spain
6 Fundació per la Bioquímica i la Patologia Molecular, 08041 Barcelona, Spain
7 Servei de Cardiología, Hospital Santa Creu i Sant Pau, 08041 Barcelona, Spain; asionis@santpau.cat (A.S.); jordisansrosello@hotmail.com (J.S.-R.)
8 CIBER de Enfermedades Cardiovasculares (CIBERCV), 28029 Madrid, Spain
9 Servei d'Urgències, Hospital Santa Creu i Sant Pau, 08041 Barcelona, Spain; aalquezar@santpau.cat
* Correspondence: mtondo@santpau.cat (M.T.); jescola@santpau.cat (J.C.E.-G.); fblancova@santpau.cat (F.B.-V.)
† These authors contributed equally to this work.

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Abstract: Impaired HDL-mediated macrophage cholesterol efflux and higher circulating concentrations of trimethylamine N-oxide (TMAO) levels are independent risk factors for cardiovascular mortality. The TMAO precursors, γ-butyrobetaine (γBB) and Trimethyllysine (TML), have also been recently associated with cardiovascular death, but their interactions with HDL-mediated cholesterol efflux remain unclear. We aimed to determine the associations between APOB depleted plasma-mediated macrophage cholesterol efflux and plasma TMAO, γBB, and TML concentrations and explore their association with two-year follow-up mortality in patients with acute ST-elevation myocardial infarction (STEMI) and unstable angina (UA). Baseline and ATP-binding cassette transporter ABCA1 and ABCG1 (ABCA1/G1)-mediated macrophage cholesterol efflux to APOB-depleted plasma was decreased in patients with STEMI, and the latter was further impaired in those who died during follow-up. Moreover, the circulating concentrations of TMAO, γBB, and TML were higher in the deceased STEMI patients when compared with the STEMI survivors or UA patients. However, after statistical adjustment, only ABCA1/G1-mediated macrophage cholesterol efflux remained significantly associated with mortality. Furthermore, neither the TMAO, γBB, nor TML levels altered the HDL-mediated macrophage cholesterol efflux *in vitro*. We conclude that impaired ABCA1/G1-mediated macrophage cholesterol efflux is independently associated with mortality at follow-up in STEMI patients.

Keywords: HDL-mediated efflux; macrophages; trimethylamine N-oxide; trimethyllysine; myocardial infarction

1. Introduction

The ability of high-density lipoprotein (HDL) particles to stimulate cholesterol efflux from macrophage foam cells, the first step of reverse cholesterol transport (RCT), is one major recognized HDL cardioprotective function [1]. Macrophage cholesterol efflux to HDL occurs via different mechanisms, termed simple aqueous diffusion, facilitated by scavenger receptor class B type I (SR-BI) and active transport induced by the transmembrane protein ATP-binding cassette transporter ABCA1 and ABCG1 (ABCA1/G1)-mediated pathways. The importance of HDL-mediated cholesterol efflux in atheroprotection emerged from a study reporting a strong inverse association between the ex vivo cholesterol efflux capacity of APOB-depleted serum, measured in cultured mouse macrophage foam cells, and the carotid intima-media thickness and likelihood of angiographical-defined coronary artery disease [2]. Despite another report found that higher cholesterol efflux was associated with an increased prospective risk of a composite cardiovascular endpoint of incident myocardial infarction (MI), stroke, or death [3], two subsequent studies confirmed an inverse association of HDL-mediated cholesterol efflux and incident coronary heart disease risk independent of HDL cholesterol (HDL-C) concentrations [4,5]. The HDL-mediated cholesterol efflux ability in mouse macrophage foam cells was further found inversely associated with cardiovascular mortality in patients with chronic coronary artery disease [6–8]. Moreover, a recent report showed that the serum cholesterol efflux capacity measured in human macrophages was also a strong predictor of all-cause mortality after a MI, but the study did not find any association with HDL-mediated efflux or ABCA1-dependent and SR-BI-mediated serum efflux capacities [9].

Trimethylamine N-oxide (TMAO), a gut microbial-derived metabolite of choline and L-carnitine, among other dietary precursors, has been associated with major adverse cardiovascular events [9–11]. Higher plasma TMAO concentrations have also been associated with higher mortality risk in patients with heart failure [12,13] and chronic kidney disease [14]. Two recent reports investigated the potential of TMAO concentrations in risk stratification after an MI. The two studies found that TMAO was a predictive biomarker of all-cause [15] and cardiovascular death [16], but only one of the studies found a role of TMAO as a predictor of reinfarction [15]. Finally, high levels of TMAO in plasma were also independently correlated with plaque rupture in patients with ST-segment elevation MI (STEMI) [17]. Beyond TMAO, γ-butyrobetaine (γBB), a major gut microbial metabolite produced from dietary L-carnitine, and its precursor Trimethyllysine (TML), have been associated with cardiovascular mortality in patients with carotid atherosclerosis [18]. TML and TMAO were also associated with major adverse cardiovascular events and all-cause mortality amongst patients presenting with acute coronary syndrome [11]. However, only TML (and not TMAO nor γ-BB) was found to predict acute MI in patients who underwent coronary angiography [19].

A potential link among TMAO, liver, and intestine cholesterol homeostasis and HDL function has been described [20]. TMAO may enhance foam cell formation by upregulating macrophage scavenger receptors, but, in experimental models, it has also been shown to deregulate enterohepatic cholesterol and bile acid metabolism and impairs macrophage RCT [21,22]. However, it has also been described that TMAO enhances macrophage cholesterol efflux, at least in part due to the upregulation of *Abca1* and *Abcg1* in peritoneal mouse macrophages [22]. Nonetheless, some studies have also reported TMAO downregulating *Abca1* gene expression in murine macrophages [23,24].

Altogether, although the prognostic value of macrophage cholesterol efflux and TMAO in predicting cardiovascular mortality in patients with MI has been reported, the association of both parameters together with other TMAO precursors has never been explored. Here, we aimed to evaluate the associations among HDL-mediated macrophage cholesterol efflux, TMAO, γBB, and TML with cardiovascular mortality in patients in the acute phase of STEMI and test whether the gut microbial-derived metabolites modulate the associations between HDL-mediated macrophage cholesterol efflux and mortality.

2. Experimental Section

2.1. Study Population and Data Collection

Blood samples from patients with STEMI (diagnosed according to the principles of the Universal Definition of Myocardial Infarction) [25,26] were obtained from a retrospective study that included 253 patients consecutively admitted to the Hospital de la Santa Creu i Sant Pau. The patients were followed for two years for cardiovascular adverse events, all-cause death, and hospital readmissions via telephone interview and/or electronic medical record review. The primary outcome of this study was cardiovascular death defined as death by cardiogenic shock, fatal MI, or heart failure. We selected a sub-sample of STEMI patients: 35 who died during admission or during follow-up and 36 who survived. In all STEMI patients, the Global Registry of Acute Coronary Events (GRACE 2.0) risk score was calculated [27]. A subgroup of 33 patients with suggested symptoms of MI but high-sensitive cardiac troponin T (hs-cTnT) concentrations below the cut-off used to define myocardial damage (14 ng/L) or not reaching an hourly increase >3.0 ng/L during three-hour serial sampling was diagnosed with unstable angina (UA) and used as the control group. All the subjects gave informed consent. The study was performed in accordance with the ethical principles set forth in the Declaration of Helsinki. UA and STEMI survivors were matched with the deceased STEMI group for age, sex, body mass index, arterial hypertension, and diabetes mellitus and smoking status.

2.2. Blood Samples and Biochemical Measurements

Blood samples were collected in EDTA anticoagulated tubes for the UA patients upon their admission and immediately before the angiographic procedure performed at the time of event in the STEMI patients. Plasma was obtained via centrifugation (10 min, at 10,000× *g*), hs-cTnT was measured in STAT-mode (Cobas e601, Roche Diagnostics, Basel, Switzerland), and aliquots were stored at −80 °C until analysis. Triglyceride, low-density lipoprotein cholesterol (LDL-C), and C-reactive protein (CRP) levels were measured enzymatically using commercial kits adapted for a COBAS 6000 autoanalyzer (Roche Diagnostics, Basel, Switzerland). Plasma HDL-C levels were measured after the precipitation of apolipoprotein (APO) B-containing lipoprotein particles with 0.44 mmol/L phosphotungstic acid (Merck, Darmstadt, Germany) and 20 mmol/L magnesium chloride (Sigma-Aldrich, Madrid, Spain). Plasma creatinine, to estimate the glomerular filtration rate (eGFR) (using the CKD-EPI formula), was analyzed with an Architect c16000 analyzer (Abbott Diagnostic, Chicago, IL, USA).

2.3. Ex Vivo and In Vitro Cholesterol Efflux Capacity

The ex vivo cellular cholesterol efflux was determined using TopFluor-cholesterol, a fluorescent cholesterol probe in which the cholesterol molecule is linked to a boron dipyrromethene difluoride (BODIPY) moiety (Avanti Polar Lipids, Alabaster, AL, USA). J774A.1 cells (7.5×10^4/well) were seeded in 48-well plates and allowed to grow for 24 h in a DMEM-supplemented medium. Next, macrophages were labeled for 1 h in a high-glucose DMEM medium (Lonza, Waltham, MA, USA) containing 0.125 mmol/L total cholesterol, where the fluorescent cholesterol accounted for 20% of the total cholesterol complexed with 10 mmol/L methyl-β-cyclodextrin (Sigma-Aldrich, Madrid, Spain), as reported in [28]. The labeled cells were subsequently equilibrated for 18 h with DMEM containing 0.2% fatty-acid free BSA (Sigma-Aldrich, Madrid, Spain) and then incubated for 24 h with 1% APOB-depleted plasma in a Roswell Park Memorial Institute (RPMI) medium. The acyl-CoA cholesterol acyltransferase (ACAT) inhibitor, Sandoz 58-035 (5 µmol/L; Sigma Aldrich, Madrid, Spain), was present during the whole experimental procedure. This experiment was performed under baseline conditions and also under experimental settings mainly stimulating the concerted ABCA1/ABCG1-dependent efflux pathways by pre-treating the cells with 2 µmol/L of the liver X receptor agonist, T0901317 (Cayman Chemicals, Ann Arbor, MI, USA), during the equilibration process or, alternatively, under experimental settings that only stimulated the ABCA1-dependent pathway by pre-treating the cells with

0.3 mmol/L of cyclic adenosine monophosphate (cAMP, Sigma-Aldrich, Madrid, Spain). The fluorescence intensity was then measured in the medium using the microplate reader, Synergy HT (BioTek Instruments, Winooski, VT, USA), at λEx/Em = 485/530 nm. The cells were solubilized with 1% cholic acid and mixed on a plate shaker for 4 h at room temperature, and the fluorescence intensity was quantified. The cholesterol efflux capacity was calculated according to the formula: [media fluorescence/(media fluorescence + cells fluorescence)] × 100. All conditions were run in duplicate. Values were normalized using an APOB-depleted plasma pool within each efflux assay.

In the in vitro study, macrophages were pre-treated with 2 μmol/L of T0901317 and then incubated with 0, 5, 20, and 40 μM of TMAO, 0, 0.5, and 2 μM of γBB or 0, 0.5, 1, and 2 μM TML (Sigma-Aldrich, Madrid, Spain) for 24 h with 1% of and APOB-depleted plasma pool. The pool of plasma samples from normolipemic patients was obtained from the Clinical Laboratory of Hospital de la Santa Creu i Sant Pau. The cholesterol efflux capacity was determined as described above and expressed as % at 24h.

2.4. Plasma TMAO, γBB and TML Determinations

First, 25 μL of human plasma was mixed and vigorously vortexed for 20 s with 300 μL of acetonitrile: methanol: water (5:4:1; *v*:*v*:*v*) containing two internal standards (IS) for quantification. The internal standards used were d_3-methylcarnitine (d_3-MeCar) to quantify the γBB and TMAO concentrations and $^{13}C_3$-TML to quantify the TML—both at 5 ppm. After 30 min of re-equilibration on ice, the samples were centrifuged for 10 min at 25,100× *g* and 4 °C, and the supernatant was transferred into the vial prior to the LC-MS analysis. To quantify the analytes in the plasma, matrix-matched calibration curves were prepared using a human plasma pool spiked with standards. The concentration ranges of the calibration curves were 0–250 μM, 0–25 μM and 0–20 μM for the TMAO, GBB and TML, respectively.

The extracts were analyzed using an ultra-high performance LC system coupled with a 6490 triple-quadrupole mass spectrometer (QqQ, Agilent Technologies, Santa Clara, CA, USA) with an electrospray ion source (LC-ESI-QqQ) working in positive mode. An ACQUITY UPLC BEH HILIC column (1.7 mm, 2.1 × 150 mm, Waters) and a gradient mobile phase consisting of water with 50 mM ammonium acetate (phase A) and acetonitrile (phase B) were used for chromatographic separation. The gradient was as follows: isocratic for 30 s at 75% B, from 0.5 to 2 min decreased to 65% B, from 2 to 2.1 min decreased to 45% B, from 2.1 to 3.9 min isocratic at 45% B, for 0.1 min raised to 75% B, and, finally, column equilibrated at 75% B until 5.5 min. The flow of the method was 0.6 mL/min. Then, 2 μL of plasma extract was injected in the LC system. The mass spectrometer parameters were as follows: drying and sheath gas temperatures of 280 °C and 400 °C, respectively; source and sheath gas flows of 20 and 12 L/min, respectively; nebulizer flow of 60 psi; capillary voltage of 2500 V; nozzle voltage of 500 V; and iFunnel HRF and LRF of 110 and 80 V, respectively. The QqQ worked in MRM mode using defined transitions. The transitions for TML, $^{13}C_3$-TML (IS), GBB, TMAO, and d_3-MeCar(IS) and the collision energy (CE(V)) were: TML 189→84(17), 189→130(30); $^{13}C_3$-TML(IS) 192→84(21), 192→130(13); GBB 146→87(16), 146→60(12); TMAO 76→58(16), 76→59(8); d_3-MeCar (IS) 165→63(16), 165→103(16).

2.5. Statistical Methods

Data is presented as the mean ± standard deviation (SD) for continuous variables and as frequencies and percentages for categorical variables. A chi-square test was used to compare the categorical data between groups. The normality of the data was analyzed using the Kolmogorov–Smirnov and D'Agostino and Pearson omnibus tests. A one-way analysis of variance (ANOVA) test was used to compare the continuous variables, and Tukey's post-test was used for comparing differences among the groups. A Kruskal-Wallis test was used to compare continuous variables not following a Gaussian distribution, and Dunn's post-test was used for comparing differences among the groups. Correlations between variables were analyzed using Pearson's correlation analysis. Multivariate analysis of

covariance (ANCOVA) was used to explore the associations with mortality, adjusting for potential confounders. The statistical software, IBM SPSS Statistics v23, and GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA) were used to perform all statistical analyses. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Study Subjects

The baseline clinical and plasma biochemical characteristics of patients admitted with UA and those with STEMI who survived or died in a two-year follow-up period are shown in Table 1. The triglyceride concentrations did not differ among the three patient groups. HDL-C concentrations were significantly lower in patients with STEMI compared to those with UA but not different between the survivors and deceased STEMI patients. The deceased STEMI patients showed significantly lower LDL-C when compared with the survivors, and the latter presented higher LDL-C concentrations than the UA patients. Logarithmically transformed plasma CRP values were higher in patients with STEMI compared with those in the UA group, and this value was higher in the deceased STEMI patients when compared with the survivors (Table 1). The eGFR was also significantly lower in the deceased STEMI patients when compared with the STEMI survivors and those admitted with UA (Table 1). Also, the GRACE risk score and hs-cTnT were higher in the STEMI deceased patients when compared with the STEMI survivors (Table 1). The percentage of STEMI patients treated with statins was 38% for survivors and 44% for deceased (p = 0.811).

Table 1. Clinical and biochemical parameters of the studied patients at admission.

	UA n **= 33**	**STEMI Survivors** n **= 36**	**STEMI Deceased** n **= 35**	p**-Value**
Age (years)	71.97 ± 3.45	72.06 ± 7.3	73.41 ± 9.86	0.6656
Sex (M/F)	17/16	20/16	19/15	0.923
Body mass index (Kg/m^2)	26.38 ± 2.86	26.66 ± 2.06	27.07 ± 4.07	0.6774
Hypertension (%)	56	72	71	0.3525
Diabetes mellitus (%)	21	25	41	0.1903
Smoking (%)	17	25	35	0.0859
Triglycerides (mmol/L)	1.07 ± 0.47	0.97 ± 0.4	1.12 ± 0.73	0.5012
HDL-C (mmol/L)	1.63 ± 0.44	1.33 ± 0.34 **	1.19 ± 0.31 ****	<0.0001
LDL-C (mmol/L)	2.21 ± 0.64	2.81 ± 1.01 **	2.12 ± 0.89 ††	0.0022
Log_{10} C-reactive protein (mg/L)	3.30 ± 0.63	3.78 ± 0.71 *	4.42 ± 0.87 **** ††	<0.0001
eGFR (mL/min/1.73 m^2)	77.74 ± 13.94	69.66 ± 18.21	51.11 ± 20.50 **** ††††	<0.0001
GRACE 2.0 risk score	ND	188.5 ± 44.23	251.6 ± 44.35 ††††	<0.0001
Log_{10} hs-cTnT (ng/L)	3.90 ± 0.17	5.36 ± 0.79 ****	6.06 ± 0.72 †††† ****	<0.0001

UA: unstable angina; STEMI: ST-segment elevation myocardial infarction; HDL-C and LDL-C: high- and low-density lipoprotein cholesterol; eGFR: estimated glomerular filtration rate by CKD-EPI equation; GRACE 2.0: Global Registry of Acute Coronary Events. ND, non-determined. Results are presented as mean ± standard deviation (SD). * p < 0.05 ** p < 0.01 **** p < 0.0001 vs. UA†† p < 0.01 †††† p < 0.0001 vs. STEMI survivors.

3.2. ABCA1 and ABCG1-Mediated Macrophage Cholesterol Efflux to APOB-Fepleted Serum Ex Vivo Is Down Regulated in Deceased STEMI Patients

The ex vivo ability of APOB-depleted plasmas in inducing macrophage cholesterol efflux was evaluated in all the groups under baseline conditions and experimental settings stimulating only the ABCA1 or both the ABCA1/G1 pathways. The baseline macrophage cholesterol efflux was significantly lower in the deceased STEMI patients when compared with the STEMI survivors and those with UA (Figure 1A). The ABCA1-mediated macrophage cholesterol efflux was also decreased in the patients who died when compared with the UA group (Supplementary Materials, Figure S1). This change was also observed after stimulating both the ABCA1/G1-dependent efflux pathways, but, in this case, the macrophage cholesterol efflux was also more decreased in the STEMI survivors when compared with the UA patients (Figure 1B).

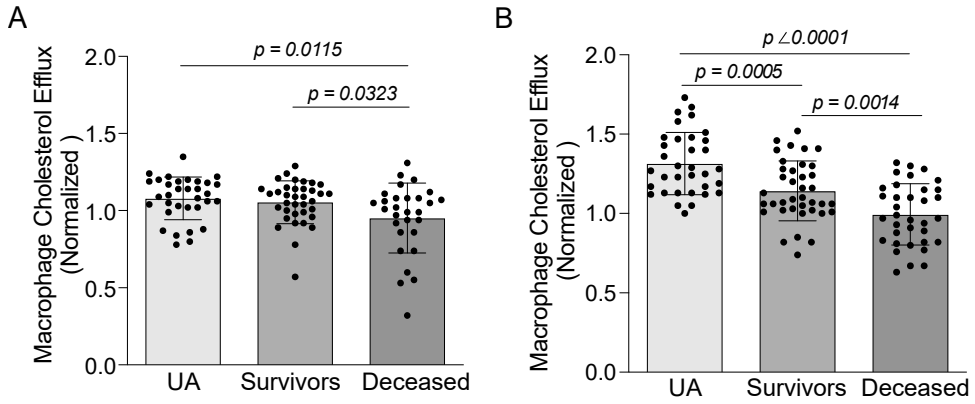

Figure 1. Macrophage cholesterol efflux is impaired in STEMI patients who died within a two-year follow-up period under baseline conditions (ANOVA p value = 0.0096) (**A**) and after activation of macrophage ABCA1/G1-dependent pathways by the LXR agonist, T090137 (ANOVA p value < 0.0001) (**B**). Values are represented as mean ± SD for UA (n = 33), STEMI survivors (n = 36) and deceased STEMI (n = 35).

3.3. Circulating Levels of TMAO, γBB, and TML Are Increased in STEMI Patients Who Died during Follow-Up

Plasma concentrations of TMAO, γBB and TML were assessed in the same samples that APOB-depleted plasma-mediated cholesterol efflux was determined. The levels of these metabolites were significantly higher in the deceased STEMI patients compared to the STEMI survivors and UA patients (Figure 2).

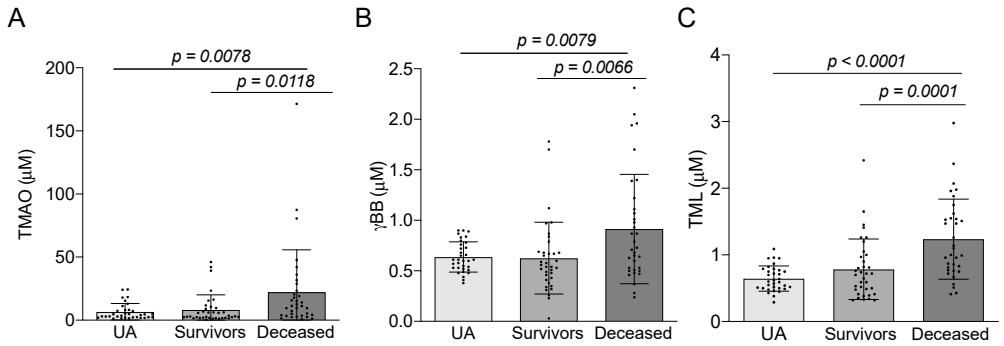

Figure 2. Plasma TMAO (ANOVA p value = 0.0040) (**A**); γBB (ANOVA p value = 0.0029) (**B**) and TML (ANOVA p value < 0.0001) (**C**) levels were higher in deceased STEMI patients than in the other two groups. Values are represented as mean ± SD in UA patients (n = 33), STEMI survivors (n = 36), and deceased STEMI patients (n = 35).

3.4. ABCA1/G1-Mediated Macrophage Cholesterol Efflux Is Independently Associated with Mortality

We further evaluated the association between APOB-depleted plasma-mediated macrophage cholesterol efflux and mortality in these patients after adjusting for the main covariates that were significantly correlated with macrophage cholesterol efflux, including HDL-C, TMAO, and eGFR (all with $p < 0.05$). The association between the ABCA1/G1-mediated macrophage cholesterol efflux and death remained significant after adjusting for these parameters in (a) of (Table 2). However, both the baseline and ABCA1-mediated macrophage cholesterol efflux were not significantly associated with death following adjustment for these factors (Supplementary Materials Table S1). Furthermore, the ABCA1/G1-mediated macrophage cholesterol efflux association with death also remained significant

when TML (adjusted R-squared = 0.469; $p = 0.009$) or γBB was included in the analyses (adjusted R-squared = 0.483; $p = 0.009$). Next, we further adjusted for other covariates that correlated with the ABCA1/G1-mediated macrophage cholesterol efflux ($p < 0.05$) which strongly predict STEMI mortality, such as hs-cTnT, GRACE score, HDL-C and CRP. The association of the ABCA1/G1-mediated macrophage cholesterol efflux with death still remained significant after adjustment in (b) of (Table 2).

Table 2. Analysis of covariance (ANCOVA) of ABCA1/G1-mediated macrophage cholesterol efflux at 24 h adjusted for (a) HDL-C, eGFR and TMAO that significantly correlated with the dependent variable or (b) hs-cTnT, GRACE score, HDL-C and CRP, factors strongly associated with mortality.

(a)					
Source	**SS**	**Df**	**Mean Square**	**F**	***p* Value**
Corrected model	2.610 *	5	0.522	18.403	0.000
Intercept	2.287	1	2.287	80.636	0.000
HDL-C	0.776	1	0.776	27.371	0.000
eGFR	0.043	1	0.043	1.509	0.222
TMAO	0.000	1	0.000	0.011	0.917
Death	0.331	2	0.166	5.838	0.004
Error	2.666	94	0.028		
Total	139.441	100			
Corrected total	5.275	99			
* R-squared = 0.495 (adjusted R-squared = 0.468)					
(b)					
Source	**SS**	**Df**	**Mean Square**	**F**	***p* Value**
Corrected model	0.755 *	5	0.151	4.652	0.001
Intercept	0.119	1	0.119	3.674	0.060
hs-cTnT	0.034	1	0.034	1.054	0.309
GRACE score	0.007	1	0.007	0.213	0.646
HDL-C	0.440	1	0.440	13.553	0.000
CRP	0.000	1	0.000	0.008	0.931
Death	0.188	1	0.188	5.804	0.019
Error	2.012	62	0.032		
Total	81.888	68			
Corrected total	2.767	67			
* R-squared = 0.114 (adjusted R-squared = 0.058)					

Also, we evaluated the association between gut microbial-derived metabolites and mortality in these patients after adjusting for the main determinant of these metabolites, eGFR, and also for the main parameter of interest, the ABCA1/G1-mediated macrophage cholesterol efflux. The association between TMAO (adjusted R-squared = 0.099; $p = 0.088$) or γBB (adjusted R-squared = 0.104; $p = 0.123$) with death did not remain significant after adjusting for these parameters. In contrast, TML retained its independent association with death after adjustment for these parameters in (a) of (Table 3). However, the independent association between TML and mortality did not persist after adjustment for hs-cTnT, GRACE score and CRP in (b) of (Table 3).

3.5. TMAO, γBB, and TML Did Not Affect APOB-Depleted Plasma-Mediated Macrophage Cholesterol Efflux In Vitro

Finally, we determined the effects of TMAO, γBB, and TML on the capacity of an APOB-depleted plasma pool to induce macrophage cholesterol efflux *in vitro*. For this purpose, the macrophages were incubated with increasing concentrations of all metabolites

during the efflux period of 24 h. Importantly, TMAO, γBB and TML did not modify the ABCA1/G1-mediated macrophage cholesterol efflux to an APOB-depleted plasma pool (Figure 3A–C).

Table 3. Analysis of ANCOVA of circulating TML levels adjusted for (a) eGFR and normalized ABCA1/G1-mediated efflux or (b) hs-cTnT, GRACE score and CRP.

(a)					
Source	**SS**	**df**	**Mean Square**	**F**	***p* Value**
Corrected model	7.423 *	4	1.856	10.054	0.000
Intercept	4.155	1	4.155	22.512	0.000
eGFR	1.415	1	1.415	7.665	0.007
Normalized ABCA1/G1-mediated efflux	0.002	1	0.002	0.011	0.917
Death	1.934	2	0.967	5.239	0.007
Error	17.535	95	0.185		
Total	103.990	100			
Corrected total	24.958	99			
* R Squared = 0.297 (Adjusted R Squared = 0.268)					
(b)					
Source	**SS**	**df**	**Mean Square**	**F**	***p* Value**
Corrected model	5.948 *	4	1.487	5.554	0.001
Intercept	0.051	1	0.051	0.191	0.663
hs-cTnT	0.005	1	0.005	0.018	0.894
GRACE score	1.733	1	1.733	6.471	0.013
CRP	1.455×10^{-5}	1	1.455×10^{-5}	0.000	0.994
Death	0.596	1	0.596	2.224	0.141
Error	17.137	64	0.268		
Total	94.710	69			
Corrected total	23.086	68			
* R-squared = 0.258 (adjusted R-squared = 0.211)					

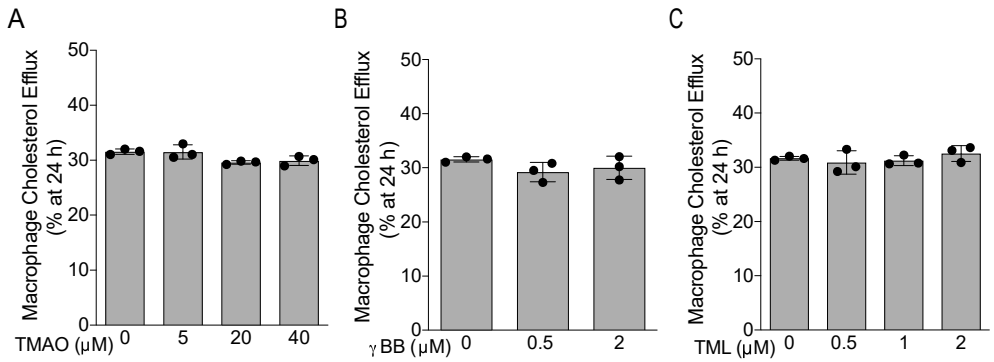

Figure 3. Effects of TMAO, γBB, and TML on ABCA1/G1-mediated macrophage cholesterol efflux to APOB-depleted plasma. The macrophage cholesterol efflux capacity was expressed as % of fluorescence released at 24 h. TMAO (**A**), γBB (**B**), and TML (**C**) levels did not affect the macrophage cholesterol efflux to the APOB-depleted plasma under the conditions stimulating the ABCA1/G1-dependent pathways. Kruskal-Wallis and Dunn's post-test did not reveal significant differences among the groups incubated with the gut microbial-derived metabolites versus the control group without TMAO, γBB, and TML. Three independent experiments were performed for each condition.

4. Discussion

Macrophage cholesterol efflux is largely induced by HDL particles. A recent meta-analysis showed that the macrophage cholesterol efflux capacity was inversely associated with the risk of cardiovascular disease and mortality, although the authors also admitted the heterogeneity among the included studies and evidence of publication bias [29]. Part of this heterogeneity could be explained by the methodology used to isolate the HDL particles; many studies have analyzed cholesterol efflux after the chemical precipitation of APOB-containing lipoproteins to isolate HDL particles from serum or plasma, while others used different approaches. Three independent studies showed that the impaired cholesterol efflux capacity of APOB-depleted serum or plasma from mouse macrophages was associated with higher cardiovascular mortality in patients with chronic coronary artery disease [6–8]. In these studies, mouse J774.A1 macrophages were incubated with cAMP to upregulate ABCA1, thereby suggesting the major contribution of a dysfunctional ABCA1-mediated pathway in this cellular model. However, a recent report failed to find any significant association between ABCA1 or SR-BI-mediated cholesterol efflux and the cardiovascular mortality of MI patients, even though it did show that their serum cholesterol efflux capacity was a strong predictor of mortality after a MI [9]. It is noteworthy that this report used human THP-1 macrophages pretreated with acetyl-LDL, which stimulates both ABCA1/G1-mediated efflux [9]. Our work adds to current knowledge that the impaired ABCA1/G1-mediated cholesterol efflux capacity from mouse J774.A1 macrophages to HDL is independently associated with mortality in STEMI patients. Our results reinforce the importance of measuring different components of macrophage cholesterol efflux capacity, integrating both the ABCA1-dependent pathway, which facilitates cholesterol release to lipid-free apoA-I/preβ-HDL, and the ABCG1-dependent efflux to mature α-migrating HDL particles. These assays better mimic the two predominant pathways of cholesterol-loaded macrophages converted into foam cells in atherosclerotic lesions [30].

Our analyses revealed that patients with STEMI who had higher plasma TMAO, γBB, and TML were more likely to experience subsequent death. However, the associations between TMAO or γBB and death were attenuated when further adjusted for other factors. Despite several reports showing that TMAO independently predicted future cardiovascular adverse events [11,31], not all reports have found independent associations between TMAO levels and cardiovascular outcomes [32–34]. The strong influence of renal function on TMAO levels could explain, at least in part, the inconsistency of TMAO in predicting adverse cardiovascular outcomes [32,33].

TML can be obtained from diet but also from endogenous production via the methylation of lysine residues in histones and, most likely, under the proteolytic degradation of other proteins; furthermore, TML is a poor precursor of TMAO [31]. Of note, the association of TML and death remained significant after adjusting for the main gut microbial-derived metabolite determinants in our study, but it was lost after adjusting for major covariates predicting STEMI mortality [35]. However, other studies have shown TML as an independent prognostic marker of major adverse cardiovascular events and mortality risk [11,19,31]. Our results do not necessarily contradict those obtained in patients with suspected stable angina pectoris [19], in subjects without evidence of acute coronary syndrome who underwent elective diagnostic coronary angiography [31], or those who had UA or adjudicated acute coronary syndromes [11]. Therefore, TML levels could provide insight regarding cardiovascular risk assessment but, at least among patients with prior STEMI, were found to not offer additional information for predicting mortality. Overall, our findings do not support the role of TML as a pure specific marker for STEMI mortality.

Previous reports have shown divergent results regarding TMAO-mediated effects on macrophage transporters involved in cholesterol efflux, specifically indicating enhancing [22], neutral [24], or downregulating effects [23]. The present study goes further and demonstrates that TMAO, γBB, and TML, at physiological levels, did not produce significant effects on macrophage cholesterol efflux, indicating that the first step in the RCT process was not affected by these three compounds. Taken together, our data does not

support any implication of TMAO, γBB, and TML in modulating the association between ABCA1/G1-mediated macrophage cholesterol efflux and STEMI mortality.

The present study has some limitations. The number of patients in our cohort was relatively modest, but it was compensated for the fact that we selected homogeneous and well-characterized patient groups, thus providing enough power to conduct our analyses.

5. Conclusions

We demonstrate that reduced ABCA1/G1-mediated macrophage cholesterol efflux is independently associated with mortality in STEMI patients. Consistent with the gradual severity of acute coronary syndrome, STEMI patients who survived presented higher ABCA1/G1-mediated macrophage cholesterol efflux than those who died. TMAO, γBB, and TML did not affect either macrophage cholesterol efflux to APOB-depleted plasma or the association between ABCA1/G1-mediated macrophage cholesterol efflux and mortality. These results motivate further research on therapeutic strategies aiming to improve the ABCA1/G1-mediated macrophage cholesterol efflux capacity.

Supplementary Materials: The Supplementary Materials are available online at https://www.mdpi.com/article/10.3390/biomedicines9101336/s1, Figure S1: Macrophage cholesterol efflux after activation of macrophage ABCA1 by cAMP, Table S1: Analysis of ANCOVA of Baseline (a) and ABCA1-mediated (b) macrophage cholesterol efflux at 24 h.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study will be available to other researchers upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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~ DISCUSSION ~

Gofman et al. were the first ones describing a negative correlation between plasma HDL levels and atherosclerotic cardiovascular disease (ASCVD) [198]. In 1977, the Framingham Heart Study confirmed the inverse association between cardiovascular risk and plasma HDL-C, as well as the direct association with cardiovascular risk and LDL-C in a large cohort of patients [181]. Large clinical trials have proved that taking statins to lower LDL-C is useful in cardiovascular risk reduction strategies [199,200]. However, patients treated with statins still have a substantial residual clinical risk and additional therapeutic strategies are needed to achieve more effective risk reduction [201]. In this context, the focus on the prevention of cardiovascular risk turned to HDL-C levels. It was hypothesized that pharmacology treatments which increase HDL-C levels could have an impact on residual cardiovascular risk. However, HDL cholesterol-raising strategies have failed to demonstrate efficacy against CVD. Among all HDL cholesterol-raising treatments, CETP inhibitors have been the most studied. However, all clinical trials failed to demonstrate reduction in cardiovascular risk or death related to HDL changes [184].

A static measurement of HDL-C does not reflect the complexity of all of the functions of HDL particles. Rather than crude HDL-C concentrations, the cumulative evidence suggests that a quantitative measure of HDL functionality may be a better predictor of cardiovascular risk. Among all the HDL functions, the ability to extract cholesterol from macrophages within atherosclerotic plaques may be crucial in HDL protection against CVD.

The results of the present thesis confirm that patients with high cardiovascular risk, specifically patients with AAA and patients with STEMI, have an impaired HDL-mediated MCE. However, this impairment did not have the same prognostic value in both of the diseases studied. MCE was not related to AAA progression; however, it was a mortality predictor in STEMI patients.

In a previous publication written by our group, we demonstrated for the first time, that HDL-mediated MCE was impaired in two independent cohorts of patients

with large AAA (>50 mm diameter). These changes were not related with HDL-C levels, supporting the concept that cholesterol transported by HDL is not a good surrogate marker of HDL functionality [202]. The presence of oxidative modifications in apoA-I residues of HDLs isolated from AAA tissues obtained after surgery in these patients was closely associated with reduced HDL-mediated MCE capacity both *in vivo* and *in vitro* [203]. Myeloperoxidase-induced modification of apoA-I is likely one of the main factors responsible for the loss of apoA-I cholesterol acceptor activity in AAA patients, by affecting apoA-I conformational stability and enhancing apoA-I displacement from HDLs and, therefore, catabolism [204]. Thus, oxidative modifications in apoA-I residues could partially explain the downregulation of HDL-mediated MCE capacity, particularly in later stages of AAA. However, clinical guidelines recommend elective abdominal aortic aneurysm repair when the diameter is ≥55 mm. Therefore, patients with a large AAA went directly into surgery; whereas patients with small/medium AAA (aorta 30 - 50 mm diameter) were monitored [11]. The clinically relevant question we aimed to answer is whether HDL-mediated MCE capacity is associated with the progression of the disease in patients with small/medium size AAA. Our study demonstrated that, apparently, HDL-mediated MCE is not a useful biomarker of progression since we reported no differences in HDL-mediated MCE in patients with AAA at different stages of progression. We did, however, validate the differences found between patients with large AAA and the control group, thereby expanding the number of patients previously reported in Martínez-López et al [202].

HDL-mediated MCE did not correlate with the diameter or growth rate of AAA. These were the two most common parameters used in AAA diagnostic and prognostic studies [205]. AAA growth rate was historically taken as a reference for measuring the progression of and for deciding on the need for surgical repair. However, this may be an incomplete reflection of the aneurysm progression. Time-to-surgery may be more useful than growth rate to estimate aneurysm progression [206]. Interestingly, when all patients with AAA were included

(small/medium and large AAA groups), and after adjusting for potential confounders, the need for surgical repair was associated with lower MCE capacity. However, significance was lost when only patients with small/medium AAA were included. This was interpreted as a biased Hazard Ratio estimation caused by the inclusion of patients with large AAA, with impaired MCE capacity, for whom surgical repair was already prescribed.

To our knowledge, our study is the first prospective study to evaluate the association of HDL-mediated MCE capacity with both AAA growth rate and/or the need for surgical repair in patients with small/medium AAA. The results of our study were in contrast to those in experimental models where the injection of different forms of human HDL had a beneficial effect on reducing the diameter and inhibiting AAA progression [207,208]. Moreover, injection of an apoA-I mimetic peptide (D4F), and overexpression of the main anti-inflammatory/antioxidant HDL enzyme paraoxonase (PON) 1, inhibited AAA progression [209,210].

HDLs display a wide variety of pleiotropic effects, including antioxidant, anti-inflammatory, anti-protease, anti-thrombotic, anti-infectious, anti-apoptotic, and vasodilatory roles that may be involved in AAA development. A previous study reported a negative association between the ratio of apoA-I/HDL-C and AAA progression and surgical repair [210]. Another study showed that the serum activity of PON1 was reduced in a small cohort of AAA patients [209]. These results suggest that other HDL functions beyond MCE, such as their antioxidant and anti-inflammatory properties, might be significant determinants of the development of AAA and would warrant further study.

Clearly, lipids have a role in AAA development, since the human AAA is characterized by the presence of cholesterol crystals and macrophage infiltration [211,212]. Moreover, histological studies performed in human AAA tissues suggested an impaired AAA cholesterol efflux, as judged by elevated apoA-I and decreased ABCA1 expression compared to normal aortic tissues [213]. AAA lesions also presented a different lipid content compared to normal aortic walls in animal

models. However, hyperlipidemia did not affect AAA development in mice, as it did not correlate with the lesion.

From these results, we can conclude that MCE is impaired in patients with large AAA but its use as a prognostic biomarker is unlikely since it was not associated with the growth rate, nor with time to surgery, in patients with an AAA with a diameter between 30 and 50mm (small/medium AAA).

Apart from the published results of this thesis, we also analyzed TMAO, which has been associated with CVDs including atherosclerosis, stroke, and thrombosis. We only found a slight increase in the small/medium AAA group compared to the control group. No differences were found between the three progression groups, and neither between TMAO and aortic diameter, growth rate, and MCE. To the best of our knowledge, there are no publications that have studied the association of gut metabolites with AAA. Recently, a study revealed that the gut microbiome in AAA mice models was different from that of control mice. AAA diameter correlated with some genera such as *Akkermansia*, *Odoribacter*, *Helicobacter*, and *Rumincoccus* [214]. However, none of these genera produce TMA. Thus, TMAO levels would not be expected to be different between AAA and control mice, as was the case in our results.

Regarding the second study, we also found that MCE was impaired in STEMI patients compared to UA patients; moreover, STEMI patients who died also had significantly reduced MCE. Indeed, MCE was associated with cardiovascular mortality, after adjustment for factors strongly associated with mortality. UA patients were used as a control group in order to simulate an inflammatory and acute background similarly to STEMI, but without myocardial injury.

The association between MCE and ASCVD has been widely studied. The first study was carried out in 2011 and concluded that MCE was inversely correlated with carotid intima-media thickness and CAD risk in middle-aged individuals [193]. These results were then validated in an independent prospective study with

ASCVD-free adults who were followed up for 9.4 years [194]. These two studies boosted interest in studying the association between MCE and CVD, in terms of major adverse cardiovascular events (MACE), cardiovascular mortality as well as all-cause mortality. However, not all the studies found similar results. In another study, MCE inversely correlated with prevalent ASCVD, but positively correlated with prospective MI, stroke, and death among older patients [215]. A recent meta-analysis showed that MCE was inversely associated with CVD risk and mortality [216]. Another subsequent meta-analysis partially agreed with these results: every standard deviation increase of MCE was associated with a 20% lower risk of MACE, also after adjusting for known cardiovascular risk factors. However, the high MCE group did not have a significantly different risk of all-cause mortality or cardiovascular mortality. Although the association of MCE with all-cause mortality was not statistically significant, the authors observed a trend towards lower mortality with higher MCE [217]. It should be noted that this meta-analysis also revealed that the performance of MCE as a prognostic indicator of cardiovascular risk among patients with CKD was limited, compared to patients with normal renal function [217]. Previous studies in CKD patients showed that MCE was not a prognostic cardiovascular risk marker [218,219]. Another study revealed that there was a significant interaction between MCE and CKD on the associations with ASCVD and CVD. In individuals without CKD, MCE was inversely associated with incident ASCVD and total CVD. Conversely, in those with CKD, MCE was directly associated with ASCVD and cardiovascular mortality and was not associated with total CVD [220]. In contrast to these results, we found an association of MCE with cardiovascular mortality, although STEMI survivors had a mildly decreased renal function and STEMI patients who died had CKD. These previous studies were carried out on patients with CKD without concomitant diseases, thereby indicating that MCE may not be a good prognostic biomarker in these patients. However, in STEMI patients with a secondary renal dysfunction, MCE seems to be a useful marker as a prognosis of cardiovascular mortality.

There is, however, heterogeneity among all these studies in previous meta-analyses. There is also evidence of publication bias. Part of this heterogeneity could be explained by the methodology used to isolate the HDL particles. Many of the studies analyzed MCE after the chemical precipitation of apoB-containing lipoproteins to isolate HDL particles from serum or plasma, while others used different approaches. Moreover, the design of the studies was diverse, including case-control or cohort studies, and prospective or cross-sectional approaches. Also, the populations studied were diverse and different donor cell and labelling methods were used.

Three independent studies showed that the impaired MCE of apoB-depleted serum or plasma from mouse macrophages was associated with higher cardiovascular mortality in patients with chronic CAD [221–223]. Concomitantly, adding MCE to a model containing traditional cardiovascular risk factors significantly increased discriminatory power and predictive ability of all-cause and cardiovascular mortality in CAD patients [221–223]. MCE was slightly but significantly higher in the ACS group compared with the stable CAD group. Despite this increase, MCE was an independent predictor of all-cause and cardiovascular mortality in both ACS and stable CAD patients, independent of circulating HDL-C levels [222].

Similarly to what we described, other studies carried out on cohorts of patients with ACS also found an impairment in MCE in patients with ACS compared to both the group with stable CAD and the healthy control group. Reduced MCE was associated with an increased relative risk of ACS, an increased risk of ASCVD outcomes (non-fatal MI and non-fatal stroke) and cardiovascular mortality after a follow-up of 3 years [224]. The impairment of MCE seems to be persistent over time, because no differences were found between the baseline and 12 weeks after the event; MCE was also significantly decreased compared to the healthy control group [225].

All of the above studies used the same type of donor cell - mouse J774.A1 macrophages - and the same labelling method - BODIPY-cholesterol. Our study used exactly the same type of donor cell and labelling method, so we are able to compare the results without having to take technical limitations into consideration. Overall, it was proved that there is an MCE impairment in CAD patients, and more specifically in those with ACS. Different mechanisms could be implicated in the impairment of MCE in ACS patients. The pro-oxidative/proinflammatory status of HDL particles, HDL particle number, HDL particle size and apoA-I levels related to HDL particles have been related to ASCVD [226,227]. We did not measure any of these parameters so, their implication in our association of MCE with cardiovascular mortality could not be ruled out.

It should be noted that MCE in all of these studies was measured using cAMP in the equilibrating period to upregulate ABCA1, thereby suggesting a major contribution of a dysfunctional ABCA1-mediated pathway in this cellular model. However, a recent report failed to find any significant association between ABCA1 or SR-BI-mediated cholesterol efflux and cardiovascular mortality of MI patients, even though it did show that their decreased serum MCE was a strong predictor of mortality after an MI [228]. It is noteworthy that this report used human THP-1 macrophages pretreated with acetyl-LDL, which stimulates both ABCA1/G1-mediated efflux. This different result could also be explained by the use of a different cholesterol acceptor, since they used total serum instead of apoB-depleted plasma or serum. HDL is the acceptor of nearly 90% of MCE, but the process is dynamic, and free cholesterol obtained from the macrophages can be rapidly transferred to LDL.

The role of LDL in efflux is controversial. Some studies found a positive correlation between LDL-C and cholesterol efflux capacity [225] while others did not [224]. One recent study carried out by our group showed that, at least in mice, a shift in macrophage-derived free cholesterol from HDL to LDL provides a significant route for macrophage cholesterol to reach the liver via the hepatic LDL receptor

[229]. This cholesterol transport, in the form of cholesteryl esters, from HDL to LDL is probably highly increased in humans with respect to rodents, due to the presence in human serum of CETP activity.

Our work adds to current knowledge that the impaired ABCA1/G1-mediated cholesterol efflux capacity from mouse J774.A1 macrophages to HDL is independently associated with mortality in STEMI patients. Our results reinforce the importance of measuring different components of macrophage cholesterol efflux capacity, integrating both the ABCA1-dependent pathway, which facilitates cholesterol release to lipid-free apoA-I/preβ-HDL, and the ABCG1-dependent efflux to mature α-migrating HDL particles. These assays better mimic the two predominant pathways of cholesterol-loaded macrophages converted into foam cells in atherosclerotic lesions [172].

Our findings that MCE is an independent predictor of cardiovascular mortality were corroborated after adjustment for strong mortality predictors, including hs-TnT, GRACE Score, HDL-C and CRP. To the best of our knowledge, no similar studies have been carried out on STEMI patients. Mody et al. examined the incremental ability of cholesterol efflux capacity to improve ASCVD risk prediction beyond validated biomarkers in clinical practice. They found that in a fully adjusted model including all traditional risk factors, prevalent coronary artery calcium, MI familiar history, and elevated hs-CRP, MCE remained inversely associated with incident ASCVD without attenuation [230].

Our analyses revealed that there is a significant increase in plasma TMAO, γBB, and TML levels in STEMI patients compared to patients with UA, and also in STEMI patients who died compared to STEMI survivors. The three gut metabolites were linked with atherosclerotic progression and cardiovascular disease in studies in both mice and humans. Of the three, the metabolite that has been studied the most is TMAO, because it constitutes the final product, whereas γBB and TML are intermediate precursors of TMA oxidation in the liver [231]. The differences found in the gut metabolites are in accordance with the differences

found in the gut microbial population of STEMI patients compared to patients with UA. This difference indicates a shift in the community composition of gut microbes that correlates with serum levels of the gut microbiota-related metabolite TMAO and the occurrence of post-STEMI cardiovascular events [232].

After Wang et al. linked TMAO with CVD in 2011, several studies have been carried out, with controversial results. While some reported that TMAO independently predicted future MACE, others did not find independent associations between TMAO levels and cardiovascular outcomes [147,233]. TMAO is almost exclusively cleared by the kidneys, so its levels are clearly influenced by renal function. The inconsistency of the studies of the association of TMAO and CVD could be explained by the differences in renal function among the individuals studied. A renal impairment confounds the association of TMAO and cardiovascular outcomes, so it should be corrected to obtain a real association [132].

In the specific context of ACS or MI, only TMAO and TML have been studied previously. In the case of TMAO, one previous study showed similar results to those obtained in our publication. TMAO was associated with MACE and cardiovascular mortality [234]. However, this significance was slightly attenuated after adjustment for strong mortality associated biomarkers including hs-TnT and CRP [234]. Conversely, TMAO levels were associated with both near-term risk of MACE and long-term mortality, among patients of two independent ACS cohorts. The association was maintained after adjustment for traditional cardiovascular risk factors including HDL-C, eGFR, and cardiac troponins [235]. TMAO levels were associated with poor prognosis, estimated as death or MI at 2 years, for patients hospitalized due to acute MI, but in this case no adjustments for potential confounders were made [236].

In our study, TML was, among the three metabolites, the only one that maintained a significant association with mortality after adjusting for the main gut microbial-derived determinants. However, the association was lost after adjustment for mortality predictors, including hs-TnT, GRACE Score, HDL-C, and CRP.

The first study published including TML revealed a dose-dependent relationship between higher TML levels and incident risk for MACE and mortality in subjects without evidence of ACS who underwent elective diagnostic coronary angiography. Moreover, TML provided independent and additive prognostic value in combination with TMAO for prediction of MACE risk. This independence seemed to be because TML can be obtained from endogenous production via lysine methylation of histones and under the proteolytic degradation of other proteins. TML obtained through the diet is less significant [121]. Furthermore, another study by the same group demonstrated that TML was independently and additively (with TMAO and traditional risk factors) associated with MACE and mortality risk in an ACS cohort [237]. Plasma TML levels were also associated with MACE, even in patients who were consistently negative for Troponin T values [237]. An independent study by another group showed that TML was associated with an increased MI risk in patients with suspected CHD, also after adjustment for traditional CVD risk factors and renal function [238]. This group also demonstrated that TML was associated with long-term all-cause mortality, as well as with cardiovascular mortality in two large prospective cohorts of subjects with or without CHD [239]. In conclusion, TML levels may provide insight for the assessment of cardiovascular risk but, at least among patients with prior STEMI, were not found to offer additional information for predicting mortality. Overall, our findings do not support the role of TML as a specific marker for cardiovascular mortality in STEMI patients.

Previous reports have shown divergent results for TMAO-mediated effects on macrophage transporters involved in cholesterol efflux, specifically indicating enhancing, neutral, or downregulating effects [149,240,241]. Our results go further and demonstrate that TMAO, γBB, and TML, at physiological levels, did not produce significant effects on MCE, indicating that the first step in the RCT process was not affected by these three compounds.

~ CONCLUSIONS ~

1. HDL-mediated MCE was impaired in patients with large AAA compared to control groups. No MCE differences were found among 3 different progression groups in patients with small/medium AAA.

2. HDL-mediated MCE was not associated with the aneurysm growth rate and/or the need for surgical repair in patients with small/medium AAA, suggesting that this major HDL functional activity may not be mechanistically involved in disease progression.

3. Patients with STEMI had an impaired HDL-mediated MCE compared to UA patients. Moreover, this impairment was higher in STEMI patients who died during the follow-up, compared to survivors.

4. ABCA1/G1-mediated macrophage cholesterol efflux is independently associated with cardiovascular mortality in STEMI patients.

5. TMAO, γBB, and TML did not affect either macrophage cholesterol efflux to apoB-depleted plasma, or the association between ABCA1/G1-mediated macrophage cholesterol efflux and STEMI mortality.

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~ ANNEXES ~

Annex 1: Trimethylamine N-oxide: a link among diet, gut microbiota, gene regulation of liver and intestine cholesterol homeostasis and HDL function

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Review

Trimethylamine *N*-Oxide: A Link among Diet, Gut Microbiota, Gene Regulation of Liver and Intestine Cholesterol Homeostasis and HDL Function

Marina Canyelles [1], Mireia Tondo [1,*], Lídia Cedó [2,3], Marta Farràs [2,4], Joan Carles Escolà-Gil [2,3,*] and Francisco Blanco-Vaca [1,3,5]

1 Hospital de la Santa Creu i Sant Pau, Servei de Bioquímica-Institut d'Investigacions Biomèdiques (IIB) Sant Pau, 08041 Barcelona, Spain; mcanyelles@santpau.cat (M.C.); fblancova@santpau.cat (F.B.-V.)
2 Institut de Recerca de l'Hospital Santa Creu i Sant Pau-Institut d'Investigacions Biomèdiques (IIB) Sant Pau, 08025 Barcelona, Spain; lcedo@santpau.cat (L.C.); mfarras@santpau.cat (M.F.)
3 CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), 08907 Barcelona, Spain
4 CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), ISCIII, 08003 Barcelona, Spain
5 Departament de Bioquímica, Biologia Molecular i Biomedicina, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
* Correspondence: mtondo@santpau.cat (M.T.); jescola@santpau.cat (J.C.E.-G.); Tel.: +34-93-5537358 (M.T.)

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Abstract: Recent evidence, including massive gene-expression analysis and a wide-variety of other multi-omics approaches, demonstrates an interplay between gut microbiota and the regulation of plasma lipids. Gut microbial metabolism of choline and L-carnitine results in the formation of trimethylamine (TMA) and concomitant conversion into trimethylamine-*N*-oxide (TMAO) by liver flavin monooxygenase 3 (FMO3). The plasma level of TMAO is determined by the genetic variation, diet and composition of gut microbiota. Multiple studies have demonstrated an association between TMAO plasma levels and the risk of atherothrombotic cardiovascular disease (CVD). We aimed to review the molecular pathways by which TMAO production and FMO3 exert their proatherogenic effects. TMAO may promote foam cell formation by upregulating macrophage scavenger receptors, deregulating enterohepatic cholesterol and bile acid metabolism and impairing macrophage reverse cholesterol transport (RCT). Furthermore, FMO3 may promote dyslipidemia by regulating multiple genes involved in hepatic lipogenesis and gluconeogenesis. FMO3 also impairs multiple aspects of cholesterol homeostasis, including transintestinal cholesterol export and macrophage-specific RCT. At least part of these FMO3-mediated effects on lipid metabolism and atherogenesis seem to be independent of the TMA/TMAO formation. Overall, these findings have the potential to open a new era for the therapeutic manipulation of the gut microbiota to improve CVD risk.

Keywords: trimethylamine; trimethylamine-*N*-oxide; intestinal microbiota; FMO3; reverse cholesterol transport; cholesterol homeostasis; atherosclerosis and cardiovascular disease

1. Introduction

Cardiovascular disease (CVD) accounts for approximately 17 million deaths worldwide each year and remains the main cause of mortality in the United States [1]. An impaired ability to eliminate cholesterol through bile acid excretion may be a risk factor for CVD development [2]. Environmental factors also play a major role in the progression of atherosclerosis and CVD [3]. Every individual has a large number of microorganisms shaping the gut microbiota, which are an intensively studied community of bacterial species [4]. The human gut microbiota represents more than 100 trillion microbes and 5000 different species, containing together around 5 million genes [5]. The combined

genomes of the microbiota contain over 100-fold more unique genes than those encoded in the human genome [6], and these microbiota genes contribute significantly to our physiology and metabolism [7]. In recent years, the concept of pathological variation in the gut microbiota as the cause of several disease states has taken greater importance thanks to data from various rodent studies suggesting that dysbiosis contributes to the pathogenesis of diseases [8,9]. In that perspective, there is significant evidence supporting a role of the gut microbiota in cardiovascular health [10] and the onset and development of complex cardiometabolic diseases such as obesity, diabetes and metabolic syndrome [11,12]. Furthermore, the gut microbiota plays a critical role in plasma lipid levels, which was mainly found in plasma triglycerides and high-density lipoprotein (HDL) cholesterol levels [13]. Based on these studies, a link between microbiota, cardiometabolic diseases and CVD can be devised.

Several studies have identified novel microbial and mammalian cometabolic pathways where the microbiota could promote CVD pathogenesis via the formation of trimethylamine-*N*-oxide (TMAO) from trimethylamine (TMA) by host's flavin monooxygenase 3 (FMO3) [14,15]. These studies reported that TMAO directly promoted atherosclerosis in mice. Additionally, as shown by two large prospective studies' analyses in humans, plasma TMAO levels predicted CVD risk [14,15]. However, the underlying pathways whereby TMAO promotes atherosclerosis require further investigation and validation.

Methods to measure TMA and TMAO in plasma and urine include liquid chromatography-mass spectrometry, proton nuclear magnetic resonance spectrometry, headspace gas chromatography, electrospray ionization tandem mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [16]. Concerning the reported reference values, the largest study performed with a healthy American population included 349 individuals and described values around 3.45 μmol/L [17]. Other studies that investigated healthy European individuals, reported values around 2.5 μmol/L in a cohort of 271 individuals [18] and values around 3.7 μmol/L in a cohort of 100 individuals [19].

In the present review, we aimed to describe and update the potential mechanisms by which TMAO production and FMO3 alter enterohepatic cholesterol and bile acid metabolism and, in consequence, impair a major HDL-mediated cardioprotective function.

2. TMA and TMAO

2.1. Metabolism of TMA and TMAO

TMAO was first described as an osmolyte of marine organism tissue [20]. TMAO is a small organic compound classified as an amine oxide and is formed in the liver from TMA. As a part of the microbial-mammalian cometabolism, TMA is generated by the action of gut microbiota using dietary precursors such as choline, choline-containing compounds, betaine or L-carnitine [16]. TMA is then converted into TMAO by FMO expressed in the liver. The intermediate γ-butyrobetaine (γBB) is formed in the conversion of L-carnitine to TMAO [21]. TMAO is then either transported to the tissues for accumulation as an osmolyte compound or, more commonly, cleared by the kidney from where TMAO is then excreted unchanged through the urine.

Among the five members of the FMO family, only FMO1 and FMO3 have the ability to oxidize TMA to TMAO—where FMO3 is the main isoform expressed in the human liver [22]. For FMO3, certain rare deleterious mutations in its gene are known to cause a reduced or absent TMAO formation, which in turn causes an accumulation of TMA. This autosomal recessive condition is called trimethylaminuria or "fish malodor syndrome" (OMIM 602079). Early reports of this condition date back to 1970 [23] and describe that individuals suffering from this disease experienced urine, sweat and breath that smelled like rotting fish.

In addition to its expression in the liver, FMO3 is also expressed in the lungs, adrenals and aorta. Interestingly, a sexually dimorphic expression pattern has been observed both in mice and humans, with females showing higher expression than males [24,25]. This gender difference may be explained by

hormonal regulation: Testosterone is responsible for the lower hepatic FMO3 expression in males and estrogen induces FMO3 expression in females [24]. However, human studies have produced divergent results on gender-related differences in plasma TMAO concentrations. For instance, several studies investigating TMAO expression in humans did not find significant sex differences in plasma TMAO levels [18,19,26], whereas others reported higher ones either in females [22,27] or in males [28,29]. In a recent study involving a cohort of 648 individuals, males were found to have significantly higher levels of TMAO, even after adjusting for confounding factors such as age and kidney function [30]. Various variables including age, body mass index and blood pressure have been proposed as a cause of these conflicting findings since they were associated positively with the levels of TMAO [18,19,26]. More studies are required to clarify this conundrum.

Fasting plasma concentrations of TMAO exhibit a wide inter- and intra-individual variation [19]. The levels of circulating TMAO are affected by several factors, which include kidney function, diet, protein transport and, as stated before, the gut microbiota. Patients with chronic kidney disease (CKD) who are on haemodialysis show about 40-fold elevated TMAO levels compared with normal controls [28,31]. However, studies linking TMAO with the risk of cardiovascular events and mortality in CKD patients rendered inconclusive results. Some authors suggest that the higher TMAO levels may be confounded with impaired kidney function and poor metabolic control rather than history, presence or incidence of symptoms or events of coronary heart disease [28]. On the other hand, one study suggests that elevated TMAO levels are strongly associated with degree of renal function in CKD and that TMAO levels normalize after renal transplantation [32]. Additionally, TMAO levels correlate with increased systemic inflammation and it is an independent predictor of mortality in CKD patients [33]. These studies also suggest that TMAO may represent a new potentially modifiable CV risk factor for CKD patients [32,33]. A recent study showed that TMAO is a prognostic biomarker of kidney function in individuals with low renal function. The authors found that the metabolites derived from the gut microbiota strongly correlated with TMAO and that the magnitude of the correlation varied with kidney function independent of age, sex and baseline glomerular filtration rate [30].

Another important cause of plasma TMAO variability is diet. Food containing TMAO or its precursors increases blood and urine TMAO levels. Red meat, eggs and dairy products are all rich in TMA precursors, and therefore, a potential source of TMAO [34]. In addition, TMA and TMAO can also be acquired directly from fish and other seafood [34]. Overall, fish seems to have the highest source of TMAO, and post-ingestion studies show a marked increase in TMAO and related metabolites when compared with other foods enriched in carnitine or choline [34]. However, inconsistencies in findings remain since the results of several long-term studies did not indicate a strong effect of diet on TMAO plasma concentrations [18,19,26]. The organic cation transporter 2 (OCT2) located on the basolateral membrane of renal tubule cells is the key uptake transporter for TMAO and it may also be a major determinant of its variability [35].

Concerning the gut microbiota, several families of bacteria from the Firmicutes and Proteobacteria phyla isolated from commensal bacteria in the human intestine have been identified as choline and carnitine consumers and, therefore, potential TMA producers [36,37]. Pathways of TMA synthesis in the intestine have been described with a specific glycyl radical enzyme (GRE) including the GRE choline TMA-lyase (cutC) and its activator GRE activase (cutD), which uses choline as a substrate [38] and also, a two-component Rieske-type oxygenase/reductase (cntA/B), which uses carnitine and its γBB derivate as a substrate [39]. In a recent study of 648 individuals following a health coaching, determinants of gut microbiota and TMAO metabolism were identified [30]. The impact of diet (enriched in either meat or vegetal foods) on both the gut microbiota's composition and TMAO was confirmed; the levels of TMAO were more affected by microbiota activity in individuals with higher kidney function. However, TMAO levels were significantly affected by the lack of TMAO filtering in individuals with low kidney function [30].

2.2. Genetic Determinants of TMAO

A great effort to find a relationship between gene expression and plasma TMAO levels was exerted over the last years. Various studies have focused on TMAO and CVD to determine the genetics that possibly underlie their relationship. Functional differences in FMO3 activity can occur in humans, secondary to variations within the *FMO3* gene. In addition to mutations causing trimethylaminuria, single nucleotide polymorphisms such as E158K and E308G have been reported to reduce FMO3 activity [40]. However, the implications of these polymorphisms on disease risk are complex and largely unknown due to inconclusive results [41–43].

Apart from *FMO3*, genome-wide association studies (GWAS) in male mice have allowed the identification of other genes related to TMAO metabolism. For instance, the *Slc30a7* gene has been found to be related to TMAO metabolism and is associated with a transporter of dietary zinc absorption (ZNT7) located on chromosome 3 [44]. Nonetheless, no significant threshold was observed in the human GWAS for the same gene [45].

A role for genetic methylation in TMAO homeostasis has recently been proposed by a study that reported an inversed association between plasma TMAO and the methylation capacity in humans [46]. However, an epigenome-wide study analyzed the methylation levels of CpG islands included in 463,995 loci finding no significant associations between methylation and circulating TMAO levels [47].

2.3. TMAO as a Plausible Contributor to Cardiovascular, Peripheral and Cerebrovascular Diseases

TMAO came recently under the spotlight due to its reported association with human CVD. In 2011, metabolomic studies identified three candidate molecules that were significantly correlated—after adjusting for traditional cardiac risk factors and medication usage—with CVD, which included the fasting TMAO, choline and phosphatidylcholine plasma levels [14]. Several years later, an association between carnitine concentration and the risk of coronary artery disease, peripheral artery disease and overall CVD was found to be affected by the microbiota metabolite TMAO [48]. The study concluded that carnitine was also an independent predictor of major adverse cardiovascular events (MACE), with TMAO as the main driver behind the association of cardiovascular risk [48]. Nonetheless, consumption of fish, which is high in TMAO, has long been associated with reduced CVD risk [49]. Other clinical trials also indicated that diets enriched in carnitine are associated with beneficial effects on CVD [50].

To better understand TMAO's role in CVD, large population studies were performed with subjects undergoing elective diagnostic coronary angiography [15,51]. A direct relationship between increased plasma TMAO levels and the increased risk of MACE was observed. Furthermore, the inclusion of TMAO resulted in an improvement of risk estimation obtained by the traditional risk factors [15]. More recently, TMAO has been related to acute coronary syndromes in two different cohorts that showed an association of high TMAO plasma levels with an increased risk of MACE and mortality [52]. Additionally, high TMAO levels were also associated with a poor prognosis since they enhanced MACE risk and reinfarction in patients that had had acute myocardial infarction [52]. Finally, TMAO was also indicated as a secondary risk stratification biomarker of acute myocardial infarction to detect low-risk patients among the high-risk group [53].

Studies that focus on specific pathologies (e.g., heart failure) have also demonstrated a higher concentration of plasma TMAO compared to their respective controls. Furthermore, this higher concentration was associated with poor prognosis for heart failure patients and, consistently, plasma TMAO levels correlated positively with other cardiac biomarkers such as B-type natriuretic peptide [54].

An increase in the all-cause mortality and an improvement in risk estimation were also observed in patients with peripheral artery disease in relation to their TMAO plasma levels [55]. Increased choline and betaine concentrations also correlated with a higher risk of MACE and CVD. However, this correlation was only significant when higher plasma levels of TMAO were observed [51].

It should be noted that not all studies have demonstrated an association between TMAO and vascular diseases. For instance, a recent study reported a reduction in TMAO plasma levels and

dysbiosis of the gut microbiota in patients that had a stroke or transient ischaemic attack [56]. The authors suggested that either the stroke event or the treatment may reduce TMAO and that the associated dysbiosis of the gut microbiota could be related with the differential role of TMAO in these pathologies [56]. Finally, other intermediate metabolites have been proposed to be involved in CVD. In patients with carotid atherosclerotic plaques, γBB and its precursor trimethyllysine were found to be associated with cardiovascular mortality [57].

The underlying mechanisms whereby TMAO contributes to CVD are not fully understood. The first hypothesis that explained the atherosclerotic role of TMAO stated a decrease in HDL levels. This was based on the inverse association between TMAO and HDL, which had been found in mice concomitant to a reduction in HDL-mediated reverse cholesterol transport (RCT, see Section 4 for more details) [14]. Nonetheless, human studies resulted in conflicting results concerning this topic as some authors reported significant lower HDL levels in high TMAO-expressing individuals with CVD [54,55], whereas others found a positive correlation between them [18]. Interestingly, a recent study reported no significant correlations between TMAO and the previously established blood markers for CVD, including the total cholesterol, low-density lipoprotein (LDL), HDL and triglycerides [30]. This might in part explain why TMAO has been found to be a prognostic marker for CVD beyond the traditional risk factors as previously indicated [52].

A recent study showed that inhibition of TMA lyases—enzymes expressed by gut microbes that convert choline to TMA—reduced atherosclerosis in mice [58]. TMAO-mediated atherosclerosis most likely occurs through multiple pathways. TMAO enhances the forward macrophage cholesterol transport via the upregulation of receptor CD36 (cluster of differentiation 36) and scavenger receptor A [14]. TMAO is also known to alter the enterohepatic cholesterol and bile acid metabolism, thereby impairing a major pathway required to eliminate cholesterol from the body, i.e., HDL-mediated RCT (see Section 4 for details) [48,59]. Other alternative mechanisms in relation to TMAO-mediated atherosclerosis include angiotensin II, which causes a prolongation of hypertension [60], the activation of nuclear factor κB signaling, which promotes vascular inflammation [61], and an enhanced platelet activation, which may promote a thrombosis effect [62].

Nonetheless, it should be noted that the findings of two recent experimental studies have cast doubts on the TMAO hypothesis [63,64]. In the first study, high doses of carnitine resulted in a significant increase in plasma TMAO levels in mice but, surprisingly, they inversely correlated with aortic atherosclerotic lesions [63]. The second study found that choline supplementation increased plasma TMAO in conventionally raised mice but not in germ-free mice. However, this treatment did not affect the atherosclerosis susceptibility [64].

3. HDL Function and CVD

The concept that HDL cholesterol protects against CVD was originally based on epidemiological data showing that low HDL cholesterol levels have a predictive factor of major adverse CVD events [65]. However, the inverse relationship between CVD risk and HDL is not strictly related to the amount of cholesterol transported by HDL, and the failure of various HDL-targeted therapies to ameliorate CVD has cast doubt on this HDL hypothesis [66,67]. A disconnection between the HDL cholesterol levels and the HDL atheroprotective functions may explain the findings obtained with therapies targeting HDL cholesterol.

HDL is mainly synthesized in the liver and small intestine, and both may secrete lipid-free apolipoprotein (apo) A-I—the main HDL protein—into circulation [68]. The interaction produced between apoA-I and the transmembrane ATP-binding cassette (ABC) A1 induces cholesterol translocation to the cell membrane [68]. As a result, apoA-I is rapidly lipidized and converted into the nascent HDL (termed preβ-HDL). Cholesterol in these nascent discoidal particles is then esterified by Lecithin: cholesterol acyltransferase (LCAT). Cholesterol can then be taken up selectively from HDL after binding to the scavenger receptor class B type I (SR-BI) in the liver, captured by liver or kidney together with the whole HDL particle, or transferred by cholesteryl ester transfer protein

(CETP) to very low density lipoprotein (VLDL) and LDL which may be later cleared by the liver via receptor-dependent pathways [68]. From there, it will be partly transformed into bile acids and removed together with cholesterol through the biliary pathway. Cholesterol and bile acids may then be finally excreted from the body in the feces. In addition to the main hepatobiliary pathway of cholesterol elimination, an alternative nonbiliary transintestinal route for cholesterol elimination has also been reported, termed the transintestinal cholesterol excretion (TICE) route. The TICE facilitates the transfer of cholesterol from the circulating plasma directly into the intestinal lumen through enterocytes [69]. All these multistep pathways have been collectively termed RCT (see Figure 1 for details). Whereas cholesterol efflux to HDL occurs in all tissues, the fraction that originates from the macrophage foam cells located in the arterial wall is considered the most critical RCT component directly related to atherosclerosis [70]. At least three cholesterol transporters are involved in the HDL-mediated macrophage cholesterol efflux: ABCA1, ABCG1 and SR-B1. ABCA1 promotes cholesterol transport to the lipid-free apoA-I, preβ-HDL and small HDL, whereas ABCG1 and SR-BI facilitate the efflux to mature α-migrating HDL [71]. It should also be noted that cholesterol efflux assays only quantify the first step of the atheroprotective RCT pathway without addressing the efficiency of the remaining RCT steps or other HDL atheroprotective properties, such as their antioxidant, anti-inflammatory and antithrombotic potential. An assay that evaluates the transfer of radiolabeled cholesterol from macrophages to feces has been widely applied to mice to determine the macrophage RCT rate of the entire pathway (Figure 1) [71]. This RCT multistep pathway, which is initiated by macrophages, is also susceptible to modulation in the liver and small intestine [69]. Studies investigating genetically engineered mice and mice treated with different RCT-enhancing therapies indicate that this major HDL antiatherogenic function is an important predictor of atherosclerosis susceptibility [72].

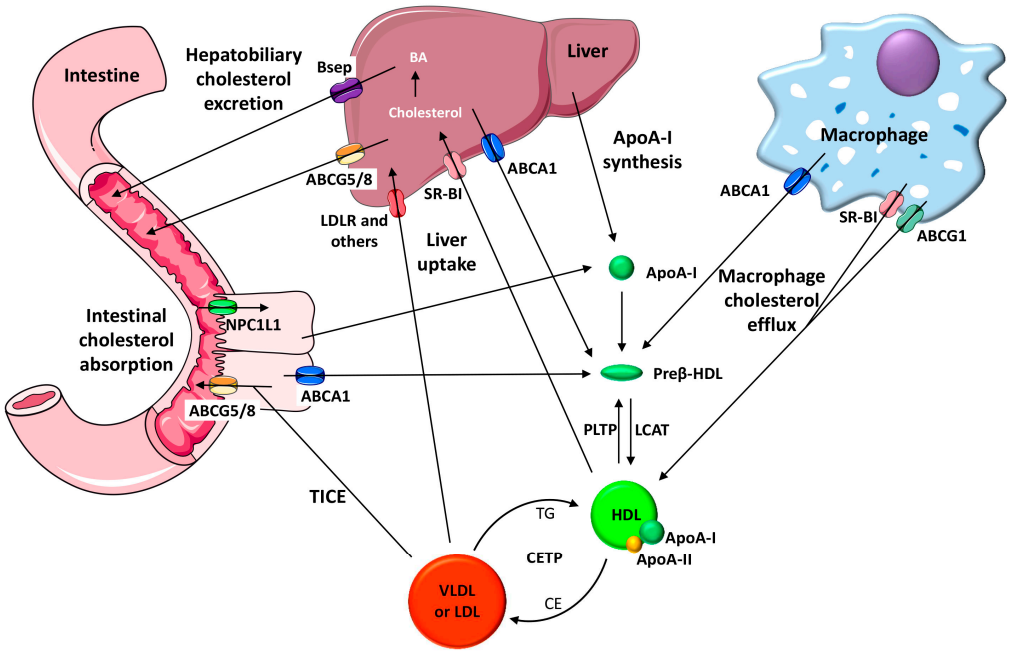

Figure 1. A schematic diagram of macrophage-to-feces reverses cholesterol transport pathways. ApoA-I is synthesized by the liver and small intestine, and it acquires phospholipids to become partially lipidated preβ-high-density lipoprotein (HDL) particles at its nascent stage. Preβ-HDL particles acquire free cholesterol from macrophages via the adenosine triphosphate-binding cassette (ABC) A1 transporter. The free cholesterol is converted into cholesteryl ester within the HDL particle by the action of Lecithin: cholesterol acyltransferase (LCAT), thereby resulting in mature HDL. ApoA-I is the major HDL protein and activates LCAT, whereas apoA-II, the second HDL protein, displaces apoA-I from the HDL particles. Both the macrophage scavenger receptor class B type I (SR-BI) and ABCG1 facilitate the cholesterol efflux process from macrophages to mature HDL. The phospholipid transfer protein (PLTP) promotes the transfer of phospholipids and free cholesterol from triglyceride-rich lipoproteins into HDL, producing a remodeling process where preβ-HDL particles can be generated. HDL cholesterol esters can be transferred to very low density lipoprotein (VLDL) or low-density lipoprotein (LDL) by the cholesteryl ester transfer protein (CETP) and be returned to the liver through the low-density lipoprotein receptor (LDLR) or other LDL and VLDL receptors. Another function of the liver is to take up HDL-associated cholesterol selectively via SR-BI. Cholesterol can be secreted into bile as unesterified cholesterol via the ABCG5/G8 heterodimer or used to synthesize bile acids (BA). The bile salt export pump (BSEP) is involved in the bile acid transport to bile. Niemann-Pick C1-like 1 (NPC1L1) is of crucial importance for absorbing macrophage-derived cholesterol in the small intestine. Cholesterol may also be excreted back to the lumen by the intestinal ABCG5/G8 heterodimer. The transintestinal cholesterol export (TICE) route promotes the flow of cholesterol from plasma to enterocytes and the intestinal lumen.

4. The Physiological Interaction between TMAO and HDL in the Context of Cardiometabolic Diseases

4.1. The Role of the Gut Microbiota in Lipid Metabolism and the Pathogenesis of CVD

Studies of gut microbiota composition and obesity have shown a direct association between Firmicutes phyla and obesity in both mice [73] and humans [74]. Dysbiosis has also been observed in other pathologies such as type 2 diabetes, where affected women had an increased *Clostridiales* colonisation at the expense of *Roseburia* [75]. A metagenomic study also showed differences in the gut

metagenome of children with type 1 diabetes versus matched controls suggesting that environmental factors may interact with the genetic susceptibility to autoimmune diabetes [76].

The role of gut microbiota in lipid metabolism and in the pathogenesis of CVD has been widely investigated. A study performed on symptomatic atherosclerotic patients revealed a greater abundance of *Collinsella* than the control group, which showed enrichment in *Bacteroides*, *Eubacterium* and *Roseburia*—these three are all genera involved in anti-inflammatory and antioxidant processes [77]. Richness in different taxonomies of gut microbiota correlated negatively with body mass index and plasma triglycerides and positively with plasma HDL cholesterol. Hence, microbiota can explain a substantial proportion of the variation in lipid profile, independently of age, sex, body mass index and genetics [13]. A comparison between germ-free versus conventionally raised mice showed increased VLDL triglyceride production rates and hepatic triglyceride levels in the conventionally raised group [78].

Beyond the effects of intestinal microbiota on triglycerides and HDL cholesterol levels, the suppression of the intestinal microbiota was correlated with enhanced macrophage-to-feces RCT [79]. This change was concomitant with an increased bile acid excretion. Since the absence of gut bacteria impairs the secondary bile acid formation, the enhanced macrophage-to-feces RCT can be explained by the accumulation of hydrophilic tauro-β-muricholic acid, which cannot be absorbed in the colon [79]. However, plasma cholesterol levels and fecal neutral sterol excretion were not affected by the absence of intestinal microbiota [79]. In line with these findings, antibiotic treatment reversed the TMAO-dependent reduction of RCT [48].

Since the bile acid pool size and its composition seem to regulate the gut microbial community structure [80], the composition of the gut microbiota appears to be a key linking feature between TMAO production, and cholesterol and bile acid metabolism. Indeed, a lower microbial diversity and a greater enrichment of *Firmicutes* (relative to *Bacteroidetes*) were detected among healthy young men who exhibited a greater postprandial increase in circulating TMAO after consuming eggs and beef [34].

Several research groups have attempted to provide a mechanistic basis for TMAO-mediated atherosclerosis, where the variation in the gut microbiota could be a direct contributor to the pathogenesis and progression of the disease. Transferring choline diet-induced TMAO production via fecal transplantation in apoE-deficient (−/−) mice resulted in an increased atherosclerosis risk [81]. In the same line, a recent study with apoE−/− mice showed that the development of atherosclerosis by microbiota was dietary dependent [64]. Altogether, these findings suggest that elevated concentrations of circulating TMAO may arise from a dysbiotic microbiota, which in turn could be the cause underlying the pathogenesis of disease and its progression. Alternatively, circulating TMAO could reflect the differences in gut microbiota composition that occurs during disease process [82].

4.2. The Role of FMO3/TMAO and Nuclear Receptors on Enterohepatic Cholesterol and Bile Acid Metabolism

Previous studies have demonstrated that the way TMAO impacts on atherosclerosis is closely connected to changes in bile acid metabolism [83]. TMAO reduced bile acid synthesis and liver bile acid transporters, effectively decreasing the bile acid pool [48]. The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that acts as a sensor of intracellular bile acid levels within the liver and intestine [84]. Activation of FXR has been shown to cause significant changes in bile acid homeostasis by altering the transcription of genes responsible for liver bile acid synthesis and intestinal uptake [85]. Indeed, FXR downregulated the hepatic cytochrome P450 7A1 (CYP7A1, cholesterol 7 α-hydroxylase)—the rate-limiting enzyme in bile acid synthesis—through a fibroblast growth factor 15/19-dependent mechanism [86]. Interestingly, FMO3 expression in the liver was upregulated by dietary bile acids through an FXR-mediated pathway (Figure 2) [22]. In vivo studies have shown that a dietary TMAO supplementation inhibited bile acid synthesis by downregulating *cyp7a1* and *cyp27a1* (Figure 2) [48]. Furthermore, mice supplemented with TMAO had a significantly smaller total bile acid pool size concomitant with a reduced expression of the multiple liver bile acid transporters organic-anion-transporting polypeptide type 1 and 2, the multidrug resistance protein 2 and the

sodium-taurocholate cotransporting polypeptide compared to control, chow-fed mice [48]. Dietary TMAO also reduced the mRNA expression of *Niemann-Pick C1-Like 1* and *abcg5/g8* and inhibited intestinal cholesterol absorption [48]. Since impaired bile acid synthesis and secretion are linked to an increased risk of CVD, the direct and indirect actions of TMAO on bile acid synthesis and excretion may represent a potential mechanism by which TMAO exerts its proatherogenic effect [48].

Recently, a gut microbial-driven pathway that balances the amount of cholesterol entering the biliary and nonbiliary pathways was identified. This study demonstrated that FOM3 inhibition diverted cholesterol into the nonbiliary TICE pathway, which resulted in the reorganization of the total body cholesterol balance [59]. The TICE is responsible for 30% of the total cholesterol loss through the feces in mice [87] and pharmacological liver X receptor (LXR) activation increased this amount up to 60% [88]. FMO3 knockdown mice presented an increased basal and LXR agonist-stimulated rate of the nonbiliary TICE pathway, which are findings that further support FMO3 as a negative regulator of the TICE (Figure 2). Moreover, knocking down FMO3 in mice strikingly reduced the expression of LXR target genes involved in the de novo lipogenesis and blunted hepatic steatosis, in contrast, it exacerbated hepatic endoplasmic reticulum stress and inflammation [59]. Overall, FMO3 activity and the TMA/FMO3/TMAO pathway seem to be major determinants for both LXR and FXR regulation as well as for the downstream liver inflammatory response (Figure 2). Therefore, all of these pathways have broad implications in sterol balance and inflammatory processes, suggesting that FMO3 has regulatory functions distinct from its enzymatic activity, being uniquely positioned among the FMO family of enzymes in impacting human disease [22,48].

Dietary TMAO has been found to normalize plasma levels of circulating TMAO in FMO3 antisense oligonucleotide (ASO)-treated mice, but neither cholesterol balance nor gene expression in the liver [48]. This indicates that the ability of FMO3 inhibitors to alter cholesterol balance, inflammation and endoplasmic reticulum stress is likely to involve several molecular mechanisms, including both the gut microbial and the gut microbe-independent mechanisms [59].

Knocking down liver FMO3 in LDL receptor-deficient mice reduced the hepatic and plasma lipid levels, bile acid pool size, liver triglyceride secretion, ketone bodies and glucose and insulin levels, and prevented atherosclerosis [89]. Global microarray expression analyses of these mice revealed that knocking down liver FMO3 downregulated 136 peroxisome proliferator-activated receptor (PPARα) target genes, possibly due to reduced hepatic concentrations of PPAR ligands, which included palmitoleate, α- or γ-linolenate, oleate and eicosapentaenoic acid [89,90]. PPARα is activated during fasting and may promote fatty acid oxidation, ketogenesis, gluconeogenesis and bile acid synthesis through transactivation of its target genes [90], thus explaining many of the FMO3-mediated effects. It is thus becoming increasingly important to identify endogenous substrates—these can include epinephrine, norepinephrine, phenethylamine, trimethylamine and tyramine or new potential substrates; and products of FMO3 that have the potential to differentially impact on the diverse phenotypes observed when manipulating this enzyme.

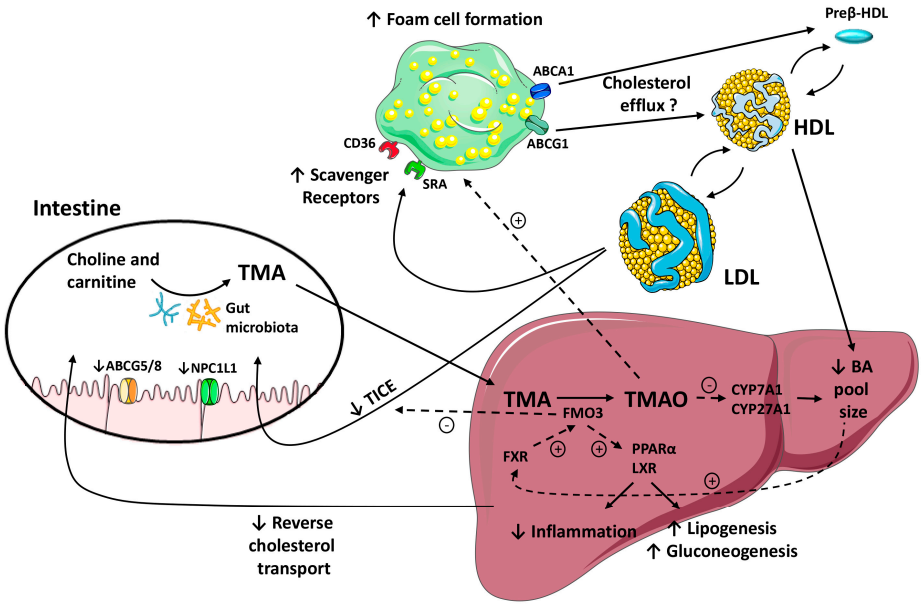

Figure 2. A schematic representation of pathways linking the gut microbiota, the formation of trimethylamine-*N*-oxide (TMAO) by flavin monooxygenase (FMO) 3 and the regulation of enterohepatic bile acid (BA) and cholesterol metabolism and macrophage reverse cholesterol transport (RCT) pathways. Black arrows indicate movement of TMA/TMAO and cholesterol through the body. Blunt-end arrows indicate activation (+) or inhibition (-) of specified receptors and transporters or pathways. Gut microbiota metabolism of choline and L-carnitine results in the formation of trimethylamine (TMA). In the liver, FMO3 converts TMA into TMAO. The potential effects of TMAO on the proatherogenic pathways include the promotion of foam cell formation by increasing macrophage scavenger receptors (↑) and the downregulation of the main liver bile acid (BA) synthetic enzymes, cyp7a1 and cyp27a1. Downregulation of these rate-limiting enzymes reduces intracellular levels of BA (↓). The BA pool size could impact the farnesoid X receptor (FXR)-mediated regulation of FMO3. In turn, this enzyme may regulate the liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR) α signaling pathways, reducing liver inflammation (↓) and promoting hepatic lipogenesis and gluconeogenesis (↓). FMO3 impairs the cholesterol flux into the nonbiliary transintestinal cholesterol export (TICE) pathway (↓). TMAO also reduces Niemann-Pick C1-Like 1 (NPC1L1) and adenosine triphosphate-binding cassette (ABC) G5/G8 expression (↓), and inhibits intestinal cholesterol absorption. Collectively, the effects of TMAO/FMO3 on BA homeostasis and TICE impair the macrophage-to-feces RCT (↓).

As stated above, elevated systemic levels of TMAO have been associated with type 2 diabetes [91]. Moreover, FMO3 was increased in obese-/insulin-resistant human subjects. Interestingly, knocking down FMO3 prevented high-fat-diet-induced obesity in mice by stimulating the beiging of white adipose tissue [91]. Furthermore, knocking down FMO3 in liver-specific insulin receptor knockout mice prevented hyperlipidemia and atherosclerosis susceptibility concomitant with an increase in LDL receptors [92]. These findings concurred with the results of a previous report demonstrating the deleterious effects of FMO3 expression in glucose tolerance in liver-specific insulin receptor knockout mice [92]. Additionally, another recent study demonstrated that maternal hypercholesterolemia exacerbates the development of atherosclerosis with a positive association of aortic lesion size with both TMAO levels and increased FMO3 mRNA expression [93]. Overall, these data strongly indicate a role for FMO3 in modulating lipid and glucose homeostasis in vivo in a dose-dependent manner and, in some cases, independently of TMA/TMAO formation.

4.3. TMAO/FMO3 and HDL Atheroprotective Functions

As stated above, several studies have demonstrated that TMAO can promote macrophage cholesterol accumulation in a microbiota-dependent manner by increasing cell surface expression of two scavenger receptors [14]. However, TMAO treatment in apoE−/− mice failed to impair the macrophage cholesterol efflux, at least in part due to upregulation of ABCA1 and ABCG1 in peritoneal mouse macrophages and increased ABCA1-dependent cholesterol efflux to ApoA-I [48]. Nonetheless, another study found downregulation of the *abca1* gene in J774.A1 murine macrophages treated with TMAO for 24 h [94] but this finding was not reproduced in another similar study [63].

Beyond the potential effect of TMAO on macrophage cholesterol efflux, mice fed with a choline- or L-carnitine-enriched diet showed an impaired macrophage-to-feces RCT in vivo [48]. This process was reverted when a broad spectrum of antibiotics was administered orally, suggesting that TMAO was the main responsible factor of altering the entire macrophage RCT pathway [48]. Consistent with this hypothesis, mice directly fed with a TMAO-containing diet al.so showed a reduced rate of macrophage-specific RCT [48]. As discussed earlier, TMAO-mediated effects on the liver bile acid synthetic pathway could be affecting the recovery of macrophage-derived cholesterol in feces (Figure 2).

A liver microarray analysis was conducted in two independent mouse models with an enhanced TICE to identify potential regulators of macrophage RCT. Less than 100 differentially expressed genes were detected within each array data set [59]. From these, the only gene that was downregulated in both models was FMO3. Consistently, when FMO3 was suppressed, the nonbiliary macrophage-to-feces RCT was enhanced [59]. However, it was not possible to determine which step of the RCT was altered because knocking down FMO3 substantially impacted the whole-body cholesterol balance [59]. Further experiments should be performed to clarify how the FMO3/TMA/TMAO pathway affects the macrophage RCT.

5. Concluding Remarks

There is significant evidence supporting the hypothesis that gut microbiota-derived production of TMAO increases the risk of atherosclerotic CVD. The concentration of circulating TMAO is determined by several main factors, including the dietary habits, gut microbiota, FMO3 activity and kidney function. Several important published works in TMAO research have used omics-techniques, mainly focusing on its involvement in CVD (see Table 1 for references of clinical studies). These technologies have been fundamental in the identification of TMAO origin and in the characterization of the unique properties of FMO3, the enzyme that ultimately synthesized TMAO. TMAO can promote atherogenesis via multiple signaling pathways. Dietary administration of choline, L-carnitine or TMAO in animals with an intact gut microbiota enhances the macrophage scavenger receptors and promotes foam cell formation. However, the diets enriched in TMAO precursors have not always been found to be proatherogenic in experimental studies. This dietary administration impairs the macrophage-to-feces RCT pathway. Furthermore, TMAO impairs the FXR-target genes *cyp7a1* and *cyp27a1*, which affect liver cholesterol, bile acid production and biliary excretion. FMO3 may also promote atherosclerosis by enhancing hepatic lipogenesis and gluconeogenesis as well as by impairing the TICE. At least a part of the FMO3-mediated effects on lipid metabolism seems to be independent of TMA/TMAO formation, suggesting multiple distinct mechanistic links between the TMAO-producing diet, FMO3 and atherogenesis (see Figure 2 for a graphical summary). Overall, and based on the highly variable plasma TMAO levels observed in human studies, it is reasonable to hypothesize that TMAO levels are affected by the intrinsic genetic factors of the host and that FMO3 activity may influence pathological outcomes via routes independent of TMAO. However, diets containing TMAO precursors provide important healthy nutrients and, thus, proposing restriction of these foods does not seem a suitable strategy. Further human studies that investigate the effects of lowering circulating TMAO on CVD are needed. Based on the promising available data regarding gut microbiota to date,

their selective manipulation could be used as a therapeutic approach in future therapies to prevent atherosclerotic CVD.

Table 1. Examples of studies using one or more multi-omics technologies investigating the pathophysiological role of TMAO.

Methods and Species	Study Findings	References
Metabolomics in humans	TMAO, carnitine, choline and phosphatidylcholine plasma levels correlated with CVD	[14,48]
GWAS in mice	The *Slc30a7* gene was related to TMAO metabolism	[44]
GWAS in humans	*SLC30A7* locus did not reach the genome-wide significance	[45]
Epigenome-wide study of DNA methylation in humans	No evidence of significant relationship between methylation markers and circulating TMAO levels	[47]
A multi-omic association study of TMAO in humans	Diet- and disease-associated metabolites were significantly associated with TMAO Proteins linked to CVD and kidney disease were correlated with TMAO	[30]
Metagenome analysis in humans	Gene-targeted sequencing allowed the quantification and characterization TMA-producing species	[37]
Metagenome analysis in humans	Gut microbiome may explain part of the population variation in plasma blood lipids	[13]
Global microarray expression analyses in mice	Knocking down FMO3 downregulated 136 PPARα target genes	[89]
Global microarray expression analyses in mice	FMO3 expression was the unique downregulated gene in mouse models of enhanced TICE	[59]

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Abbreviations

ABC	ATP-binding cassette
ASO	Antisense oligonucleotide
CD36	Cluster of differentiation 36
CETP	Cholesteryl ester transfer protein
CKD	Chronic kidney disease
CVD	Cardiovascular disease
FMO	Flavin monooxygenase
FXR	Farnesoid X receptor
GWAS	Genome-wide association studies
HDL	High-density lipoprotein
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein
LXR	Liver X receptor

MACE	Major adverse cardiovascular events
mRNA	Messenger ribonucleic acid
Npc1L1	Niemann-Pick C1-Like 1
OCT2	Organic cation transporter 2
PPAR	Peroxisome proliferator-activated receptor
SR-BI	Scavenger receptor class B type I
TICE	Transintestinal cholesterol excretion
TMA	Trimethylamine
TMAO	Trimethylamine-*N*-oxide
VLDL	Very low-density lipoprotein
γBB	γ-Butyrobetaine

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Annex 2: Impaired HDL (high-density lipoprotein)-mediated macrophage cholesterol efflux in patients with abdominal aortic aneurysm—brief report

Impaired HDL (High-Density Lipoprotein)-Mediated Macrophage Cholesterol Efflux in Patients With Abdominal Aortic Aneurysm—Brief Report

Diego Martínez-López, Lídia Cedó, Jari Metso, Elena Burillo, Annabel García-León, Marina Canyelles, Jes S. Lindholt, Monica Torres-Fonseca, Luis Miguel Blanco-Colio, Jesús Vázquez, Francisco Blanco-Vaca, Matti Jauhiainen, Jose Luis Martín-Ventura,* Joan Carles Escolà-Gil*

Objective—The ability of HDL (high-density lipoprotein) to promote macrophage cholesterol efflux is considered the main HDL cardioprotective function. Abdominal aortic aneurysm (AAA) is usually characterized by cholesterol accumulation and macrophage infiltration in the aortic wall. Here, we aim to evaluate the composition of circulating HDL particles and their potential for promoting macrophage cholesterol efflux in AAA subjects.

Approach and Results—First, we randomly selected AAA and control subjects from Spain. The AAA patients in the Spanish cohort showed lower plasma apoA-I levels concomitantly associated with low levels of plasma HDL cholesterol and the amount of preβ-HDL particles. We determined macrophage cholesterol efflux to apoB-depleted plasma, which contains mature HDL, preβ-HDL particles and HDL regulatory proteins. ApoB-depleted plasma from AAA patients displayed an impaired ability to promote macrophage cholesterol efflux. Next, we replicated the experiments with AAA and control subjects derived from Danish cohort. Danish AAA patients also showed lower apoA-I levels and a defective HDL-mediated macrophage cholesterol efflux.

Conclusions—AAA patients show impaired HDL-facilitated cholesterol removal from macrophages, which could be mechanistically linked to AAA.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:2750-2754. DOI: 10.1161/ATVBAHA.118.311704.)**

Key Words: aneurysm ■ apolipoprotein ■ cells ■ cholesterol ■ macrophages

Circulating human HDL (high-density lipoproteins) include a highly complex and heterogeneous array of subpopulations that are continuously remodeled by plasma factors.[1] The ability of HDL particles to promote macrophage cholesterol efflux is the most extensively reported cardioprotective function associated with HDL.[2] ABC (ATP-binding cassette) A1 mediates cholesterol efflux to lipid-poor preβ-HDL and small HDL particles,[3] whereas another ABC transporter, ABCG1, facilitates cholesterol efflux to large-HDL particles.[4] In 2 human trials, the stimulation of the HDL-dependent cholesterol efflux from J774.A1 macrophage cells mediated by the ABCA1 has been found to be inversely associated with the incidence of atherothrombotic cardiovascular disease events.[5,6]

Two recent meta-analysis have demonstrated a potential role of lipoproteins in the pathogenesis of abdominal aortic aneurysm (AAA).[7,8] We have previously reported in an epidemiological study that HDL cholesterol levels in AAA patients are lower than in patients with aortoiliac occlusive disease.[9] HDL cholesterol levels also predicted aneurysmal growth rate in a population-based prospective cohort study of AAA detected by mass screening.[9] However, HDL cholesterol levels do not necessarily reflect the functional dynamics of HDL to promote macrophage cholesterol efflux.[1] Macrophage infiltration, cholesterol accumulation, and ensuing cholesterol crystals are usually present in the human AAA wall.[10,11] Moreover, LXR (liver X receptor) α and ABCA1 mRNA and protein

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From the Laboratorio de Patología Vascular y CIBER de Enfermedades Cardiovasculares (CIBERCV), FIIS-Fundación Jiménez Díaz-Universidad Autónoma, Madrid (D.M.-L., E.B., M.T.-F., L.M.B.-C., J.L.M.-V.); Institut d'Investigacions Biomèdiques (IIB) Sant Pau, Barcelona, Spain (L.C., A.G.-L., M.C., F.B.-V., J.C.E.-G.); CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain (L.C., F.B.-V., J.C.E.-G.); Minerva Foundation Institute for Medical Research and National Institute for Health and Welfare, Genomics and Biomarkers Unit, Biomedicum, Helsinki, Finland (J.M., M.J.); Elitary Research Centre of Individualized Medicine in Arterial Disease (CIMA), Department of Cardiothoracic and Vascular Surgery, Odense University Hospital, Denmark (J.S.L.); Cardiovascular Proteomics Laboratory, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), CIBER de Enfermedades Cardiovasculares (CIBERCV), Madrid (J.V.); and Departament de Bioquímica, Biología Molecular i Biomedicina, Universitat Autònoma de Barcelona, Spain (F.B.-V., J.C.E.-G.).

*J.L. Martín-Ventura and J.C. Escolà-Gil are joint senior authors.

Correspondence to Joan Carles Escolà-Gil, PhD, Institut d'Investigacions Biomèdiques (IIB) Sant Pau and CIBERDEM, Antoni M. Claret 167, Barcelona 08025, Spain, Email jescola@santpau.cat; or Jose Luis Martín-Ventura, PhD, Laboratorio de Patología Vascular y CIBER de Enfermedades Cardiovaculares (CIBERCV), FIIS-Fundación Jiménez Díaz-Universidad Autónoma, Madrid, Av Reyes Católicos 2, Madrid 28040, Spain, Email jlmartin@fjd.es

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Nonstandard Abbreviations and Acronyms	
AAA	Abdominal aortic aneurysm
ABC	ATP-binding cassette
CETP	cholesteryl ester transfer protein
CRP	C-reactive protein
HDL	high-density lipoproteins
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LXR	Liver X receptor
PLTP	phospholipid transfer protein

levels were decreased in the AAA media compared with the normal media layer, pointing to possible deregulation of the cholesterol efflux mechanism in AAA.[11] In the present study, we have evaluated the composition of circulating HDL and their potential for promoting macrophage cholesterol efflux in AAA patients and normolipidemic control subjects.

Materials and Methods

Data Sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Design and Participants

Initially, we randomly selected 20 AAA patients (aortic size >50 mm, confirmed with abdominal ultrasound) and normolipidemic control subjects (aortic size <30 mm) originating from Spain. Samples were obtained from the biobank of Fundación Jimenez Diaz. A second experiment with AAA patients and control subjects from the Danish cohort and matched with similar age and statin use was conducted to replicate and validate the main findings obtained from the Spanish cohort. Samples were derived from the VIVA trial (Viborg Vascular; URL: http://www.clinicaltrials.gov. Unique identifier: NCT00662480). The study was performed in accordance with the ethical principles set forth in the Declaration of Helsinki and approved by the institutional review committee of each Institution; the subjects studied gave informed consent.

Lipid, Lipoprotein Apolipoprotein Analyses

Plasma total cholesterol, triglycerides, and apoA-I were determined enzymatically and by an immunoturbidimetric assay, respectively, using commercial kits adapted to a COBAS 501c autoanalyzer (Roche Diagnostics). HDL cholesterol levels were measured in plasma obtained after precipitation of apoB-containing lipoprotein particles with phosphotungstic acid and magnesium ions (Roche Diagnostics). LDL (low-density lipoprotein) cholesterol levels were calculated with the Friedewald equation. Total HDL was isolated by sequential ultracentrifugation at density 1.063 to 1.21 kg/L, and lipids and apoA-I were determined as described above. Free cholesterol and phospholipids were evaluated with reagents from Wako Diagnostics. The amount of apoA-I in preβ-HDL particles was quantified with 2-dimensional crossed immunoelectrophoresis in individual plasma samples and in the plasma samples incubated for 6 hours at 37°C in the presence of a lecithin-cholesterol acyltransferase (LCAT) inhibitor (1 mmol/L iodoacetate).[12]

Lipid Transfer Proteins and Enzymes

PLTP (phospholipid transfer protein) activity was measured with a radiometric assay using [^{14}C]phosphatidylcholine liposomes as donors and HDL_3 as acceptor in the presence of individual plasma samples.[13] CETP (cholesteryl ester transfer protein) activity was analyzed by a radiometric method as a transfer/exchange of radiolabeled [^{14}C]cholesteryl oleate between exogenously added human LDL and HDL_2.[14] LCAT activity was measured using the lipoproteins of plasma as substrate and expressed as molar esterification rates.[15] Alternatively, LCAT activity was measured with a radiometric method using radiolabeled reconstituted apoA-I-discoidal HDL particles as substrate and measuring the amount of radiolabeled cholesterol esters generated as described in more detail earlier.[16]

Cholesterol Efflux Assays

The cholesterol efflux capacity of apoB-depleted plasma samples (equivalent to 5% of plasma) was determined by using J774.A1 [^{3}H]-cholesterol-labeled mouse macrophages.[17] After 4 hours of incubation in the presence of apoB-depleted plasma, efflux of cholesterol was determined and expressed as [^{3}H]-cholesterol medium/([^{3}H]-cholesterol cells+medium)×100. Spanish and Danish samples were assayed in duplicate in 2 independent batches by using 6-well plates. In each experiment, some AAA cases and control samples were always included in the same plate to minimize the effects of intra-plate variation. This experiment was also performed under experimental settings, which stimulated mainly ABCA1-dependent cholesterol efflux by treating the cells with 0.3 mmol/L cyclic adenosine monophosphate or, alternatively, stimulated concerted ABCA1/ABCG1-dependent efflux pathways by treating the cells with 2 µmol/L T0901317 compound, a well-known LXR agonist.[17]

Statistical Analyses

Data are presented as mean±SEM for continuous variables and as frequencies and percentages for categorical variables. Fisher exact test was used to compare the categorical data between groups. The normality of the data was analyzed using the Kolmogorov-Smirnov test. The student unpaired *t* test was used to compare the continuous variables. Correlations between variables were analyzed using Pearson correlation analysis. Multivariate logistic regression models were analyzed to explore the association between efflux and AAA taking into account potential confounders, such as age, statins, HDL cholesterol, LDL cholesterol, apoA-I, preβ-HDL apoA-I levels, and triglycerides. The statistical software R (http://www.r-project.org) and GraphPad Prism 5.0 software (GraphPad, San Diego, CA) were used to perform all statistical analyses. A P value of <0.05 was considered to represent a significant difference in all the analyses.

Results

Defective Preβ-HDL Formation and HDL-Mediated Macrophage Cholesterol Efflux in Spanish Patients With AAA

Clinical characteristics of the Spanish cohort are shown in Table 1. The AAA patients in the Spanish cohort showed lower plasma levels of HDL cholesterol and apoA-I, which were concomitant with low triglyceride and LDL cholesterol levels (Table 1). Basal levels of preβ-HDL particles were 3-fold lower in AAA patients, and were kept at very low levels, even on incubation of plasma at 37°C in the presence of an LCAT inhibitor, as compared with the control group (Table 1). This incubation approach measures plasma potential to generate preβ-HDL. However, the activities of the main HDL metabolism associated lipid transfer proteins and enzymes (PLTP, CETP, and LCAT) were similar in both groups (Table 1). Pearson correlation test did not show any association between these plasma HDL remodeling proteins and preβ-HDL levels (r=−0.13, −0.28, and −0.08 for PLTP, CETP, and LCAT, respectively; P>0.05). Furthermore, the analysis of mature α-mobile HDL composition only revealed

Table 1. Plasma and HDL Parameters in the Spanish Cohort

	Controls (N=20)	AAA Patients (N=20)	*P* Value
General information			
Age, y	65±0.02	70.6±1.36	<0.001
Sex (N/% of men)	20/100	18/90	0.487
Diabetes mellitus (N/%)	7/35	4/25	0.480
Hypertensive (N/%)	13/65	11/55	0.748
Smokers (N/%)	8/40	7/35	1.000
Statins (N/%)	5/25	14/70	0.010
Plasma lipid, lipoprotein profile, and HDL parameters			
Total cholesterol, mmol/L	5.35±0.20	3.03±0.21	<0.001
Triglycerides, mmol/L	1.62±0.18	1.02±0.12	0.009
LDL cholesterol, mmol/L	3.35±0.19	1.78±0.16	<0.001
HDL cholesterol, mmol/L	1.27±0.06	0.79±0.06	<0.001
Mature HDL apoA-I, g/L	1.29±0.04	0.89±0.04	<0.001
Preβ-HDL apoA-I, g/L	0.29±0.02	0.09±0.01	<0.001
Generation of preβ-HDL apoA-I, g/L 6 h	0.50±0.03	0.14±0.01	<0.001
Macrophage cholesterol efflux, %	12.39±0.39	9.26±0.29	<0.001
Master HDL remodeling lipid transfer proteins and enzymes, nmol/mL per hour			
PLTP activity	6387±380	6549±311	0.743
CETP activity	29.31±1.20	31.94±2.43	0.285
LCAT activity	34.13±4.90	34.39±5.49	0.972
Mature HDL composition (mass %)			
Triglycerides	4.01±0.23	5.13±0.41	0.023
Phospholipids	33.39±0.48	33.26±0.51	0.857
Free cholesterol	3.50±0.07	3.54±0.11	0.743
Esterified cholesterol	16.50±0.25	16.42±0.51	0.886
ApoA-I	42.61±0.35	41.66±0.49	0.120

Data are presented as mean±SEM for continuous variables and as frequencies and percentages for categorical variables. AAA indicates abdominal aortic aneurysm; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; and PLTP, phospholipid transfer protein.

Table 2. Plasma and HDL Parameters in the Danish Cohort

	Controls (N=21)	AAA patients (N=21)	*P* Value
General information			
Age, y	68.7±0.52	69.5±0.73	0.373
Sex (N/% of men)	21/100	21/100	1.000
Diabetes mellitus (N/%)	1/4.8	2/9.5	1.000
Hypertensive (N/%)	8/38.1	11/52.4	0.536
Smokers (N/%)	5/23.8	7/33.3	0.734
Statins (N/%)	7/33.3	11/52.4	0.350
Plasma lipid, lipoprotein profile, and HDL parameters			
Total cholesterol, mmol/L	5.55±0.16	4.45±0.12	<0.001
Triglycerides, mmol/L	1.41±0.16	1.27±0.12	0.505
LDL cholesterol, mmol/L	3.17±0.20	2.24±0.14	<0.001
HDL cholesterol, mmol/L	1.74±0.09	1.64±0.05	0.331
Mature HDL apoA-I, g/L	1.63±0.07	1.38±0.05	0.008
Preβ-HDL apoA-I, g/L	0.33±0.03	0.27±0.02	0.081
Generation of preβ-HDL apoA-I, g/L 6 h	0.64±0.04	0.56±0.03	0.112
Macrophage cholesterol efflux, %	12.71±0.68	10.41±0.46	0.008

Data are presented as mean±SEM for continuous variables and as frequencies and percentages for categorical variables. AAA indicates abdominal aortic aneurysm; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

a moderate enrichment of triglycerides in HDL derived from the Spanish AAA patients (Table 1). Also, the inflammatory CRP (C-reactive protein) levels were similar in AAA patients and controls (4.27±1.36 versus 3.48±0.66 mg/L; *P*=0.609).

Next, we determined macrophage cholesterol efflux to apoB-depleted plasma, which contains mature HDL, preβ-HDL particles, and HDL regulatory proteins, thereby permitting optimal HDL remodeling and cholesterol flux to HDL particles. ApoB-depleted plasma from Spanish AAA patients displayed an impaired ability to promote macrophage cholesterol efflux (Table 1). These differences were also observed under experimental settings, which stimulated mainly ABCA1-dependent cholesterol efflux (14.23±0.51% in controls versus 10.80±0.62% in AAA patients; *P*<0.001) or that, alternatively, stimulated concerted expression of ABCA1/ABCG1 (25.29±0.56% in controls versus 21.39±1.00% in AAA patients; *P*=0.002).

A potential limitation of the study in the Spanish cohort was the increased age of AAA patients together with the higher percentage of patients under statin treatment. However, an inverse association between cholesterol efflux and AAA was observed, even after adjusting by age and statin intake (*P*=0.011).

Danish AAA Patients Show Impaired Macrophage Cholesterol Efflux

To validate/replicate the association of cholesterol efflux and preβ-HDL levels with AAA, a new experimental setup was conducted with AAA and control subjects derived from Danish cohort matched with similar age and statin use (Table 2). Danish AAA patients also showed lower LDL cholesterol and apoA-I levels, but HDL cholesterol levels were similar in both groups. Preβ-HDL particles levels tended to be lower in Danish AAA patients; however, this trend did not reach statistical significance (Table 2). More importantly, Danish AAA patients showed impaired macrophage cholesterol efflux (Table 2), thereby emphasizing that this major HDL cardioprotective function was altered independently of HDL cholesterol levels, age, and statin use.

We also evaluated different multivariate logistic regression models to take into account potential confounders. An inverse association between cholesterol efflux and AAA was observed, even after adjusting for age, statin use and lipid, apoA-I, and lipoprotein levels (*P*≤0.016 for all logistic regression models).

Discussion

Human AAA wall is characterized by the presence of cholesterol crystals and macrophage infiltration.[10,18] Cholesterol crystals could promote AAA dilation and growth towards rupture by inducing smooth muscle cell death and an immune-inflammatory response. The presence of cholesterol crystals has been associated with a decrease in smooth muscle cell density in atherosclerotic plaques, suggesting a link between smooth muscle cell death and cholesterol crystals formation.[19] Cholesterol crystals also induce inflammasome activation,[20] and this has been shown to be involved in the development of experimental AAA.[21,22] Furthermore, cholesterol accumulation as a result of an impaired cholesterol efflux sensitizes macrophages to a more proinflammatory state.[23] Two experimental studies have raised the concept that injecting reconstituted HDL, consisting of native apoA-I and phospholipids, or the apoA-I mimetic peptide D4F have potential to reduce AAA.[9,24] Infusion of reconstituted HDL or D4F increase preβ-HDL levels and promote macrophage cholesterol efflux,[25,26] thereby indicating that HDL-mediated macrophage cholesterol efflux could attenuate AAA progression. In the present study, we show, for the first time to our knowledge, that HDL from AAA patients has an impaired ability for promoting cholesterol efflux from macrophages.

We previously reported that plasma apoA-I levels were lower in AAA patients compared with those of healthy subjects.[9,27] In the latter report, the percentage of small α-mobile HDL particles was moderately but significantly lower in AAA patients than in control subjects. These small HDL particles represented <10% of the total HDL pool of particles and the percentage of intermediate and large α-mobile HDL did not differ between the 2 groups.[27] These findings are in line with these new results in AAA patients with large AAA diameter (aortic diameter >50 mm) in which the amount of nascent preβ-HDL particles was low, but only moderate changes were found in the mature α-mobile HDL particles. The lack of association among the plasma factors involved in HDL remodeling and preβ-HDL particle levels in AAA patients indicates that the low preβ-HDL levels may be rather attributable to an impaired de novo formation of preβ-HDL or an enhanced clearance of lipid-free apoA-I, thereby reducing the potential to form apoA-I-containing mature α-mobile HDL. Also, it is possible that some inherent particle properties of HDL of AAA patients that is not detected based on major HDL component analysis attenuate particle interconversion to preβ-HDL. This view needs further verification.

Defects in macrophage cholesterol efflux in both Spanish and Danish AAA patients was even higher than what we recently found in Spanish familial hypercholesterolemic patients without any treatment.[28] However, we did not find any correlation between macrophage cholesterol efflux and AAA diameter in AAA patients. Because we only included AAA patients with large aortic diameter in this study, the narrower distribution of values could likely contribute to the loss of this correlation between AAA diameter and macrophage cholesterol efflux. Further studies are needed to establish the association between HDL-mediated macrophage cholesterol efflux and AAA presence and progression in larger cohorts.

In conclusion, the present study shows that AAA patients have low levels of plasma apoA-I and preβ-HDL particles and impaired macrophage cholesterol efflux. This major HDL functional alteration in AAA patients could be mechanistically linked to AAA.

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Disclosures

None.

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Highlights

- This is the first study to provide evidence that HDL (high-density lipoprotein)-mediated macrophage cholesterol efflux is impaired in patients with an abdominal aortic aneurysm.
- This major HDL functional alteration could be mechanistically linked to abdominal aortic aneurysm.
- Abdominal aortic aneurysm patients show low levels of preβ-HDL particles.