PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER

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PhD Thesis Dissertation

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UNIVERSITAT ROVIRA i VIRGILI

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I STATE that the present study, entitled "**Peripheral artery disease: the search for a biological marker"** presented by **Anna Hernández Aguilera** for the award of the degree of Doctor, has been carried out under my supervision at the **Department of Medicine and Surgery** of this University.

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"Scientists say that we are made of atoms, but a little bird told me that we are made of stories"

"Si me caí es porque estaba caminando. Y caminar vale la pena, aunque te caigas"

Eduardo Galeano (1940 – 2015)

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A la meva família

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Hi ha tanta gent a qui he d'agrair la seva col·laboració en algun moment o altre de la tesi que segur que m'oblidaré d'algú.

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Abbreviations

α-SMA: Alpha-smooth muscle actin, acetylated N-terminal
ABI: Ankle-Brachial index
ACKR: Atypical chemokine receptors
AhR: Aryl hydrocarbon receptor
ALI: Acute limb ischemia
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
BCAA: Branched-chain amino acids
BMI: Body mass index
C4M: MMP-mediated type IV (alpha 1) collagen degradation
CAD: Coronary artery disease
CCL2: Chemokine (C-C motif) ligand 2
CCR2: Chemokine (C-C motif) receptor 2
CLI: Critical limb ischemia
CRP: C-reactive protein
CRPM: Specific fragment of MMP-1, -3, -8, -9, CatS/K, ADAMTS1-mediated degradation of C-reactive protein
CV: cardiovascular
DAB: 3, 3 -diaminobenzidine
DARC: Duffy antigen receptor for chemokines
DSPG: Dermatan sulfate proteoglycan
EC: Endothelial cells
ECM: Extracellular matrix
eNOS: Endothelial nitric oxide synthase
ER: Endoplasmic reticulum
FDA: Food and Drug Administration
GAG: Glucosaminoglycan
HDL: High-density lipoprotein
HSPG: Heparan sulfate proteoglycan
IC: Intermittent claudication
ICAM-1: Intercellular adhesion molecule-1
IL: Interleukin
IMR or I/M: Intima/Media Ratio

IMT: Intima-Media Thickness
IQR: Interquartile range
iTRAQ: Isobaric tag for relative and absolute quantification
KSPG: Keratan sulfate proteoglycan
Lam-a5: Specific fragment of MMP-9-mediated degradation of alpha 5 chain of laminin
LDL: Low-density lipoprotein
LTQ: Linear trap quadropole
MCP-1: Monocyte chemoattractant protein 1
MIM: Specific fragment of MMP-9 and -12-mediated degradation of mimecan
mmLDL: Minimally-modified LDL
MMP: Matrix metalloproteinase
MS: Mass spectrometry
NF-kβ: Nuclear factor kappa beta
NHANES: National Health and Nutrition Examination Survey
oxLDL: oxidized LDL
PAD: Peripheral artery disease
PCA: Principal component analysis
PDGF: Platelet-derived growth factor
PON: Paraoxonase
PPAR: Peroxisome proliferator-activated receptor
ROS: Reactive oxygen species
SMC: Smooth muscle cell
T2D: Type-2 diabetes
TCA: Tricarboxylic acid
TIMP: Tissue inhibitors of metalloproteinases
tPA: Tissue plasminogen activator
VCAM-1: Vascular cell adhesion protein-1
VCANM: Specific fragment of MMP-8 and -12-mediated degradation of versican
VEGF-a: Vascular endothelial growth factor-A
vWF: Von-Willibrand factor
WHO: World Health Organization

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ABSTRACT

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Abstract

Peripheral artery disease (PAD) is a common manifestation of systemic atherosclerosis affecting peripheral arteries. Despite affecting more than 200 million patients in industrialized countries, PAD is often underdiagnosed. The first stages of the disease are asymptomatic and thus, to perform an early diagnose becomes difficult. Patients can undergo different treatment according disease severity: management of cardiovascular risk factors, exercise, rehabilitation and pharmacological intervention. In advanced stages, vascular specialists usually perform invasive surgical procedures.

The ankle-brachial index (ABI) is the most used test for PAD diagnosis although it presents some limitations. It is an easy technique based on the ratio between the pressure of ankle arteries and the pressure of brachial artery of the arm. There are additional circulating biomarkers of diagnosis, mainly based on cardiovascular risk factors, inflammation or oxidative process; they are not exclusively for PAD but for atherosclerosis in general.

Inflammation, oxidation, vascular remodeling and mitochondrial dysfunction play an important role in the development of atherosclerosis. We hypothesized that an increased knowledge on these processes would provide circulating biomarkers for the disease as well as possible therapeutic strategies.

In **Study 1** (*International Journal of Molecular Sciences*), we performed a tissue characterization by investigating the immunohistochemical expression of paraoxonases (PON) and chemokines in arteries of PAD patients. Results showed that PON1 and PON3 were increased in

Abstract

atherosclerotic arteries, probably by PPARγ- and NFκβ-mediated pathways, suggesting an oxidative stress prevention and foam cell formation during atherosclerosis. Chemokine (C-C motif) ligand 2 (CCL2) and atypical chemokine receptors (ACKR) DARC and D6 were also significantly increased in affected arteries, although they did not show a uniform distribution among artery layers. This increased expression of CCL2 may be related to the inflammatory conditions within the artery wall. The expression of ACKRs, which bind to CCL2 but do not exert signal transmission, may modify chemokine availability and cell migration.

Extracellular matrix (ECM) turnover is also involved in vascular remodeling. In **Study 2** (*Journal of Vascular Surgery*), by combining histological and proteomics approaches, we confirmed a significant artery remodeling in PAD patients. Atherosclerotic arteries showed a specific protein profile in which ECM-related components were underexpressed, suggesting a possible degradation of the ECM. We then measured a panel of ECM fragments of degradation (neo-epitopes) by ELISA in serum samples of PAD patients. Neo-epitopes of versican degradation (VCANM) and type IV collagen degradation fragments (C4M) showed potential as biomarkers to segregate patients across the spectrum of PAD.

Impaired metabolism and mitochondrial dysfunction may also predispose to disease. In **Study 3** (*Translational Research*), we used a targeted metabolomics methodology to assess the plasma metabolome of PAD patients. Many of measured metabolites were connected not with PAD, but with associated comorbidities (hypertension, type-2 diabetes, dyslipidemia), age or body mass index. We discarded these metabolites as PAD biomarkers, and focused on the 6 remaining metabolites directly

Abstract

related to PAD. (Iso)citrate and glutamate showed the best discriminant capacity not only between control volunteers and PAD patients, but also for an early detection of the disease.

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INTRODUCTION

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1. CARDIOVASCULAR SYSTEM

In humans, cardiovascular system is a closed and double system consisting of heart and blood vessels by which blood is carried to the tissues. Its main function is to maintain cellular homeostasis by allowing the exchange of breathing gases, nutrients, water, hormones and metabolic waste products between tissues and vessels [1–4].

Blood vessels are divided into arteries, veins and capillaries. The wall of arteries and veins has a similar structure with some minor differences meanwhile capillary structure is less complex [5]. Traditionally, the artery is constituted by 3 concentric layers, laminas o *tunicas*: *Intima, Media* and *Adventitia* [1,4,6,7].

1.1 Tunica Intima

The *intima* is the deepest layer of the artery wall composed by a single layer of endothelial cells (ECs) located on a thin basal lamina. The sub-endothelial layer have variable thicknesses [8]. ECs constitute the luminal side of the vessel wall [9]. Commonly, these elongated and flat-shaped cells can suffer changes depending on the direction of blood flow and communicate to each other through a network of three types of junctions: a) Tight junctions, which regulate the delivery of substances across the endothelium; b) Adherent junctions, which control circulating cells by acting on endothelium permeability; c) Gap junctions, which allow the exchange of ions and metabolites between cells [10,11]. Endothelial dysfunction is known to be an important factor in atherosclerosis initiation and progression [4,9,12,13] The *intima* layer is mainly composed of type I collagen, and of type III and IV collagen in less proportion and with different orientation patterns. The sub-endothelial

layer contains mainly smooth muscle cells (SMC) and collagen fibrils [14]. The internal elastic lamina determines the limit between *tunica intima* and *tunica media*. This elastic lamina is usually prominent in muscular arteries and thin in elastic arteries. The main functions of this *tunica* are the regulation of material transport between blood and tissues, the regulation of platelet function and the modulation of vascular tone [3,9,13].

1.2 Tunica Media

The *media* is located between the *intima* and the *adventitia* and is the most important layer of the arteries regarding mechanical properties. It is delimited by the internal elastic lamina and the external elastic lamina. *Tunica media* is mainly composed of smooth muscle cells (SMCs) that are normally distributed in a complex linkage with elastin and collagen [15]. SMCs have a spindle shape, an elongated nucleus and adopt a particular orientation in arteries. In elastic arteries, SMCs are separated by elastin. ECM components are synthesized by SMC. These SMCs are essential for the vasculature: they modulate arterial structural changes and diameter to endure blood flow dynamics and maintain blood pressure. SMCs can suffer phenotype changes, shifting from contractile to synthetic, and then display different cell shape, marker expression and proliferative and migration rates [16]. This change on SMC phenotype from contractile into synthetic stimulates cell proliferation and invasion of the *tunica intima*, characteristic of atherosclerosis [12,17,18]. This *tunica* is responsible of the elasticity and contraction of the vessel [19].

1.3 Tunica Adventitia

The *adventitia* is the most external layer of the artery wall. It is mainly composed of fibroblasts, macrophages, collagen and matrix [8]. Depending on the localization and function of the vessel wall, this *tunica* exhibits variable levels of thickness. Collagen is one of the main constituents, offers support and strength to the arterial wall and prevents an excessive distension of the vessels. Other characteristic parts of the *adventitia* are nerve fibers and *vasa vasorum*, which is an intravascular organization of small vessels [15]. They act as a nutritive support to arteries when nutrient diffusion from the *intima* is not possible due to excessive thickness. *Vasa vasorum* may have an important role in the initiation and progression of atherosclerosis, as it is involved in inflammatory cell infiltration [20].

2. ARTERIOSCLEROSIS

Arteriosclerosis is a Greek-derived term meaning "hardening of the arteries" [21]. It is one of the top contributors to mortality in Western countries [22–25]. Sections of the arterial tree with high or oscillatory endothelial stress, placed near branch points and along inner curvatures, are the most vulnerable to suffer stiffening, thickening and loss of elasticity [2,26]. Since 1954, arteriosclerosis classification has been divided in three lesion types [27]:

a) ***Atherosclerosis***. In this case, the atheroma is the characteristic lesion in large and elastic muscular arteries. The lesion is mainly located in the intima, where lipids, connective tissue, calcium deposits, inflammatory cells and matrix proteins can be found.

b) ***Mönckeberg medial calcific sclerosis***. It is a calcification development that involves only the tunica *media* of the arteries. The arterial lumen is not generally affected, although this kind of arteriosclerosis can coexist with atherosclerosis.

c) ***Arteriolosclerosis***. The lesion takes place in small arterial vessels with 1 or 2 layers of SMC and it is classically linked with hypertension and diabetes mellitus.

Although this classification is currently being used, there is still controversy, as some authors believe terms do not describe exactly observed facts [21,28].

3. ATHEROSCLEROSIS

Atherosclerosis is a progressive and multifactorial disease related to chronic inflammation in which a disturbed equilibrium in the arteries leads to plaque development [29–31]. Coronary artery disease (CAD), cerebrovascular disease, atherosclerotic kidney disease and peripheral artery disease (PAD) are different kinds of atherosclerotic diseases [7]. Although their symptoms are different, there are common risk factors including genetic conditions and inappropriate lifestyle, which can be the origin of the disease [6,30,32]. Pathogenic mechanisms for cardiovascular diseases are not completely understood, mainly due to their complexity. Initial stages of atherosclerosis are asymptomatic and starts during childhood or adolescence, although its impact takes place at advanced ages [7,9,33–35].

3.1 Risk factors

Cardiovascular risk factors, which can be genetic and environmental, are thought to be responsible for the onset of the disease. In 2009, the World Health Organization (WHO) showed estimations that 8 modifiable risk factors (tobacco, sedentary lifestyle, raised blood pressure, raised blood glucose and cholesterol, alcohol consumption, high body mass index and poor consumption of fruit and vegetables) contributed for 61% of all cardiovascular disease deaths [36].

The most important cross-sectional and longitudinal epidemiologic studies performed in the general population providing evidence on the role of these risk factors were The Framingham Study [37] and Project SCORE [38]. The identification of these risk factors allowed the subsequent development of mathematical models to calculate cardiovascular risk in patients [39]. The established risk factors can be classified as modifiable, which can be treated or changed, or non-modifiable, which cannot be changed (**Table 1**) [7,32,40,41].

3.2 Theories of atherogenesis

Many theories have been proposed in order to explain the initiation of atherosclerotic lesion, although there are numerous common steps in all of them: I) Proliferation of intimal smooth muscle cells; II) Synthesis of excessive extracellular components; III) Accumulation of lipids around cells; and IV) Entrance of monocytes/macrophages into affected area [42].

Table 1. Main cardiovascular risk factors [31].

Traditional cardiovascular risk factors	
Non-modifiable	**Modifiable**
Age Gender Familiar antecedents	Smoking Obesity Hypertension ↑LDL-cholesterol and ↓ HDL-cholesterol Type 2 diabetes / Insulin resistance Metabolic syndrome High-fat diet ↑ CRP Sedentary lifestyle
Other emergent cardiovascular risk factors	
↑ Triglycerides ↑ Lipoprotein (a) Dense and small LDL particles ↑ Apolipoprotein B and ↓ apolipoprotein A-I ↑ total cholesterol/HDL-cholesterol ↑ Serum homocysteine Endothelial dysfunction Prothrombotic factors Inflammatory markers (IL-6, CCL2…) ↑ Carotid IMT	

LDL: Low-density lipoprotein; HDL: High-density lipoprotein; CRP: C-reactive protein; IL: Interleukin; CCL2: Chemokine (C-C motif) ligand 2; IMT: Intima-Media thickness.

The Lipid theory was based on the finding that lipids accumulate in the *intima*. An increased plasma low-density lipoproteins (LDL) concentration and an altered permeability of the endothelium may favor LDL-cholesterol accumulation and the subsequent inflammatory processes [7,43].

The Hemodynamic theory suggests that altered hemodynamics causes lesions in the artery wall. Oscillating flow may also modify endothelial permeability [2,7].

The Fibrin Incrustation theory was proposed on 19th century and was based on a possible reorganization of the thrombus. This theory postulated that when a thrombus is formed on the luminal side of the artery it can be incorporated into the plaque and transformed into fibrous tissue, increasing plaque mass and size [7]. Due to some inadequacies, this theory has evolved and now is focused on the general process of thrombogenesis.

The Nonspecific Mesenchymal Hypothesis describes the importance of SMC. Stimuli to the arterial wall induce the migration of SMC (here Mesenchymal) from the *media* to the *intima*. Then they proliferate and produce connective tissue with proteoglycans and collagen [7,12].

The Response to Injury Hypothesis is considered an improved version of the Nonspecific Mesenchymal Hypothesis only applicable in some context. This theory postulates that an initial injury of the arterial wall may strip the endothelium and induce platelet adherence. In turn, those platelets secrete platelet-derived growth factor (PDGF) and this induces SMC migration to the *intima*, where they proliferate and synthesize connective tissue [7].

3.3 Pathogenesis: Initiation and progression of atherosclerosis

Traditionally, LDL accumulation has been proposed as the principal initiation event for the atherosclerotic process (**Figure 1**) [30,31,44]. These LDL particles bind to intimal proteoglycans, and then the endothelium becomes more leaky. The increased expression of

lipoprotein-binding molecules improves the capacity to retain LDL [22,26].

After departing the blood, LDL particles accumulate in the intima where they are exposed to oxidative modifications by macrophage lipoxygenase and myeloperoxidase and progress from minimally modified LDL (mmLDL) to oxidized LDL (oxLDL) [45,46]. OxLDL deposition in the arterial wall enhances a chronic inflammatory response that starts with the production of chemotactic molecules, such as monocyte chemoattractant protein 1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), which induces monocytes to enter the plaque and differentiate into macrophages [18,23,31]. Cell adhesion molecules expressed on endothelial surface mediate this recruitment: Vascular cell adhesion protein-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin among others [31].

Macrophages express scavenger receptors that can recognize oxLDL and imbibe them so becoming the prototypical cell in atherosclerosis: lipid-laden macrophages or foam cells [6,23,31]. Foam cells form a typical yellow-colored fatty streak, the first sign of atherosclerosis [22,26]

Atherosclerosis

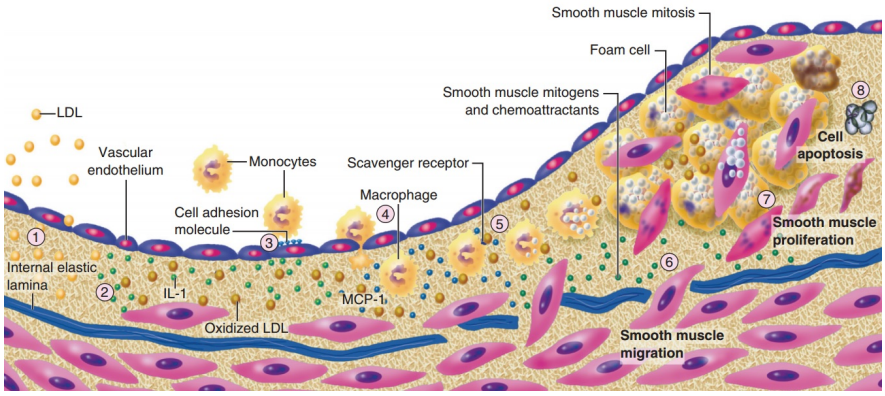

Figure 1. Schematic of the evolution of the atherosclerotic plaque. 1, Accumulation of lipoprotein particles in the *intima* (yellow spheres). 2, Oxidative stress can induce local cytokine elaboration. 3, These cytokines increase expression of adhesion molecules from leukocytes that cause their attachment and chemoattractant molecules that direct their migration into the *intima*. 4, Blood monocytes entering the artery wall in response to chemoattractant cytokines. 5, Scavenger receptors mediate the uptake of modified lipoprotein particles and promote the development of foam cells. 6, SMCs migrate from the *media* to the *intima*. 7, SMCs can then divide and elaborate extracellular matrix, promoting extracellular matrix accumulation in the growing atherosclerotic plaque. 8, In later stages, fibrosis continues, accompanied by SMC death, yielding a fibrous capsule surrounding a lipid-rich core that also may contain dying or dead cells and their detritus. Extracted from Libby P. (2015).

Different immune cells migrate into the plaque and release diverse types of cytokines that contribute to plaque progression or regression, depending on their immunologic role. Inflammatory and pro-atherogenic cytokines increase macrophage recruitment, foam cell formation and apoptosis. M1 macrophages, in turn, contribute to the oxidant environment by producing hydrogen peroxide, superoxides and myeloperoxidases, which initiate endoplasmic reticulum (ER) stress [18]. Extracellular matrix has also an important role in atherosclerosis, as ECM macromolecules breakdown and accumulate in atheroma [6]. This contributes to the growth of the necrotic core and the lesion is then called fibroatheroma [47].

A subsequent evolution of the atheroma involves SMC, which play a critical role in the progression of atherosclerosis [6,18]. In response to proinflammatory cytokines, growth factors, oxLDL and other stimuli, vascular SMC suffer a phenotypic dedifferentiation: from the quiescent, contractile phenotype to an activated, synthetic phenotype [6,12,18]. They become proliferative and migratory, increasing the production of ECM proteins and invading *tunica intima*. Those migratory SMC differ from those in the normal arterial *tunica media* [6]. SMC replication and accumulation in the atherosclerotic plaque is also accompanied by SMC death. However, SMC play a vital role in the formation of the protective fibrous cap, characteristic of later stage atheroma [18].

In progressive atherosclerotic lesions, it is common to find calcifications, as necrotic core act as a focus for calcium granules in a process that shares mechanisms with bone formation [6]. When the necrotic core fully calcifies, then calcifications represent the major part of plaque volume [47].

Advanced atherosclerotic plaques can lead to ischemia or plaque rupture and thrombosis (**Figure 2**). Ischemic symptoms take place due to the progressive narrowing of the lumen. During plaque rupture, lipids and tissue factor react with blood components, and they induce the coagulation cascade with the subsequent platelet adherence and thrombosis [31].

Atherosclerosis

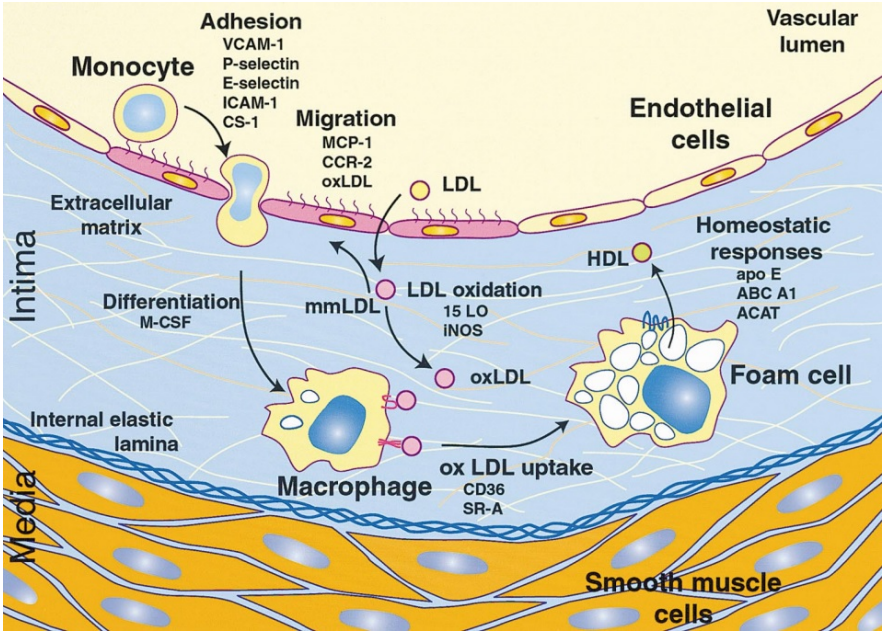

Figure 2. Plaque Rupture and Thrombosis. Necrosis of macrophage and smooth muscle cell–derived foam cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol. Macrophage secretion of matrix metalloproteinases and neovascularization contribute to weakening of the fibrous plaque. Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets, and the formation of a thrombus. Extracted from Glass C.K. and Witztum J.L. (2001).

4. PERIPHERAL ARTERY DISEASE (PAD)

Peripheral artery disease is caused by the lack of perfusion in the extremities as atherosclerotic plaque builds up in peripheral arteries, which mainly carry blood to the limbs [48,49]. It affects approximately 27 million people in Europe and United States [50]. The Edinburgh Artery Study described that 209 out of 1519 (14%) apparently healthy volunteers developed PAD during a follow-up period of 17 years [51]. Despite its common occurrence, PAD is often underdiagnosed and to prevent derived consequences becomes impossible [49,52]. The progression of the disease is normally silent, so preventive measures

cannot be applied [49,52–54]. In PAD patients, the overall amount of tissue affected of atherosclerosis is far superior to that in CAD, due to the size of the arteries involved. This fact is of great importance when developing diagnostic and therapeutic strategies [55–57].

4.1 Definition and classification

The disease can be defined anatomically or functionally, thus dividing patients into asymptomatic and symptomatic. Anatomically, it can be defined as atherosclerotic arterial disease. Functionally, it is defined as arterial narrowing causing intermittent claudication, exercise limitation or even tissue loss [58]. PAD has a wide range of clinical manifestations, from asymptomatic disease and intermittent claudication (IC) to critical limb ischemia (CLI) [59].

Many classification schemes have been proposed to characterize the severity of the disease. The most commonly used classifications are those proposed by Fontaine and Rutherford (**Table 2**) [58,59].

Table 2. Fontaine and Rutherford classification systems for PAD.

Fontaine		**Rutherford**	
Stage	**Symptoms**	Stage	Symptoms
I	Asymptomatic	**0**	Asymptomatic
IIa	Mild claudication	**1**	Mild claudication
IIb	Moderate claudication	**2**	Moderate claudication
		3	Severe claudication
III	Ischaemic rest pain	**4**	Ischaemic rest pain
IV	Ulceration or gangrene	**5**	Minor tissue loss
		6	Major tissue loss

Mild claudication is defined when symptoms appear after ambulating >200m and moderate claudication is defined when symptoms appear after ambulating <200m.

4.2 Risk factors for PAD

Age

The prevalence of this disease increases with age after 40 years old [7]. Young population have more risk for PAD not only for age, but also for other risk factors, meanwhile people over 70 are at increased risk for PAD only for aging [60–64].

Gender

Peripheral artery disease is generally more prevalent in men compared with women. This has been classically explained for the protective role of women hormones. In fact, after menopause, rates of PAD for women and men become similar [7,65].

Familiar history and genetic factors

Patients with familiar antecedents of cardiovascular disease seem to be at major risk for PAD. The concrete contribution of genetics and environmental factors are still not clear, but are in active research. Families with identified early onset atherosclerosis have an increased risk for PAD, although there is not genetic marker identified [57,66].

Smoking

The association between smoking and peripheral artery disease is well established. It is known that cigarette smoke influences the development and progression of atherosclerosis by inducing endothelial damage, arterial smooth muscle proliferation, thrombophilia, inflammation, and other metabolic abnormalities

[67–70]. In fact, smoking seems to be a higher risk factor for PAD than for CAD [71–73].

Hypertension

There is a strong association in men and women between the development of atherosclerosis and hypertension. In hypertensive patients, the risk of developing symptomatic PAD (intermittent claudication) was twice that in patients without hypertension in the Framingham study [74,75]. Moreover, the National Health and Nutrition Examination Survey (NHANES) study revealed that hypertensive patients have a higher prevalence of asymptomatic PAD [63].

Hyperlipidemia

Patients with abnormal concentration in total cholesterol, LDL, triglycerides and lipoprotein (a) have an increased risk for cardiovascular disease. PAD patients are more likely to have higher levels of triglycerides, cholesterol, lipoprotein (a), and very low-density lipoprotein, compared with healthy individuals [76–78]. On the contrary, the levels of high-density lipoprotein (HDL) cholesterol and apolipoprotein A-I and A-II, the so-called "protective" lipoproteins, are lower in these patients [79]. Lipoprotein (a) is another important risk factor for PAD. Patients with premature PAD have lipoprotein (a) levels higher than controls [80]. A fasting cholesterol concentration higher than 270 mg/dL was associated with an increased incidence of intermittent claudication in the Framingham study [75].

Progression and incidence of PAD may be decreased with a properly treatment of hyperlipidemia.

Diabetes

Diabetes is another coronary artery disease risk equivalent. In the Framingham Heart Study, diabetes increased the risk for developing symptomatic PAD [75]. At initial diagnosis, patients with diabetes have more advanced arterial disease and minor outcomes compared to non-diabetic patients [81,82]. The risk of atherosclerosis is also increased with a poor glycemic control [83].

4.3 Symptoms and diagnostics

Symptomatic PAD patients usually present intermittent claudication, which is a muscle pain during exercise that is calmed by rest [58,84]. Pain is usually felt in the calf, buttock and foot, distal to the arterial occlusion. Estimated walking distances are used to measure the severity of the disease, as it may serve as an indicator of improvement or deterioration [84]. Normally, symptoms appear over months or years and if they show rapid exacerbation, this may indicate new vessel occlusion.

Critical limb ischemia, the most severe manifestation of PAD, is characterized by ulceration, gangrene or rest pain for more than two weeks that affects the toes or foot of the affected limb [59,84]. In this stage, gradual ischemic conditions promote angiogenesis of collateral vessels to maintain limb perfusion [59].

Due to acute thrombosis of a rupture atherosclerotic plaque, PAD patients are at risk of developing acute limb ischemia (ALI). It is manifested with the 6Ps: pain, pallor, pulseless, perishing cold, paresthesia and paralysis [59,84]. There are no collateral vessels in ALI to maintain blood flow, so this threatens limb integrity [59].

The usual evolution of PAD is to follow a gradual progression, from asymptomatic to intermittent claudication and CLI. However, more than half of patients with CLI did not show ischemia six months earlier [84].

To perform the diagnosis of PAD, vascular specialists perform physical examinations and questionnaires, supported by non-invasive and invasive techniques [58,59,84]. Physical examination should focus on the arterial system: auscultation and palpation of the abdomen, palpation and gradation of femoral, popliteal and tibialis pulses… Moreover, limbs should be inspected to detect ulcers, calluses or xanthomas. Specific questionnaires, developed to detect symptomatic PAD, consist of six questions and a diagram to indicate the location of the pain [58].

The anche-brachial index (ABI) is a non-invasive, inexpensive, simple test used to diagnose PAD based on the assessment of lower extremities hemodynamics [59,85]. It represents the ratio of the highest ankle pressure in each leg, obtained at the *dorsalis pedis* and posterior tibial arteries with a Doppler probe, referred to the highest brachial artery pressure (**Table 3, Figure 3**). Although vascular specialists have been employing ABI for some time, there is a lack of standardization for the method of measuring ABI and results do not always reflect a real

situation [56,85]. There is a diminished sensitivity in patients with medial calcification due to hypertension, diabetes or chronic kidney disease [59]. Moreover, some PAD patients have normal ABI values [86].

Table 3. Reference values for ankle-brachial test.

Ankle-brachial indices	
1.0 – 1.3	Normal
0.9 – 1.0	Borderline
0.7 – 0.9	Mild
0.4 – 0.7	Moderate
<0.4	Severe

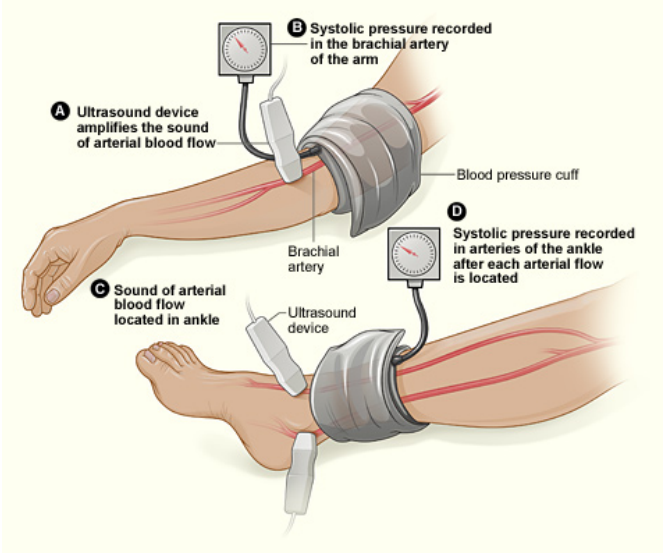

Figure 3. Ankle-Brachial Index test.

Regarding anatomical definition, arteriography is the golden standard, as it has allowed better diagnosis and image definition. Due to the risk of nephrotoxicity that traditional iodinated contrast agents have, carbon dioxide arteriography has also been developed [58].

4.4 Treatments

PAD treatment can be divided in four strategies: management of cardiovascular risk factors, exercise and cardiovascular rehabilitation, pharmacologic intervention and invasive strategies (**Figure 4**) [57–59].

Figure 4. Four main PAD therapeutic strategies.

In addition to required medications to modify risk factors (antihypertensives, cholesterol lowering agents…), antiplatelet medications and agents that increase the distance to claudication can be used [58,84].

If risk factor modification, exercise programs and pharmacologic treatment fail, then invasive interventions are considered [58,59]. They can be endovascular (angioplasty or stenting) or surgical. For patients with intermittent claudication, specialists perform angioplasty or stenting to a single arterial segment. Surgical bypass is only needed in some special cases, but especially appropriate in patients with critical limb ischemia. When revascularization is not successful or not considered, primary amputation is the choice [59,84].

4.5 Current and emerging biomarkers

The Food and Drug Administration (FDA) describes a biomarker as a defined characteristic that is measured which is an indicator of normal

biological processes, pathogenic processes or response to interventions (including therapeutics) or exposure [87].

There are some evidenced and established biomarkers for PAD, although not exclusively for it, as all cardiovascular diseases share common risk factors and pathophysiology [88]. The majority of circulating biomarkers for PAD have a role in inflammatory or oxidative processes, are modulators of angiogenesis, or involved in the thrombosis cascade [57,88]. Moreover, the list is still growing up, as the emerging "omics" (mainly metabolomics and proteomics) allow the possibility to propose novel circulating biomarkers (**Table 4**).

Table 4. Main established and emerging biomarkers for PAD.

Pathway	Biomarkers
Inflammation	CRP IL-6 β2-microglobulin
Chemokines and endothelial activation	VCAM-1 ICAM-1 P-selectin CCL2 CD40 ligand
Thrombosis cascade	vWF tPA Fibrinogen
Angiogenesis	VEGF-A Angiopoietin-2
Oxidative stress and other biomarkers	Homocysteine Protein carbonyls PON1 Isoprostanes

CRP: C-reactive protein, IL: interleukin; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; CCL2: chemokine (C-C motif) ligand 2; vWF: Von-Willibrand factor; tPA: tissue plasminogen activator; VEGF-A: vascular endothelial growth factor-A, PON1: paraoxonase-1. Adapted from Hazarika S. 2017 and Fort-Gallifa I. 2016.

5. VASCULAR REMODELING

Vascular remodeling is an important process that affects the pathophysiology and clinical manifestation of common conditions [89]. It is an active process that involves structural changes in four cellular processes: cell growth, cell death, cell migration and synthesis or degradation of ECM [90]. Vascular remodeling starts with the detection of signals due to changes in hemodynamic conditions (sensors) and the transduction of signals to adjacent cells. Then, depending on the signal, there is a synthesis and release or activation of substances to influence one of the four aforesaid processes [90].

The endothelium is the key player in vascular remodeling, as it have a prominent role in assessing hemodynamic and humoral signals and eliciting biological responses [89,90]. Endothelial dysfunction and extracellular matrix turnover are important parts of this remodeling [89,90].

5.1 Endothelial dysfunction

Endothelial dysfunction plays a key role in the initiation of atherosclerotic process [91]. Vascular endothelium is a highly selective semipermeable layer of single-cell located between blood stream and vessel wall. It responds to hemodynamic and humoral stimuli and regulates vascular tone [13].

Physiologic and pathophysiologic stimuli can provoke changes in endothelial permeability. Those changes are believed to be an early event and contribute to disease progression in atherosclerosis (**Figure 5**),

as the dysfunctional endothelium may allow LDL particles to enter the vessel wall [13,91].

Proposed mechanisms of endothelial dysfunctions are related to oxidative stress, inflammation, infection, vitamin D deficiency and shear stress [9,89]. Oxidative stress seems to be the most common underlying mechanism for endothelial dysfunction. Most of cardiovascular risk factors are associated with the up-regulation of oxidative stress and reactive oxygen species (ROS), promoting among others endothelial nitric oxide synthase (eNOS) uncoupling. In this uncoupled state, eNOS becomes a ROS generator. Because of the causal relationship between oxidative stress and inflammation, vascular inflammation signaling pathways are amplified and many inflammatory markers have been associated with endothelial dysfunction in atherosclerosis [9,92–94].

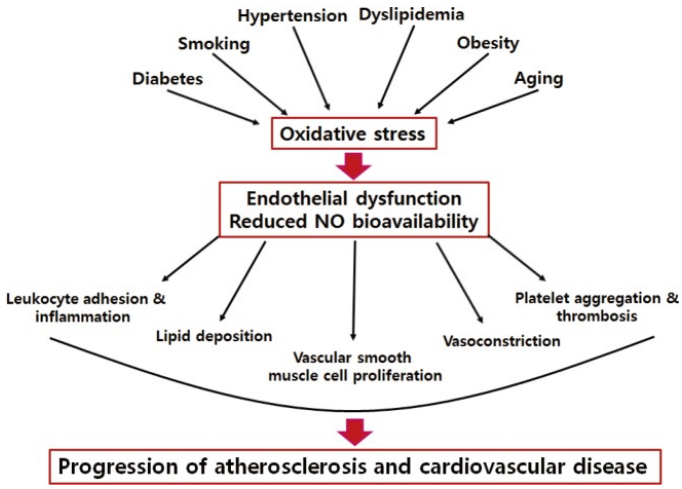

Figure 5. Progression from risk factors to atherosclerosis through oxidative stress and endothelial dysfunction. Extracted from Park K-A *et al*. (2015).

5.2 Extracellular matrix

Inside artery structure, extracellular matrix (ECM) has a remarkable role as it represents more than half of the wall mass and is an essential component of the cardiovascular system [95,96]. ECM is synthesized by medial SMC and by adventitial fibroblasts and is implicated in the control of many important functions of the heart and vessels: it maintains structural uprightness of vascular network, originates a scaffold for cell junction and function and mediates cell adhesion and cell-cell contacts [8]. Moreover, ECM controls remodeling during inflammation, vascular injury and growth, acts as a reservoir of cytokines, proteases and growth factors, provides physical support and maintains the integrity of the tissue [97].

ECM constituents are mainly collagens, elastin, fibronectin, microbifrils (mainly fibrillins), abundant amorphous or soluble proteoglycans, and leucine-rich glycoproteins, which are differently distributed among artery layers (**Figure 6**) [96].

Elastin, the most prominent component of the arterial wall, is a hydrophobic and insoluble protein. It contributes to the elasticity and mechanical integrity of the arteries and regulates SMC proliferation and phenotype [8]. It represents the 90% of elastic fiber total weight and the 50% of dry weight of the arterial wall [98]. Elastin gene encodes for tropoelastin, a precursor form of elastin. The mature form of elastin has a half-life of 40 years and aging or disease provokes its degradation.

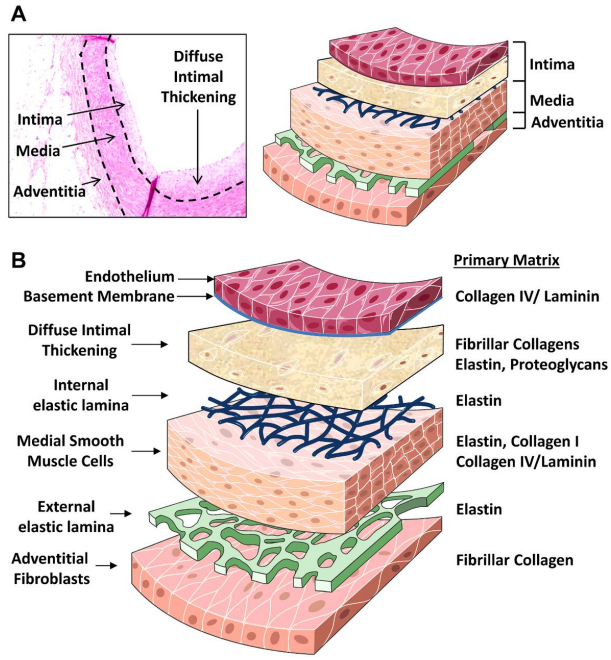

Figure 6. Vascular matrix by arterial layer. (**A**) Representation of the arterial intima, media and adventitia in a human coronary artery. (**B**) Changes in vascular matrix components among different arterial layers. Extracted from Yurdagul A. *et al* (2016).

Collagen is a 300nm triple helix protein that normally consists of two identical chains and an additional one varying in its chemical composition. Collagen is a big family of proteins that includes 24 different subtypes whose function is to limit excessive vessel distension [91]. Post-transcriptional modifications are needed for the maturation of collagen. During arterial injury, there is an unbalance between the proportion of the different types of collagen [99].

Laminin and proteoglycans are other ECM proteins with important functions in blood vessels. Laminins are a big family of high cross-like heterodimeric glycoproteins involved in the development of the endothelium [96,100]. Proteoglycans are complex macromolecules involved in the interaction between the endothelium and lipoproteins

[23,96]. Their structure is composed of a protein core linked to glycosaminoglycan (GAG) chains [101]. They participate in many biological functions in arterial wall: ECM assembly, cell proliferation and regulate vascular permeability and lipid metabolism among others. Depending on the GAG type, they are divided into four types: chondroitin sulfate proteoglycans (CSPG), heparan sulfate proteoglycan (HSPG), dermatan sulfate proteoglycan (DSPG) and keratan sulfate proteoglycan (KSPG) [8,23].

ECM is a dynamic structure, constantly subjected to remodeling through the synthesis and degradation of its main components, and changes in its architecture to ensure a correct function of the tissue. It plays an essential role in the development of many pathological states. It is known that arterial stiffening is produced for the alteration of ECM composition and architecture [91]. Matrix metalloproteinases (MMPs) are the main mediators of a healthy ECM remodeling but are also implicated in the formation of atherosclerotic plaque formation because foam cells generate oxidative stress which enhance MMPs expression [97,98,102,103]. This excess of MMPs removes extracellular matrix and permits an easier diapedesis of inflammatory cells. MMPs also promote SMCs proliferation and the generation of monomeric collagen, which enhances atheroma growing [99,103].

5.3 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) is a multi-gene family of zinc and calcium-dependent endopeptidases capable of cleaving ECM components [104]. Most of MMPs are released as zymogens, latent precursors that are proteolytically activated in the extracellular space

[103]. Although its protein structure depends on the subgroup, MMPs are generally composed of five domains: a signaling peptide, a hydrophobic propeptide domain, a catalytic domain, a hinge region and a hemopexin-like domain [47]. Classification can be proposed according their structure and the substrate they can hydrolyze, with at least seven subgroups of MMPs described [105].

According to the substrate specificity, they can be classified as:

a) MMP-1, -8, -13 (Collagenases): cleave type I, II and III collagen.
b) MMP-2, -9 (Gelatinases): cleave heat denatured collagen (gelatin).
c) MMP-3, -10 (Stromelysins)
d) MMP-7, -26 (Matrilysins)
e) Membrane-type MMP (MMP-14, -15, -16, -17, -24, -25): activate MMPs and digest type I, II and III collagen.
f) Other MMP, including MMP-12 (metalloelastase).

MMPs biological activity is strictly controlled, because of its protein degradation potential. There are three different levels of regulation: gene transcription and translation, pro-MMPs (zymogens) activation and MMP inhibitors. Transcription process of MMPs can be inhibited by corticosteroids, retinoids and sex hormones but, on the contrary, inflammatory cytokines, hormones and growth factors may enhance its function. The activation of zymogens is mediated by an enzymatic activator of MMPs, plasmin. Its regulation influences the amount of activated MMPs [97]. Endogenous inhibitors can also modulate the activity of MMPs. Two of the most important inhibitors are α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs)

[104,105]. The MMP/TIMP balance is believed to be a key factor in the regulation of the proteolytic activity of the individual MMPs [104].

MMPs are involved in ECM remodeling, concretely in the proteolysis of its components [103,105]. They mediate for a healthy ECM remodeling during development, tissue morphogenesis and repair due to inflammatory processes (**Figure 7**) [103]

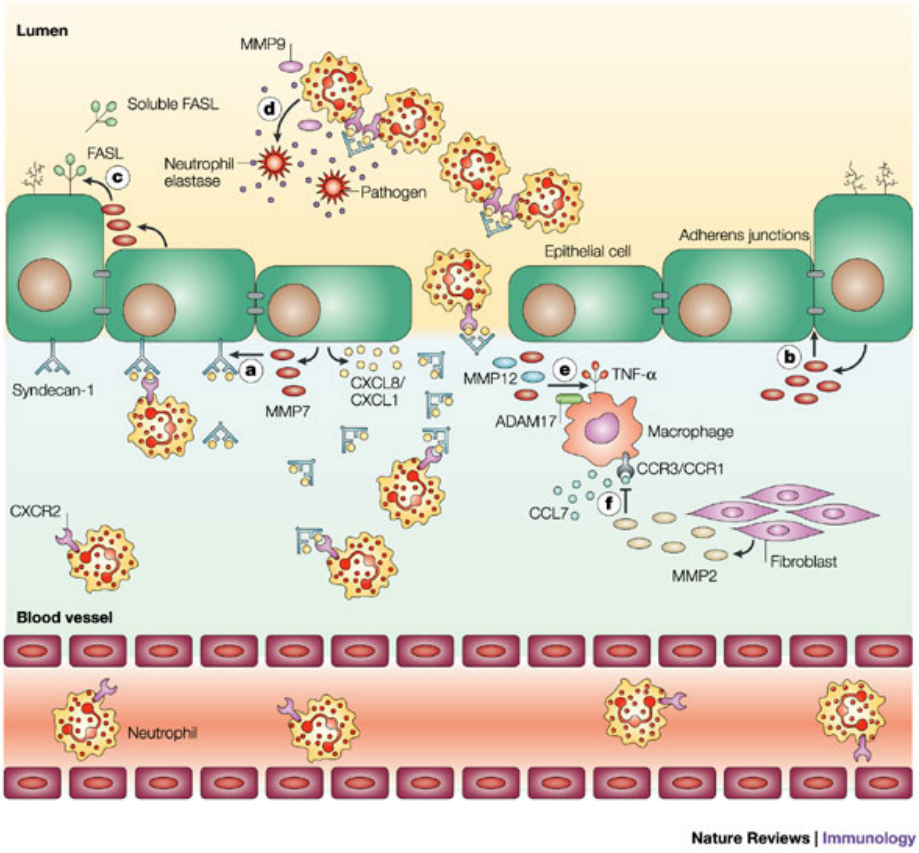

Figure 7. Injury initiates a coordinated response to repair the damaged tissue and to defend against infection. Almost all resident cells participate in these processes and contribute to the regulation of inflammation. This occurs partly through the specific activity of a variety of matrix metalloproteinases (MMPs) that are produced by these cells. Extracted from Parks W.C. *et al*. (2004).

6. ATHEROSCLEROSIS AND INFLAMMATION

In recent years, non-communicable diseases such as obesity and atherosclerosis have related to inflammation [6]. Inflammation is one of the most studied processes today because of its dual and contradictory role in the organism. It is considered to be the response of the immune system to cellular or tissue damage caused by pathogens, physical, chemical or mechanical agents. This response is intended to ensure the survival of the species by its ability to manage infections or repair damage. However, a continuous and chronic inflammatory process produces a totally opposite effect in the organism, with a malfunction of many tissues and organs, and a consequently alteration in energy homeostasis [106,107].

In the pathophysiology of complex diseases such as obesity or atherosclerosis, there are interconnections between metabolic pathways and the immune system. Cytokines and chemokines play an essential role in these connections. Proper coordination of cytokines and other components such as hormones or cells ensures a correct energy balance in an organism subjected to various metabolic conditions [108–110].

Atherosclerosis has been defined as an immune-mediated disease because of immune activation and cytokine signaling in the atherosclerotic plaque [111]. Chronic inflammation has been shown to transform an atherosclerotic plaque into an unstable and vulnerable lesion [109,110]. Thus, inflammatory markers could play a key role as biological markers efficient in predicting future cardiovascular problems.

Specifically, CCL2 is one of the main effectors of chronic inflammation, and is also associated with different diseases such as cancer, type 2 diabetes and cardiovascular diseases [110,112–115].

6.1 Chemokines and cytokines

Chemokines are small proteins (60-100 amino acids) that are part of the chemoattractant cytokine family. They are characterized by the presence of four cysteine residues that form two disulfide bridges. Its secretion is induced by numerous signals, such as proinflammatory cytokines. Its main function is to regulate cell traffic, having an important role in the selective recruitment of monocytes, neutrophils and lymphocytes to the site of inflammation. They create a chemical gradient known that allows cells to move to the sites with the highest concentration of chemokines. These chemokines are responsible for chemotaxis and macrophage accumulation in the fatty streak [116–118].

There are four subfamilies depending on the number and location of the cysteine residue at the N-terminal end of the protein. According to the systematic nomenclature, we have [108,118]:

- CXC chemokines (α-chemokine): They are present on chromosome 4q13 and are formed by two cysteine separated by an amino acid.
- CC chemokines (ß-chemokine): they are related to homeostatic process rather than to the inflammatory process. They have two attached cysteine and can be found on chromosomes 17q11 and 12.
- CX_3C chemokines (δ-chemokine): they act on the endothelium stimulating chemotaxis.
- C Chemokines (γ-chemokine): they act as CX3C chemokines.

6.2 Chemokine (C-C motif) ligand 2 (CCL2)

Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1) is a potent chemotactic factor for monocytes. In humans, CCL2 is made up of 76 amino acids, has a weight of 13kDa and is located on chromosome 17q11.2, encoded by the *CCL2* gene.

CCL2 is produced constitutively or by induction of different factors such as oxidative stress. Different cell types, including endothelial cells, fibroblasts, epithelium and SMC synthesize this chemokine although its main production of CCL2 is due to monocytes/macrophages. It is generally secreted in two predominant forms, as results of different O-glycosylation, although this does not affect its ability to induce monocyte migration. The amino-terminal region is crucial for its biological activity [117,119]

This chemokine regulates the migration and infiltration of monocytes, T lymphocytes and Natural Killer cells. CCL2 mediates the adhesion of monocytes to the vascular endothelium and its extravasation towards the lesion zone. In addition, CCL2 regulates the expression of ß2 integrin on the surface of monocytes and promotes adhesion of monocytes to extracellular matrix proteins [117,119].

CCL2 expression occurs in different tissues during the progression of diseases related to inflammation, such as atherosclerosis. Agents such as IFN-γ, TNF-α and PDGF among others regulate its expression at the transcriptional level. They also have inhibitors that decrease their activity, such as retinoic acid or estrogens [119].

CCL2 mediates the infiltration of monocyte into atherosclerotic lesions by its interaction with CCR2, although it can also bind to other nonspecific receptors, known as atypical chemokine receptors (ACKR) [111,118]. CCL2 is upregulated in atherosclerotic plaques. *ApoE*$^{-/-}$ mice also lacking CCR2 and LDLr$^{-/-}$ mice lacking CCL2 have decreased atherosclerotic lesion size [111,120,121]. The monoclonal antibody MLN1202 has been tested, as it inhibits CCL2 binding to CCR2. It significantly decreased CRP levels after dosing, confirming than chemokine signaling is an interesting therapeutic target for atherosclerosis [122].

6.3 Chemokine (C-C motif) receptor 2

CCR2 is a seven-transmembrane G-protein-coupled receptor located on the lipid membrane of cell surface [111,119]. When CCL2 binds to CCR2, it triggers a set of cellular reactions that produces inositol triphosphate, with subsequent calcium release and protein kinase C activation. This causes NF-kβ activation, which regulates specific gene transcription. CCR2 also activates proteins of Rho family, responsible for leukocyte migration [119].

6.4 Atypical Chemokine Receptors (ACKR)

Atypical chemokine receptors (ACKRs), also known as silent or promiscuous receptors, are a subfamily of chemokine receptors with a strong structural resemblance to "classical" receptors, but unlike them, they are not coupled to G proteins [123–125].

These silent receptors can modify the availability and signaling of chemokines: when the ligand binds to the receptor, signal transmission does not occur and consequently there is no cellular response. They act favoring the transfer of cytokines through the endothelial barriers and act as traps to moderate inflammatory responses [126,127].

The most important silent receptors are DARC (ACKR1) and D6 (ACKR2), which show different specificity and distribution [124].

DARC receptor (ACKR1)

Duffy antigen receptor for chemokines (DARC) is a rhodopsin-line seven-helix transmembrane receptor. It has a broad range of affinities between chemokines, with at least attraction to 20 different CC and CXC-chemokines [124]. Its expression takes place on the surface of vascular endothelial cells. DARC lacks the DRYLAIV motif, which is necessary for G protein coupling [126,127]. However, it binds to chemokines and there is an internalization of both the receptor and the ligand by transcytosis, causing the removal of chemokines [126].

D6 receptor (ACKR2)

Chemokine binding protein 2, also known as D6 or ACKR2, is a member of the rhodopsin-like family, with seven transmembrane domains. It has high affinity for 14 inflammatory chemokines of the CC subfamily [126,128]. Its expression takes place in compartments of lymphatic endothelial cells and placenta, although it is also located in trophoblasts, leukocytes, macrophages and dendritic cells. D6 do has the DRYLAIV motif but has a DKYLEIV altered motif [124,126–128]

7. ATHEROSCLEROSIS AND OXIDATION

Oxidative stress plays a very important role in atherogenesis, since it starts with oxidative modification of LDL in the arterial wall by ROS. Atherosclerosis risk factors increase the production of ROS mainly by macrophages and SMC [129,130].

The production of ROS induces the first step of atherosclerosis: endothelial dysfunction. OxLDL are easily captured by macrophages compared to non-oxidized LDLs. On the other hand, increased ROS production reduces NO production and its absence results in vasoconstriction, platelet aggregation and neutrophil adhesion to the endothelium. ROS also affect the expression of adhesion molecules, since hydrogen peroxide impairs the expression of these molecules. ROS interfere in the proliferation and migration of SMC, apoptosis of endothelial cells, activation of metalloproteinases and alteration of vasomotor activity [129].

There are different cellular mechanisms that protect the body from oxidative stress. Paraoxonase enzymes (PON) are noteworthy. These enzymes are linked to HDL, because they are responsible for their antioxidant properties. They delay the oxidation of LDL and thus inhibit the initiation and progression of atherosclerosis [130–133].

In humans, the PON family gene includes three members with similar structural homology (about 65%) and located adjacent to chromosome 7: *PON1*, *PON2* and *PON3*. They show antioxidant properties, play an important role in maintaining a low oxidative state in the blood

circulation and, therefore, are important for the prevention of atherosclerosis [132,134].

The name of the PON family comes from its ability to hydrolyze the ester O-P bond of organophosphates and pesticides, including paraoxon (a metabolite of paration, diazoxon and other gases such as sarin and soman). Thereby, the body is protected from poisoning.

PON1 is mainly present in serum coupled to HDL. There are small amounts in VLDL and chylomicrons but there is no PON1 in LDL. PON3 is also located in serum associated with HDL, although PON1 prevail in human serum. PON1 and PON3 protect serum lipids from oxidation. On the other side, PON2 is exclusively intracellular [131,134].

7.1 Paraoxonase 1

PON1 is a serum esterase calcium-dependent enzyme, with a molecular weight of 43-45 kDa and composed of 354 amino acids [135]. *PON1* gene is on chromosome 7, between q21.3 and q22.1. Several genetic polymorphisms have been characterized in human PON1, being the most important those at positions 55 (a leucine (L) for methionine (M) at position 55) and 192 (a glutamine (Q) for arginine (R) at position 192) in *PON1* gene [136]. Its secretion is done mainly in the liver, but it can be found also in the brain, lungs, heart, kidney and serum, where circulates coupled to HDL particles [132,136,137]. PON1 is known to downregulate CCL2 (***Annex 1***) [138].

When PON1 is linked to HDL, it inhibits the oxidation of LDL thus protecting them from oxidative stress and decreasing foam cell

formation and atherosclerosis development (**Figure 8**). In fact, it is known that *PON1*$^{-/-}$ mice are highly susceptible to atherosclerosis [132].

Although its physiological substrates have not yet been identified, PON1 hydrolyze a broad range of substrates thus exhibiting different activities: paraoxonase activity (organophosphorus), lactonase activity (lactones) and arylesterase activity (phosphorus arylesters). Still sharing the same active site, different residues are involved in each activity [139–141].

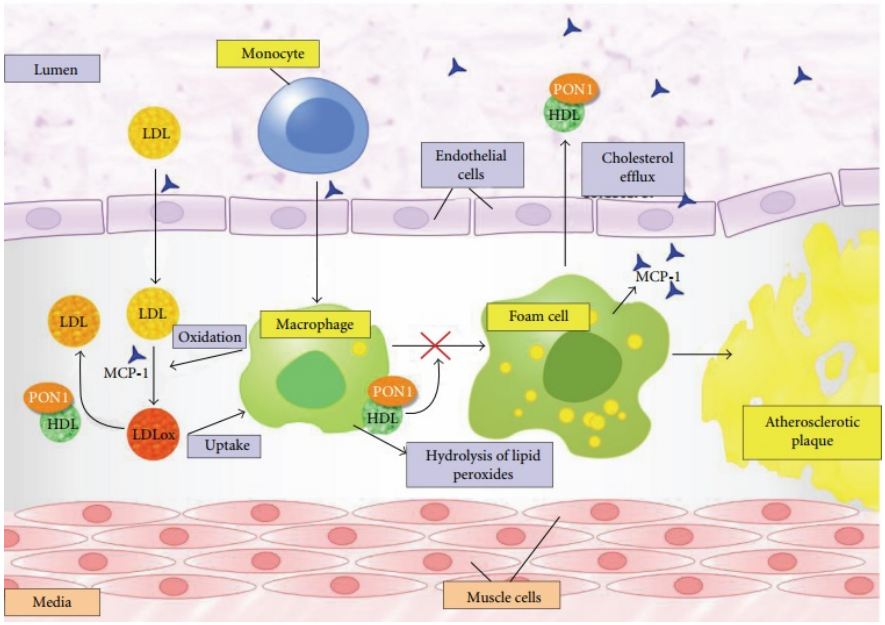

Figure 8: The protective role of PON1 in atherosclerosis. In an oxidant and inflammatory environment, circulating monocytes are activated to become macrophages. oxLDL particles are internalized into macrophages, which are transformed into foam cells. PON1 hydrolyzes oxidized lipids in LDL, inhibiting the development of atherosclerosis. PON1 favors cholesterol efflux from macrophages. Extracted from Camps J. *et al* (2012).

7.2 Paraoxonase 2

PON2 is an intracellular protein not present in circulation and considered the oldest member of the PON family [142]. It has a molecular weight of 44kDa and has only lactonase activity, although PON2 can hydrolyze bacterial products [143]. Its gene expression is detected different human tissue.

PON2 is thought to regulate mechanisms linking endoplasmic reticulum stress, atherosclerosis development and mitochondrial dysfunction [144–147].

7.3 Paraoxonase-3

PON3 is the newest member of PON family and the less characterized. It is a 354-aminoacid protein with a molecular weight of 44kDa. It is bound to HDL in lower concentration and has only lactonase activity [148,149]. Function and cell-type association are similar to PON2 [150–152]. Both human PON1 and PON3 delay LDL oxidation *in vitro*, although PON1 is more effective [132,133]. Several polymorphisms have been recently described, mainly located in the promoter region and still with an unclear physiological role.

UNIVERSITAT ROVIRA I VIRGILI
PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
Anna Hernández Aguilera

HYPOTHESIS AND AIMS

UNIVERSITAT ROVIRA I VIRGILI
PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
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Hypothesis

Inflammation, oxidation, vascular remodeling and mitochondrial dysfunction are implicated in the development of atherosclerosis. Understanding these processes in affected tissues and the assessment of related circulating markers may provide possible therapeutic strategies and suggest potential biomarkers for an early diagnose or disease severity.

Aims

→ To increase knowledge on the pathophysiological processes involved in atherosclerosis.

→ To analyze the expression of inflammatory (CCL2 and associated receptors) and oxidative stress (PON1 and PON3) markers in atherosclerotic and healthy arteries.

→ To assess the specific proteome of diseased arteries.

→ To identify candidate circulating biomarkers of disease severity based on ECM-degradation products.

→ To explore energy metabolism status in plasma of PAD patients.

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PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
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RESULTS

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STUDY 1

Immunohistochemical analysis of paraoxonases and chemokines in the arteries of patients with peripheral artery disease

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ABSTRACT

Oxidative damage to lipids and lipoproteins is implicated in the development of atherosclerotic vascular diseases, including peripheral artery disease (PAD). The paraoxonases (PON) are a group of antioxidant enzymes, termed PON1, PON2, and PON3 that protect lipoproteins and cells from peroxidation and, as such, may be involved in protection against the atherosclerosis process. PON1 inhibits the production of chemokine (C-C motif) ligand 2 (CCL2) in endothelial cells incubated with oxidized lipoproteins. PON1 and CCL2 are ubiquitously distributed in tissues, and this suggests a joint localization and combined systemic effect. The aim of the present study has been to analyze the quantitative immunohistochemical localization of PON1, PON3, CCL2 and CCL2 receptors in a series of patients with severe PAD. Portions of femoral and/or popliteal arteries from 66 patients with PAD were obtained during surgical procedures for infra-inguinal limb revascularization. We used 8 normal arteries from donors as controls. PON1 and PON3, CCL2 and the chemokine-binding protein 2, and Duffy antigen/chemokine receptor, were increased in PAD patients. There were no significant changes in C-C chemokine receptor type 2. Our findings suggest that paraoxonases and chemokines play an important role in the development and progression of atherosclerosis in peripheral artery disease.

1. INTRODUCTION

Lower-extremity peripheral artery disease (PAD) is an important health problem that is associated with severe impairment of different arterial territories. Indeed, PAD is a predictor of substantial coronary and cerebral vascular risk [1,2]. The disease prevalence increases with age and, in people over the age of 55 years, it is estimated to be about 20% [3-6]. Atherosclerosis affects wide portions of numerous arteries in the lower extremities of PAD patients. This is the effect of a sustained and silent progression of the disease in which appropriate and effective prevention measures are applied too late, or not implemented at all [3-8].

Oxidative damage to lipids and lipoproteins is implicated in the development of atherosclerotic vascular diseases, including PAD [9-10]. The paraoxonases (PON) are a group of antioxidant enzymes that protect lipoproteins and cells from peroxidation and are involved in the atherosclerosis process and, consequently, in vascular diseases [11]. The PON family contains three enzymes: PON1, PON2 and PON3, the genes of which are located adjacent to each other on chromosome 7q21-22 [12, 13]. PON1 and PON3 are found in many tissues, as well as in blood, where they are associated with high-density lipoproteins (HDL). Conversely, PON2 is exclusively intracellular [14-17]. Pioneer studies reported that oxidized low-density lipoprotein uptake by macrophages in tissue culture and *in vivo* increases the production of the inflammatory chemokine (C-C motif) ligand 2 (CCL2). The consequence is the stimulation of arterial fatty streak formation, which is the progenitor of atheroma. PON1 has been shown to inhibit these alterations [18-20]. Chemokines, CCL2 in particular, are central to the vascular inflammatory

response in mediating monocyte recruitment into the arterial wall [21-22]. We have previously reported that PON1 and CCL2 are ubiquitously distributed in mouse tissues, suggesting a joint localization and combined systemic effects [23]. Clinical data suggest that circulating CCL2 concentrations or serum PON1 activity are important biomarkers of a variety of diseases involving inflammatory response to an increased oxidative stress [24-29].

Previous studies from our group found that serum PON1 activity and concentration were significantly lower, and CCL2 concentration higher, in PAD patients compared to controls, while the combination of plasma CCL2 and PON1-related variables, discriminated controls from patient almost completely [30]. In addition, we observed an increase in serum PON3 concentration in PAD patients, relative to the healthy population [31]. However, data on the protein expression of these molecules at the lesion level in patients with PAD are scarce. The aim of the present study was to quantify the immunohistochemical localization of PON1, PON3, CCL2 and CCL2 receptors in a wide series of patients with severe PAD.

2. EXPERIMENTAL SECTION

2.1. Study population

Patients with clinically diagnosed PAD were recruited from the outpatient clinics of *Hospital Universitari Joan XXIII*. Diagnosis was with standard clinical assessments including measurement of the ankle-brachial index (ABI), non-invasive imaging, and angiography when indicated. Symptoms of chronic ischemia were detected using the Fontaine classification, the standardized physician-administered questionnaire that seeks to identify the presence of calf discomfort on

exertion, such as walking uphill or walking rapidly [51]. Exclusion criteria from our study were the presence of acute ischemia, signs of infection, renal failure, liver disease, cancer, or autoimmune disease. Portions of femoral and/or popliteal arteries from patients were obtained during surgical procedures for infra-inguinal limb revascularization (*n*=66). All patients were at Stages III and IV of the Fontaine classification. Eight normal arteries obtained from accident victims and stored at the Blood and Tissue Bank of Catalonia *(Banc de Sang i Teixits*, Barcelona, Spain) were used as controls (http://www.bancsang.net/es/donants/donacio_teixits.html). All tissues (patients and controls) were kept at –80ºC until thawed for processing. After thawing, the tissues were rinsed in phosphate buffer to remove residual blood and placed in at least 10 volumes of buffered formalin using a standard protocol for embedding tissue in paraffin wax for subsequent histology slide preparation. Three sections per slide were used for histological and immunohistochemical analyses. A peripheral blood sample was also obtained from each patient (and control individual) at the time of the surgery for biochemical and hematological measurements. The hospital's Ethics Committee and Institutional Review Board approved the procedures of the study protocol, and written informed consent was obtained from the participants prior to entry into the study (10-04-29/4proj3; 11-10-27/10proj1).

2.2 Biochemical analyses

Serum concentrations of glucose, cholesterol, HDL cholesterol, triglycerides, fibrinogen, C-reactive protein, total proteins, and complete blood cell counts were performed by standard methods in the *Hospital Universitari Joan XXIII*. LDL cholesterol concentrations were estimated

using the Friedewald formula. Serum concentrations of PON1 and PON3, and EDTA-plasma concentrations of CCl2 were determined by ELISA as previously reported [30,31]. Serum concentrations of 8-isoprostanes were analyzed by Enzyme Immunoassay (Cayman Chemical Co., Ann Arbor, Michigan, USA). Serum PON1 lactonase activity was analyzed by measuring the hydrolysis of 5-thiobutyl butyrolactone [27].

2.3 Histological and immunohistochemical analyses

Sections, of 4-μm thickness, were stained with hematoxylin-eosin for arterial histology. Masson's trichrome stain (Masson's Trichrome Goldner with light green, Bio Optica, Milano, Italy) was used to assess the structure and extent of fibrosis. Alizarin Red staining (Sigma-Aldrich, Steinheim, Germany) was used to identify the sites of micro-crystalline, or non-crystalline, calcium phosphate salts. The *intima* and *media* thicknesses were measured in all histological sections as an estimate of the extent of atherosclerosis. Antibodies against PON1 and PON3 were raised in rabbits using peptides derived from specific sequences of mature PONs, as previously reported [52-54]. PON1 and PON3 antibodies were used at a dilution of 1/50 and 1⁄300, respectively. A previous study already demonstrated that these antibodies were highly specific for PON1 and PON3 [54]. Commercial primary antibodies were purchased: CCL2 (dilution 1/200), CCR2 (dilution 1/100), and D6 (dilution 1/500) from Abcam plc (Cambridge, UK); antibodies against DARC (dilution 1/200) from Abnova (Taipei, Taiwan); and antibodies against CD68 from Dako (Glostrup, Denmark). The appropriate biotinylated secondary antibodies (antirabbit, antimouse or antigoat; purchased from Vector Laboratories Inc., Burlingame, CA, USA) were used at a dilution of 1:200. Detection was performed with the ABC peroxidase system (Vector

Laboratories) and DAB peroxidase substrate (Dako). The times of the detection reactions were 4 min. for PON1 and PON3, 1 min. for DARC, 1.5 min. for CCR2 and D6, 10 min. for CCL2, and 5 min. for CD68. All immunohistochemical sections were counterstained with Mayer's hematoxylin. Negative control samples were processed identically to the test samples except that the primary antibodies were omitted from the incubation. The positively-stained area was quantified automatically for each antibody using an image analysis system (AnalySIS®, Soft Image System GmbH, Olympus Corp., Munster, Germany), and expressed as percentage of the total area. Initially the colors of the images that have been stained to the molecule of interest were defined. Once these colors were defined, they were automatically detected in all samples. The software analyzed the stained area in relation to the total image area, which is termed phase analysis. The rationale for this method is described in more detail in the Supplementary Methods, and is also available at ftp://ftp.ccmr.cornell.edu/utility/FEI%20temp/AnalySIS%20docs/Getting%20Started.pdf. This is a semi-quantitative analysis that measures areas and not intensities. The coefficient of variation is lower than 10%. This method is commonly accepted and has been employed previously in several immunohistochemical studies by our group and other authors [23,28,40,54-58]. Inter-assay coefficients of variation were as follows: PON1, 9.6%; PON3, 7.3%; CCL2, 4.5%; CCR2, 5.3%; D6, 6.4%; DARC, 7.1% (n = 20 for each variable).

2.4 Statistical analyses

Significance of difference between groups was assessed by the Mann-Whitney *U* test. Results are expressed as medians and IQR

(Interquartile Range). All statistical analyses were performed with the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc, IBM Corp, Chicago, IL, USA).

3. RESULTS

Patients with PAD did not significantly differ from the control group in age and gender distribution. The patient group had a significantly higher percentage of smokers, and lower serum cholesterol and low-density lipoprotein (LDL) cholesterol concentrations. We did not observe any significant difference in any of the other standard biochemical and hematological variables. The circulating levels of CCL2 and 8-isoprostanes (a marker of oxidative stress) were significantly increased in PAD, while serum PON1 concentrations and activities were decreased (Table 1). CRP protein levels were not significantly increased in our patients, a finding probably related to that they were treated with salicylates and antiplatelet agents.

The histological and immunohistochemical analyses of the peripheral arteries revealed that PAD patients had a significantly thicker *tunica intima* relative to the *tunica media* of the artery wall (termed the I/M ratio). There were significant increases in the percentage positive staining for PON1, PON3, CD68 antigen (a marker of macrophages), CCL2, and also in the CCL2 receptors termed chemokine-binding protein 2 (CCBP2, also termed D6), and Duffy antigen/chemokine receptor (DARC). We did not observe any significant change in C-C chemokine receptor type 2 (CCR2) staining relative to controls (Table 2). Similar results were obtained when smokers were excluded from the PAD group (Table S1).

Results

Table 1. Selected descriptive characteristics and laboratory variables in participants.

Parameter	Control (n=8)	PAD (n=66)	*p*-value
Clinical characteristics			
Age, years	66 (30 – 76)	70 (62 – 77)	0.223
Male, n (%)	5 (62.5)	55 (85.9)	0.094
Smokers, n (%)	1 (14.3)	16 (31.4)	**0.048**
Complete blood count			
Red blood cells, $x10^{12}$/L	4.32 (3.18 – 4.47)	3.67 (3.14 – 4.24)	0.449
Hemoglobin, g/dL	12.46 (9.99 – 13.28)	10.85 (9.45 – 12.93)	0.468
Leukocytes, $x10^{9}$/L	9.22 (8.58 – 10.17)	9.89 (7.44 – 12.20)	0.668
Platelets, $x10^{9}$/L	227.5 (163.7 – 246.2)	312.5 (199.0 – 419.0)	0.080
Biochemical variables in serum or plasma			
Glucose, mmol/L	5.77 (5.11 – 6.77)	6.38 (5.11 – 8.83)	0.406
Total cholesterol, mmol/L	4.77 (3.87 – 6.39)	3.39 (2.90 – 4.47)	**0.030**
HDL – cholesterol, mmol/L	1.24 (0.98 – 1.40)	0.93 (0.83 – 1.20)	0.074
LDL – cholesterol, mmol/L	3.54 (3.11 – 4.42)	1.95 (1.68 – 2.69)	**0.001**
Triglycerides, mmol/L	1.47 (1.13 – 2.15)	1.31 (1.00 – 1.87)	0.449
Fibrinogen, g/L	5.51 (4.48 – 7.54)	6.96 (5.34 – 8.11)	0.237
C-reactive protein, mg/L	6.1 (0.6 – 7.2)	8.1 (2.7 – 16.0)	0.147
Total proteins, g/L	65 (55 – 68)	60 (55 – 69)	0.743
CCL2, ng/L	373.4 (255.2 – 431.8)	622.8 (472.7 – 898.4)	**< 0.001**
PON1, mg/L	75.4 (56.7 – 143.8)	25.2 (18.4 – 35.8)	**< 0.001**
PON3, mg/L	1.95 (1.51 – 2.50)	1.73 (1.43 – 2.27)	0.490
8-isoprostanes, ng/L	14.2 (2.0 – 37.2)	100.8 (37.6 – 314.7)	**< 0.001**
PON1 lactonase activity, U/L	5.69 (5-02 – 6.29)	3.04 (2.11 – 3.73)	**< 0.001**

The bold numbers highlight the statistically significant differences

Table 2: Differences in selected variables between control individuals and PAD patients

Parameter	Control (n=8)	PAD (n=66)	*p*-value
IMT (mm)	1.00 (0.70 – 1.30)	1.29 (1.00 – 1.74)	0.150
I/M ratio	0.16 (0.13 – 0.65)	2.10 (1.33 – 3.22)	**<0.001**
% PON1 staining	1.70 (1.54 – 3.72)	11.19 (7.25 – 20.81)	**<0.001**
% PON3 staining	0.55 (0.22 – 0.73)	3.25 (2.01 – 4.37)	**<0.001**
% CCL2 staining	2.26 (0.36 – 3.65)	30.75 (9.63 – 44.41)	**<0.001**
% CCR2 staining	18.29 (7.02 – 27.56)	22.99 (13.21 – 42.71)	0.263
% CD68 staining	1.10 (0.65 – 2.88)	4.57 (2.40 – 9.24)	**0.007**
% D6 staining	0.83 (0.22 – 12.9)	41.21 (24.55 – 58.39)	**<0.001**
% DARC staining	3.29 (2.01 – 5.06)	37.26 (18.06 – 51.85)	**<0.001**

IMT: *Intima-Media* thickness. Results are shown as medians (IQR). Staining for chemokine (C–C motif) ligand 2 (CCL2), C–C chemokine receptor type 2 (CCR2), cluster of differentiation 68 (CD68), Duffy antigen/chemokine receptor (DARC), chemokine-binding protein 2 (D6), paraoxonase-1 (PON1) and paraoxonase-3 (PON3) were measured as the area of positive staining and expressed as percentage of the total area examined using the image analysis system (see text for details). The bold numbers highlight the statistically significant differences.

Affected arteries had severe alterations compared to the normal artery histology (Figure 1). The intima was thicker and had extensive deposits of cholesterol and inflammatory cells. Calcium deposits were clearly identified in the media. Masson's trichrome stain was used to evaluate the arteries' architecture which, in affected arteries, highlighted an infiltration of smooth muscle cells from the media into the intima, or perhaps a loss of muscle cells from the media and increase in connective tissue, and greater obstruction of the arterial lumen.

Results

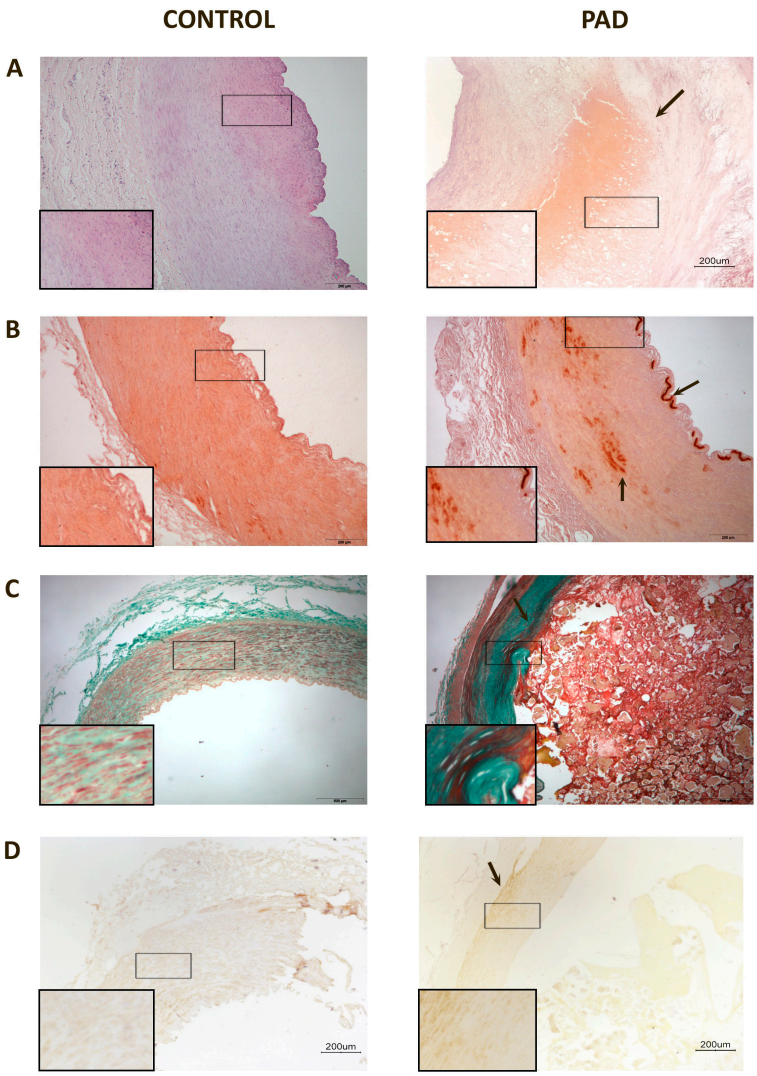

Figure 1. Representative histological images of peripheral arteries; (**A)** Arteries stained with Hematoxylin-Eosin. The *intima* in affected arteries was thicker and replete with cholesterol deposits and inflammatory cells (arrow). Magnification 20x; (**B)** Alizarin Red staining to detect the presence of calcium. There were calcium deposits in affected arteries located, mainly, in the *media* and, in some cases, calcium was observed in the internal elastic lamina (arrows). Magnification 20x; (**C)** Masson's Trichrome stain showing, in affected arteries, an infiltration of smooth muscle cells from the *media* to the *intima* (arrow). The lumen shows partial obstruction. Magnification 40x. (**D)** Actin staining to detect the presence of smooth muscle cells. The arrow shows the area of infiltration of these cells from the *media* to the *intima*. Magnification 20x. The inserts show higher magnification (100x) images of the indicated areas.

In normal arteries, PON1 expression was low and located in the *intima* and in the *adventitia*. PON3 expression was imperceptible. Conversely, in the arteries of PAD patients, PON1 and PON3 expression were higher. PON1 presented two types of localization: 1) when the *intima* was only moderately enlarged, PON1 was located in the *adventitia* vessels and the *media*; 2) when the *intima* was disorganized and with cholesterol deposits, PON1 was found surrounding the cholesterol crystals at the site of the lesion. In affected arteries, PON3 was found in the *adventitia* or in the injury sites of the *intima* (Figure 2). Areas of CD68 staining had a similar spatial distribution than those of paraoxonases and CCL2 (Supplementary Figures 1 and 2).

In normal arteries, CCL2 was mildly expressed in the *adventitia*, while CCR2 was found mostly in the *media*, with weaker expressions in the *adventitia* and *intima*. CD68, D6 and DARC expressions were mild. Conversely, the arteries of PAD patients had higher expressions of CCL2, CD68, D6 and DARC. CCL2 was found mostly in the *adventitia* while CCR2 was found mostly in the *media*, with weaker expressions in the *adventitia* and *intima*, as found in normal arteries. CD68 expression was observed mostly in the thickest areas of the *intima*. DARC was located mostly in the *media*, although it could also be found in the *adventitia* and/or *intima* of the vessels. D6 was found mostly in the *adventitia* (Figure 3).

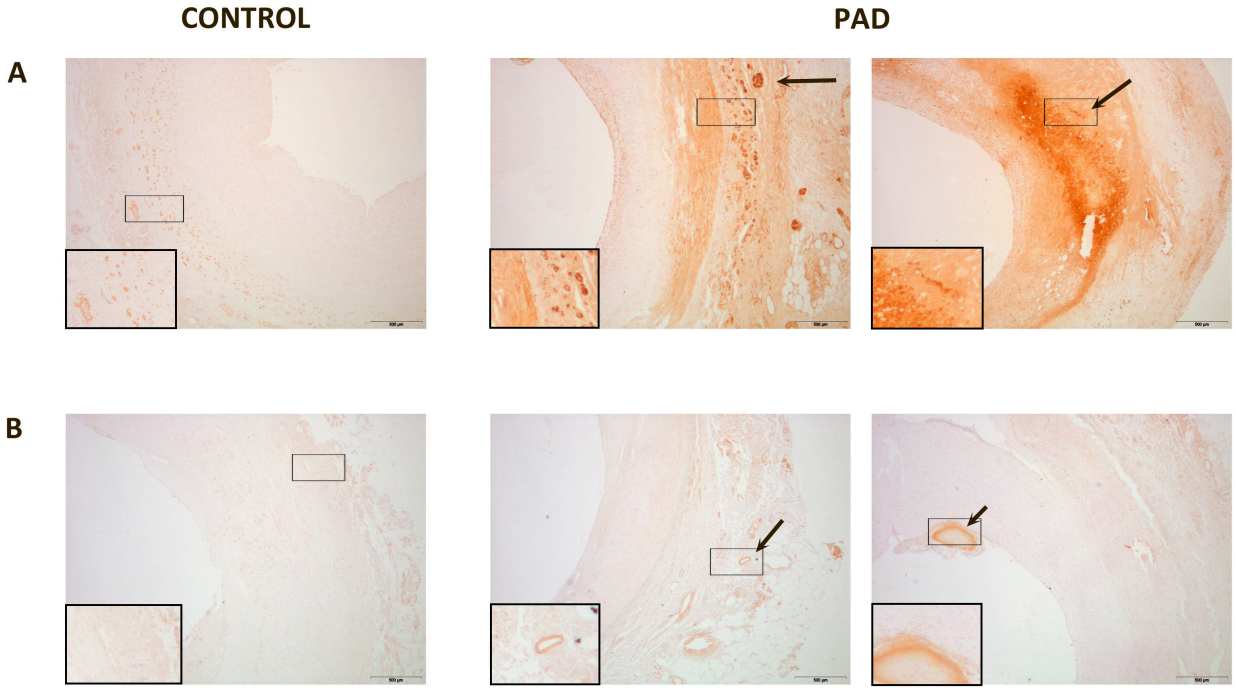

Figure 2. Representative immunohistochemical images for paraoxonase-1 (PON1) and paraoxonase-3 (PON3) staining of peripheral arteries; (**A)** PON1 expression in normal artery was almost undetectable, and located in the *media* and *adventitia*. PON1 had two types of localization in affected arteries: when the *intima* was not very thick, PON1 was located in the *adventitia* and *media* of the vessels (arrow). When the *intima* was disorganized and with cholesterol deposits, PON1 was expressed in the lesion site (arrow); (**B)** PON3 expression was undetectable in normal tissue whereas, in affected arteries, PON3 was located in the *adventitia* or in the injury sites of the *intima* (arrow). Magnification x20. The inserts show higher magnification (100x) images of the indicated areas.

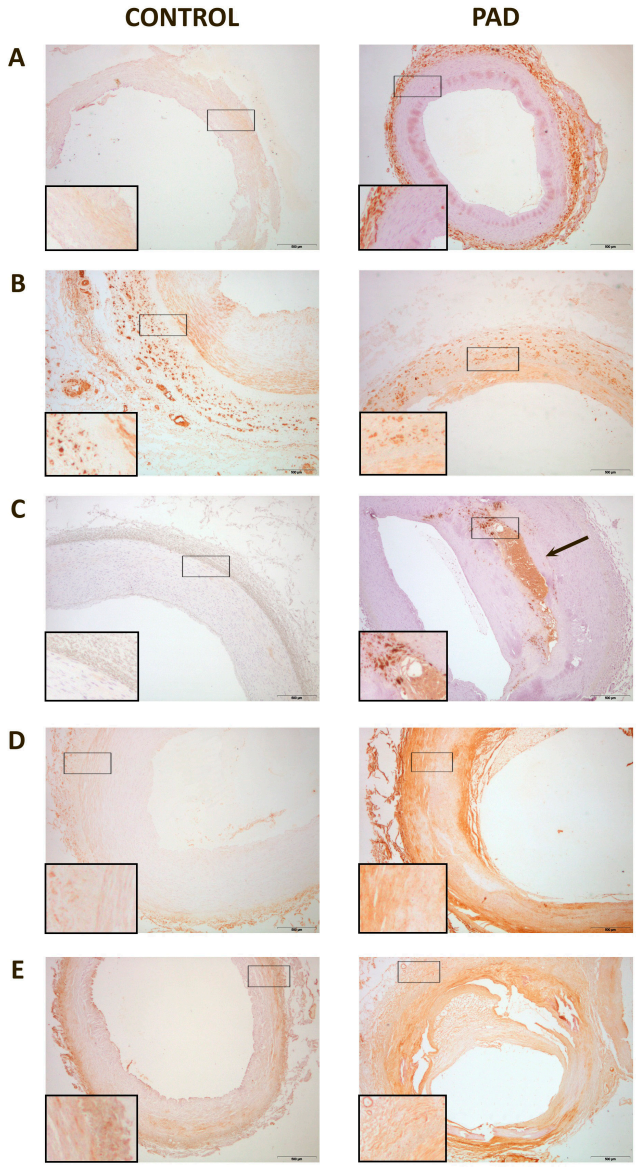

Figure 3. Representative immunohistochemical images for inflammatory markers in peripheral arteries: (A) Chemokine (C–C motif) ligand 2 (CCL2) was expressed in the adventitia in normal and affected arteries (arrow); (B) C–C chemokine receptor type 2 (CCR2) was expressed, mainly, in the media in normal and affected arteries. However, it can also be found in the intima and in adventitia of the vessels (arrow); (C) Cluster of differentiation 68 (CD68) was mildly expressed in control arteries while, in affected arteries, the expression was higher and located, mainly, in the intima (arrow); (D) Chemokine-binding protein 2 (D6) expression was found, mainly, in the adventitia; (E) Duffy antigen/chemokine receptor (DARC) was found, mainly,

in the media, although it was observed as well in the adventitia and/or intima of some vessels. Magnification 20×. The inserts show higher magnification (100×) images of the indicated areas.

4. DISCUSSION

The present study shows (by immunostaining) that paraoxonases, CCL2 and several CCL2 receptors are increased in peripheral arteries with indications of atherosclerosis. This could be a response to increased cellular oxidative stress as well as the migration of monocytes. In PAD patients, we observed an increased CD68 staining which is a specific marker of macrophages. Macrophage mitochondrial oxidative stress plays a major role in atherosclerosis via mechanisms involving the NF-κB-CCL2 pathway [32]. Paraoxonases prevent oxidative stress by reducing the amount of oxidized LDL in the circulation as well as the vessel wall. This, in turn, reduces monocyte infiltration into the vessel wall and, as such, is anti-inflammatory [33, 34]. The protein expression of this enzyme has been observed in many tissues in humans [35] and mice [23]. PON1 reduces macrophage oxidation of LDL as well as macrophage oxidative stress, and increases cholesterol efflux from macrophages to high-density lipoprotein (HDL), thus reducing foam cell formation and, as a consequence, the development or progression of atherosclerosis. Therefore, the increase in PON1 staining found in this study could indicate that a protective response to increased oxidative stress was occurring in the macrophages of the diseased arteries. For example, it is of considerable note that PON1 expression was found surrounding cholesterol deposits in severely diseased arteries, and which strongly supports the hypothesis of a protective role for this enzyme i.e. that PON1 infiltrates the arterial tissue to combat the deposition of the

atherosclerosis-promoting cholesterol. The physiological role of PON3 is still unclear. Results from the present study support previous findings from our group showing increased serum PON3 concentrations in patients with PAD [31]. Studies on cellular expression of this enzyme and the elucidation of its athero-protective role are scarce and inconclusive. PON3 has lactonase but not paraoxonase activity [36, 37]. Previous studies reported that PON3 attenuates the oxidation of LDL *in vitro* [38] and that the overexpression of human PON3 decreased atherosclerosis and adiposity in mice [39]. Although the increase in PON3 protein expression in the arteries of PAD patients is quantitatively small, it needs to be taken into account that PON3 is about 100 times more potent per mg of protein than PON1, in protecting LDL against lipid peroxidation [36]. Hence, the increase in the enzyme's expression in these patients could be of clinical relevance.

In the peripheral circulation, decreased PON1 activities are associated with increased concentration of CCL2 [30], and *in vitro* studies found that PON1 inhibits the production of CCL2 induced by oxidative stress in endothelial cells [20]. However, this inverse relationship is not confirmed at tissue level. Indeed, both molecules are ubiquitously expressed in most tissues and are located in close proximity to one another, suggesting some manner of coordinated function [23,40]. Results of the present study, and previous others, show that the expression of both proteins is increased in the arteries of patients with atherosclerosis [40]. This observation would suggest that the variations in PON1 and CCL2 concentrations in plasma do not necessarily correlate with their roles at cellular level. Perhaps PON1

protein expression is increased in diseased arteries to counteract oxidative stress and CCL2-induced inflammation. However, this hypothesis has to be confirmed by further studies.

CCL2 is likely to have considerable impact on PAD since the biological function of this chemokine is to induce monocyte migration and, as well, because the arteries with moderate atherosclerosis appear to accumulate CCL2 in response to a variety of pro-inflammatory stimuli [24,30,41-44]. Atherosclerosis is an inflammatory disease, and the consensus is that CCL2 is involved in its pathogenesis [45]. In the present study, we found increased CCL2 expression in the arteries of PAD patients, together with an increased expression of two of the CCL2 receptors i.e. D6 and DARC. D6 and DARC belong to the poorly-understood chemokine receptors collectively known as atypical or silent. These are G-protein coupled receptors that do not activate conventional signaling events. Conversely, they may internalize, degrade or transport ligands (i.e. they have the potential to create clinically relevant chemokine patterns in tissues) [46]. Their levels of expression have not been explored previously in diseased arteries of PAD patients. The availability of CCL2 may be complicated by potential effects induced by differential expression of the specific receptor CCR2 and the presence of these atypical chemokine receptors. We observed that the expression of these receptors was increased in diseased arteries, and that their histological distributions are not uniform. A pathogenic role is likely, and data suggest that atypical chemokine receptors modify chemokine availability in PAD. Although these receptors have no involvement in cell migration, their modulatory effect on inflammatory response is likely.

Previous studies from our group also reported increased PON1 and PON3 expressions in aortas from patients undergoing coronary or aortic artery bypass grafting [40]. This is relevant, because it suggests that, despite the atherosclerosis burden is higher in PAD, changes inside the tissue are similar at a molecular level. The mechanisms underlying the increased PON1 and PON3 immunohistochemical staining in the arteries of PAD patients cannot be ascertained from the present investigation, but these patients had oxidative stress, as indicated by the elevated serum 8-isoprostanes concentration. Oxidative stress stimulates PPARγ and NFκB-related pathways [47], and these molecules have been reported to stimulate the expression of paraoxonases [48,49]. However, this increase is in an apparent contradiction with the decrease in the serum levels of the enzymes, and a possible explanation could be an increase in PPARδ expression and decreased PON1 proteolysis. This is the case in a rat model of liver fibrosis that our group published a few years ago [50]. Rats with CCl_4-induced liver fibrosis had oxidative stress and increased PPARδ gene expression. These alterations were associated to an inhibition of the HDL synthesis and, consequently, a decreased PON1 secretion to the extracellular medium. In addition, the hepatic levels of the protease cathepsin B were decreased, leading to an inhibition of protein degradation. Thus, hepatic PON1 levels were elevated as a consequence of the combination of a decreased HDL secretion, and to an inhibition of lysosomal protein degradation. To ascertain if the same phenomena occur in the arteries of PAD patients requires further studies, but the strong decrease in HDL-cholesterol concentrations observed in our patients is in agreement with this hypothesis.

A caveat of the present study is that we could not analyze PON2 in the arteries of PAD patients. This enzyme plays an important role in the intracellular protection against oxidative stress [14], and new investigations focused in PON2 and PAD should be further pursued.

5. CONCLUSION

In conclusion, PON1 and PON3, CCL2 together with the D6 and DARC receptors are increased in the arteries of patients with PAD. The findings suggest that these molecules play an important role in the development and progression of atherosclerosis in peripheral artery disease.

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UNIVERSITAT ROVIRA I VIRGILI
PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
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STUDY 2

Identification of candidate biomarkers of disease activity in peripheral artery disease by targeting the extracellular matrix

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ABSTRACT

Background and objective: The prevalence of peripheral artery disease (PAD) is high (20-25%) in the population older than 65 years, and patients frequently do not present for treatment until the disease is advanced. Circulating markers of disease activity might provide patients with a key opportunity to be treated. The established role of matrix metalloproteinases (MMPs) in vascular remodeling and their association with atherosclerosis progression is the basis on which to explore the feasibility of detecting blood-specific peptides generated during the degradation of the extracellular matrix (ECM).

Methods: A combined histological and non-targeted proteomic approach using liquid chromatography and tandem mass spectrometry was used to assess the protein profile in arterial specimens from patients undergoing elective surgery. We then selected a panel of neo-epitopes, likely indicating ECM turnover, and measured them by enzyme-linked immunosorbent assays in serum samples from a cohort of 195 PAD patients who were in a stable state and exhibited different disease activity.

Results: Histological and proteomic analyses confirmed the structural disorganization of affected arteries. Several proteins (14 out of 81) were identified as differentially expressed in diseased arteries; most of them were related to ECM-components and the difference in expression was likely due to an imbalance in vascular remodeling. Multivariate analyses suggest that severe lesions in PAD patients may have a specific proteome. Targeting selected neo-epitope fragments in the serum revealed that some but not all fragments had potential value in the clinical management of PAD. Notably, the detection of

neo-epitopes from fragments of MMP-mediated degradation of versican and collagen type IV segregated patients with mild/moderate PAD (intermittent claudication, Fontaine I-II) from those with severe PAD (critical limb ischemia, Fontaine III-IV).

Conclusion: We propose novel non-invasive candidate biomarkers that may be clinically useful across the PAD spectrum.

1. INTRODUCTION

Systemic atherosclerosis is a progressive, age-related disease underlying the most common causes of death. The prevalence of peripheral artery disease (PAD) is high, with over 200 million afflicted patients in industrialized countries, and is further increasing due to the convergent epidemics of diabetes and obesity [1,2]. Lesions in the arteries of the lower extremities represent a clinical spectrum encompassing asymptomatic and underdiagnosed illnesses as well as symptomatic disorders in which the initial manifestations are either intermittent claudication (IC) or critical limb ischemia (CLI) [3,4]. The challenge is to establish non-invasive biomarkers for predicting patients likely to progress to CLI and for improving success in offering preventive medical management.

Changes in lumen caliber are major determinants of the course of PAD symptoms, and wall remodeling in peripheral arteries of a certain size and length seems to be a crucial process to understand the reaction of old and damaged tissue to atherosclerotic injuries [5]. Mechanistic knowledge is incomplete, but the major role of the extracellular matrix (ECM) in providing a mechanical scaffold and support to cell migration is undisputed [6]. The dynamic regulation of the ECM is governed by the balance between synthesis and degradation of ECM components, which is context-specific and involves the correct functioning of cytokines, enzymes such as matrix metalloproteinases (MMPs) and growth factors [7-9]. We now know that atherosclerosis-associated remodeling is the complex response to inflammatory cells, lipid deposition and mechanic or shear-dependent stimuli, which are responsible for changes in ECM composition and for

disrupted cytoskeletal architecture [10-14]. Here we provide an insight into the proteome composition and the relative expression of ECM components in severely affected peripheral arteries and we test the hypothesis that a directed choice of neo-epitopes may provide clinically useful non-invasive biomarkers in PAD patients.

2. MATERIALS AND METHODS

Participants and study design

The local Ethics Committee and Institutional Review Board approved the procedures involved in this study (Epinols/12-03-09/3proj6). Written informed consent was obtained from all participants before inclusion. Patients (n=195) were consecutively enrolled men selected from among those attending our Department of Vascular Surgery and with an established diagnosis of PAD according to Fontaine classification [15]. Patients with infected lesions, evidence of neoplastic disease, chronic kidney disease, liver disease or inflammatory disease (or receiving anti-inflammatory drugs) were not included. Ankle-brachial index (ABI) was measured per standard technique in both lower limbs and non-invasive imaging techniques or arteriographies were performed according to the standard of care. Serum was collected at the time of inclusion and stored at -80ºC until analyses. Patients were followed up every 3 months for 1 year and there was no mortality during this period. No patient was included postoperatively or lost to follow-up but some patients needed infrainguinal limb revascularization (n=18). These patients were invited to participate in a case-control study (Inflamet/15-04-30/4proj6) that required donating portions of diseased artery for proteomic analysis that were compared with healthy artery samples obtained from road

accident victims of a similar age range (66-70 years). New informed consent was obtained from either the participants or a next of kin.

Histological examination

To examine tissue morphological features, serial sections of tissue were obtained from samples fixed in 10% neutral-buffered formalin and embedded in paraffin. Hematoxylin and eosin staining (Sigma-Aldrich, Steinheim, Germany) was used to identify different cellular structures. Masson's trichrome staining (Bio Optica, Milano, Italy) was used to assess collagen fibers, smooth muscle cells, nucleus and cytoplasm and Sirius red staining (Direct Red 80, Sigma-Aldrich, Steinheim, Germany) was used to identify collagen fibers. Images were obtained at x200 magnification and the Intima/Media ratio (IMR) was obtained by dividing the thickness of the intima by the thickness of the media measured using an optical microscope (Nikon, Eclipse E600, Madrid, Spain) equipped with image analysis.

Proteomics

We have previously used these methods to analyze the protein secretion profile of carotid atherosclerotic plaques [16]; ancillary methods and specific details may be found in supplementary material and methods, S1. Briefly, sample arteries were cut into pieces and homogenized in the presence of type 1 collagenase (Sigma-Aldrich, Steinheim, Germany). Following different rounds of centrifugation and chemical treatment, precipitated proteins were vacuum-dried and dissolved in 0.5 M triethylammonium bicarbonate, pH= 7.2, to be sequentially denatured, reduced and alkylated. For digestion, samples were incubated with sequencing-grade trypsin overnight at 37 °C. We

used a mass spectrometry approach for quantification by performing isobaric tag for relative and absolute quantitation (iTRAQ) labeling using iTRAQ 8-plex reagent kits (SCIEX, Madrid, Spain), as previously described [17]. Labeled peptides were then purified using a SCX column (Strata® SCX 55um, 70Å, Phenomenex), desalted and concentrated through a C18 Sep-Pak column (Waters, Bedford, MA, USA) and analyzed by using a C-18 reversed phase nano-column coupled to a trap nano-column for real time ionization and peptide fragmentation on a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA). To identify proteins, information was obtained from tandem mass spectra with the aid of Proteome Discoverer, version 1.4.0.288, from Thermo Fisher Scientific. All MS and MS/MS samples were analyzed using Mascot (Thermo Fisher Scientific; version 2.4.1.0). Protein quantification was performed by comparing the peak intensity of the reporter ions in the MS/MS spectra to that of the selected peptides to assess the relative abundance of the peptides. Normalized concentrations of selected proteins were used to assess the increased or decreased expression of proteins in PAD arteries.

Enzyme-linked immunosorbent assays (ELISA)

Methods involved in monoclonal antibody development and technical evaluation of the assays were essentially similar to those recently described [18]. Specific details may be found in supplementary material and methods, S2. The list of selected neo-epitopes from the MMP-degraded proteins is shown in Table S1, and also in the supplementary information and the references therein. This selection was made after confirming that these neo-epitopes were

consistent with proteomic data and pathway analysis according to the ConsensusPathDB-human platform [19].

Statistical analysis

The Kolmogorov-Smirnov test was used to assess normal distribution of the variables. We used the Mann-Whitney U test to compare non-parametric variables, Student's t-test for parametric variables and contingency tables and the chi-square test for categorical variables. For multiple comparisons, the Kruskal-Wallis test or Analyses of Variance (one-way ANOVA) was used. The results were expressed as median and interquartile range or percentage of the total participants. For proteomic analyses, principal component analysis (PCA) and hierarchical clustering analysis were performed using the Mass Profiler Professional software v.12.1 (Agilent Technologies). Only proteins that appeared in more than 70% of the samples were considered, and the PANTHER system (www.pantherdb.org) was used for functional classification. We used the Benjamini–Hochberg method to avoid false positives in differences due to multiple testing. Analyses with receiving operating characteristics (ROC) curves and binary logistic regression were performed using the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc., IBM Corp, Chicago, IL, USA). MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) was used to generate scores/loading plots, heatmaps and random forest analysis.

3. RESULTS

The clinical characteristics and laboratory measurements (Table I) suggest that the cohort of patients used for this study is representative of the clinical spectrum of PAD patients seeking attention in our

facilities. The high prevalence of cardiovascular risk factors and associated treatment, including the fact that most were smokers, did not significantly affect severity of disease, with the possible exception of a lower prevalence of diabetes in Fontaine I patients. However, age was a significant factor in establishing disease severity and a major consideration in further analyses.

In a case-control study combining histology and proteomics, we first evaluated the differences in the integrity of arterial tissue and signs of vascular remodeling, in severely lesioned and normal arteries (Figure I). Atherosclerosis was evident in all samples from the PAD patients. The *tunica* intima was disorganized and thicker, and the presence of lipid vacuoles and cholesterol crystals and other histologic features (Figure 1A) were consistent with the higher ($p<0.0001$) intima/media ratio observed in PAD patients (2.10 [1.33-3.22]) with respect to that of similarly aged donors of healthy arteries (0.16 [0.13-0.65]). Furthermore, smooth muscle cells normally located in the media were also present in the intima of atherosclerotic arteries (Figure 1B) and the distribution of collagen fibers was disrupted (Figure 1C).

Table I. Clinical characteristics, complete blood count and biochemical characteristics of PAD patients segregated by Fontaine classification

	Fontaine I N=11	**Fontaine II N= 41**	**Fontaine III N=34**	**Fontaine IV N=109**	**p-value**
Clinical characteristics					
Age (years)	55 (50 – 69)	70 (59.25-75)	63 (55-69.25)	71 (64-77)	<0.001
BMI (kg/m^2)	28.9 (23.05-31.16)	27.3 (23-29.4)	25.5 (22.25-27.9)	24 (22-27.8)	ns
Diabetes (%)	10	69.4	45.5	79.8	<0.001
Hypertension, (%)	50	63.2	57.6	75	ns
Dyslipidaemia, (%)	55.6	41.7	24.2	36.7	ns
Complete blood count					
Red blood cells, x10^{12}/L	5.11 (4.41-5.4)	4.48 (3.95-4.79)	4.29 (3.74-4.53)	4.00 (3.34-4.59)	<0.001
Hemoglobin, g/dL	14.6 (13.23-16.35)	13.1 (11.5-15.2)	13.57 (12.02-14.07)	11.5 (10.5-13.5)	0.02
Leukocytes, x10^9/L	7.44 (6.85-10.23)	7.51 (6.3-9.42)	7.61 (6.39-9.56)	8.35 (6.4-10.1)	ns
Platelets, x10$^{9/L}$	217.25 (186-243.5)	219 (183-268)	252 (200.5-333.65)	270 (209.5-343)	0.011
Biochemical variables					
Total-cholesterol, mmol/L	4.06 (2.84-5.65)	4.04 (3.72-4.74)	3.95 (3.37-4.47)	3.77 (3.1-4.51)	ns
HDL-cholesterol, mmol/L	0.8 (0.72-1.14)	1.1 (0.86-1.29)	1.1 (0.87-1.26)	0.92 (0.74-1.14)	ns
LDL-cholesterol, mmol/L	2.04 (1.4-3.32)	2.41 (1.94-3.4)	2.2 (1.73-2.81)	2.18 (1.72-2.83)	ns
Triglycerides, mmol/L	1.56 (1.18-4.53)	1.51 (1.14-2.56)	2.35 (1.87-3.47)	1.97 (1.37-2.86)	ns
Glucose, mmol/L	6.69 (4.1-7.64)	5.93 (4.96-8.82)	5.59 (4.62-7.49)	5.77 (4.59-7.6)	ns
ALT, U/L	19 (12.14-35)	21 (16-26)	22 (16-40)	21 (13-32)	ns
Gamma-GT, U/L	27.5 (16.94-39.8)	28 (18-47)	24 (17.25-43)	31.5 (17-48.8)	ns
AST, U/L	21 (12-27)	19 (16-22)	20 (14.75-31)	19 (15-30)	ns
Fibrinogen, g/L	4.07 (3.6-5.48)	4.84 (4.12-6.43)	5.39 (4.27-6.11)	5.82 (4.44-7.78)	ns

BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ALT: Alanine Aminotransferase; AST: Aspartate aminotransferase. Non-parametric variables are shown as median and IQR (25-75%). Qualitative variables are expressed as (%) of total participants. Multiple comparisons between groups using Kruskal-Wallis test.

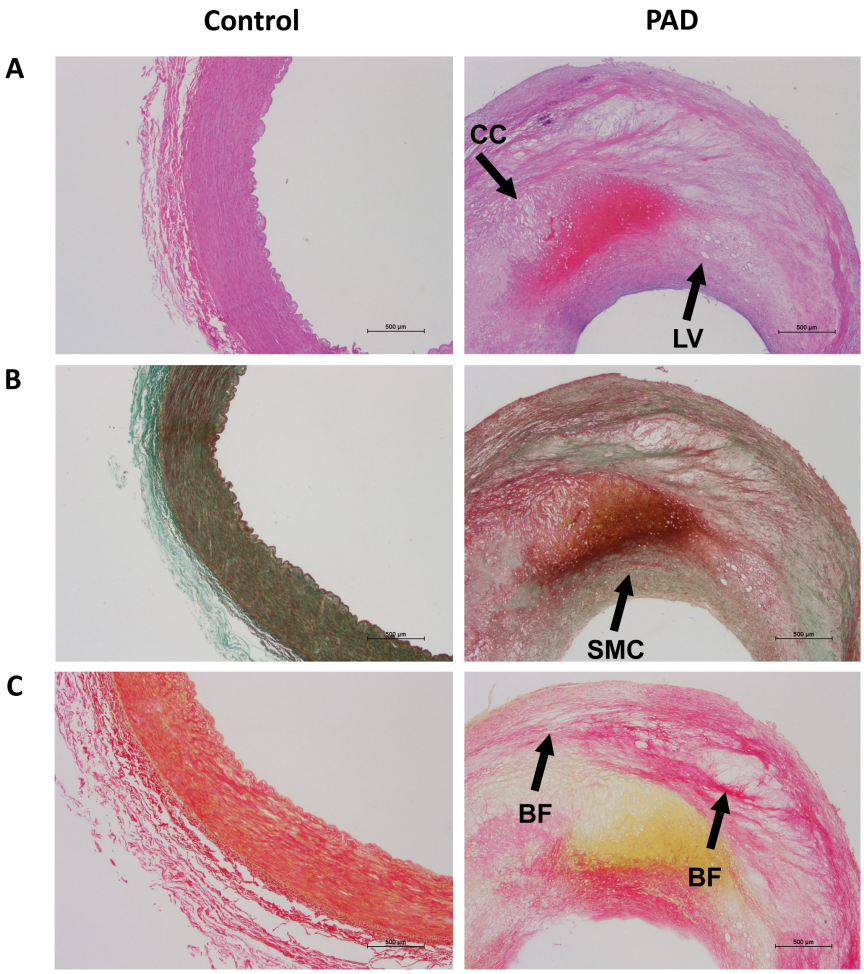


Figure 1: Representative micrographies of peripheral arteries from control group and PAD patients (x20). Hematoxylin & Eosin (A), Masson's Trichrome staining (B), Sirius Red staining (C) were performed in arteries from both groups. CC: Cholesterol crystals; LV: Lipid vacuoles; SMC: Smooth muscle cells; BF: Broken fibers of collagen.

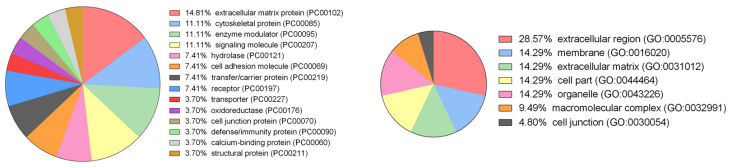
Results

Our untargeted proteomics approach identified and quantified 81 proteins present in both control and diseased arteries and in more than 70% of the samples (Table S2). However, once filtered, corrected and normalized, we identified a unique subset of proteins (n=14) with statistically significant differences between diseased and healthy arteries and therefore with the potential to represent specific biomarkers (Figure 2A). Putative functions of these proteins in atherosclerosis, according to the literature, are listed in Table S2. Notably, most of these proteins were ECM or cytoskeletal components (Figure 2B) suggesting that vascular remodeling provides a specific target that might be used to explore progression of atherosclerosis. Hierarchical clustering analyses and principal component analyses strongly suggest that severe disease in PAD patients may have a specific proteome, as illustrated in Figure 2C-E.

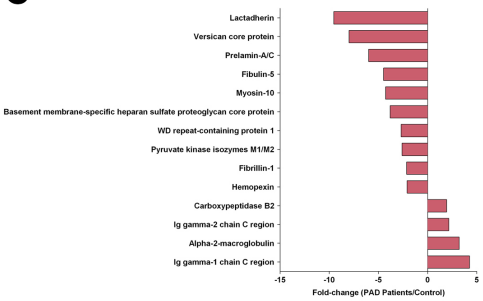
A

Swiss-Prot	Gene name	Proteins	Control (n=3)	PAD Patients (n=4)	*p-value*
P01023	A2M	Alpha-2-macroglobulin	1.328	3.014	**0.017**
P98160	HSPG2	Basement membrane-specific heparan sulphate proteoglycan core protein	-0.301	-2.232	**0.045**
Q96IY4	CPB2	Carboxypeptidase B2	1.291	2.248	**<0.001**
P35555	FBN1	Fibrillin-1	0.417	-0.670	**<0.001**
Q9UBX5	FBLN5	Fibulin-5	0.453	-1.709	**<0.001**
P02790	HPX	Hemopexin	1.289	0.230	**<0.001**
P01857	IGHG1	Ig gamma-1 chain C region	0.677	2.768	**0.017**
P01859	IGHG2	Ig gamma-2 chain C region	0.995	2.110	**0.017**
Q08431	MFGE8	Lactadherin	0.503	-2.751	**<0.001**
P35580	MYH10	Myosin-10	-0.464	-2.560	**0.017**
P02545	LMNA	Prelamin-A/C	-0.094	-2.676	**<0.001**
P14618	PKM	Pyruvate kinase isozymes M1/M2	0.381	-0.991	**<0.001**
P13611	VCAN	Versican core protein	0.730	-2.266	**0.017**
O75083	WDR1	WD repeat-containing protein 1	-1.519	-2.950	**<0.001**

B



C



D

E

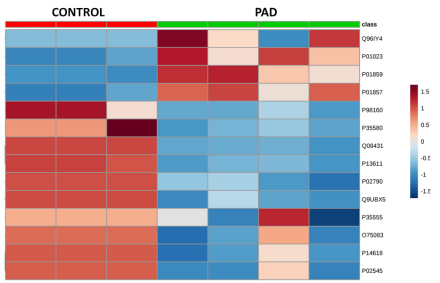


Figure 2: (A) Proteins showing statistically significant differences between control group and PAD patients. (B) Protein class (Left) and cellular component (Right) percentage of selected proteins obtained by PANTHER system. (C) Representation of the fold-changes obtained using normalized concentrations of selected proteins in both groups. D) Principal Component Analysis and E) Heatmap diagram of proteomics results. Q96IY4: Carboxypeptidase B2; P01023: Alpha-2-macroglobulin; P01859: Ig gamma-2 chain C region; P01857: Ig gamma-1 chain C region; P98160: Basement membrane-specific heparan sulfate proteoglycan core protein; P35580: Myosin-10; Q08431: Lactadherin; P13611: Versican core protein; P02790: Hemopexin; Q9UBX5: Fibulin-5; P35555: Fibrillin-1; O75083: WD repeat-containing protein 1; P14618: Pyruvate kinase isozymes M1/M2; P02545: Prelamin-A/C

Results

Normalized concentrations identified some proteins that were either underexpressed or overexpressed in atherosclerotic arteries indicating the delicate balance between production and degradation or removal of proteins in ECM turnover. Nevertheless, we assumed an imbalance favoring degradation of ECM proteins to select candidate neo-epitopes (Table S1) to be measured in the serum of PAD patients with validated ELISA tests.

Median and IQR values (Table II) indicated that measurements of specific fragments of MMP-8- and MMP-12-mediated degradation of versican (VCANM), MMP-9-mediated degradation of alpha 5 chain of laminin (Lam-a5) and MMP-mediated degradation of type IV collagen (C4M) had discriminative value in the clinical presentation of PAD patients. This was further confirmed by using random forest analyses, but Lam-a5 levels failed to discriminate patients with IC from those with CLI and were not considered in further analyses. Serum VCANM concentration decreased progressively, was correlated with clinical severity, and the analysis of ROC curves displayed a high sensitivity and specificity to distinguish between Types I and IV patients (Figure 3A, B). A similar discriminative value was obtained for C4M concentrations, but this variable increased according to disease severity (Figure 3C, D). The combination of both potential biomarkers provided specificity higher than 90% to discriminate between patients with mild IC and those with CLI (Figure 3E).

Table II. Differences in selected neo-epitopes between PAD patients segregated by Fontaine classification.

	Fontaine I N = 11	**Fontaine II N = 41**	**Fontaine III N = 34**	**Fontaine IV N= 109**	**p-value**
VCANM	1800 (1640 – 1900)	1610 (1375 – 1830)	1530 (1055 – 1810)	1250 (1080 – 1560)	<0.001
C4M	16530 (13720 -21710)	21480 (16860 – 30120)	24790 (18095 – 31940)	31730 (22415 – 45165)	<0.001
Lam-a5	5610 (4630 – 8490)	6660 (4855 – 9810)	6130 (3928 – 8278)	8710 (6755 – 11960)	<0.001
CRPM	7620 (5690 – 11520)	9380 (6780 – 14100)	8645 (6615 – 11590)	9970 (7775 – 12430)	ns
α-SMA	3870 (2830 – 4900)	3620 (2770 – 5570)	3355 (2283 – 4513)	3600 (2430 – 5100)	ns
MIM	7600 (3600 – 20370)	7430 (3770 – 12090)	6770 (2795 – 13635)	8070 (3885 – 13290)	ns

Results are expressed in pg/mL and as median (IQR range) for non-parametric variables. VCANM: Specific fragment of MMP-8 and -12-mediated degradation of versican; C4M: MMP-mediated type IV (alpha 1) collagen degradation; Lam-a5: Specific fragment of MMP-9 mediated degradation of alpha 5 chain of laminin; CRPM: Specific fragment of MMP-1, -3, -8, -9, CatS/K, ADAMTS1-mediated degradation of C-reactive protein; α-SMA: Alpha-smooth muscle actin, acetylated N-terminal; MIM: Specific fragment of MMP-9 and -12-mediated degradation of mimecan. Multiple comparisons between groups using Kruskal-Wallis test.

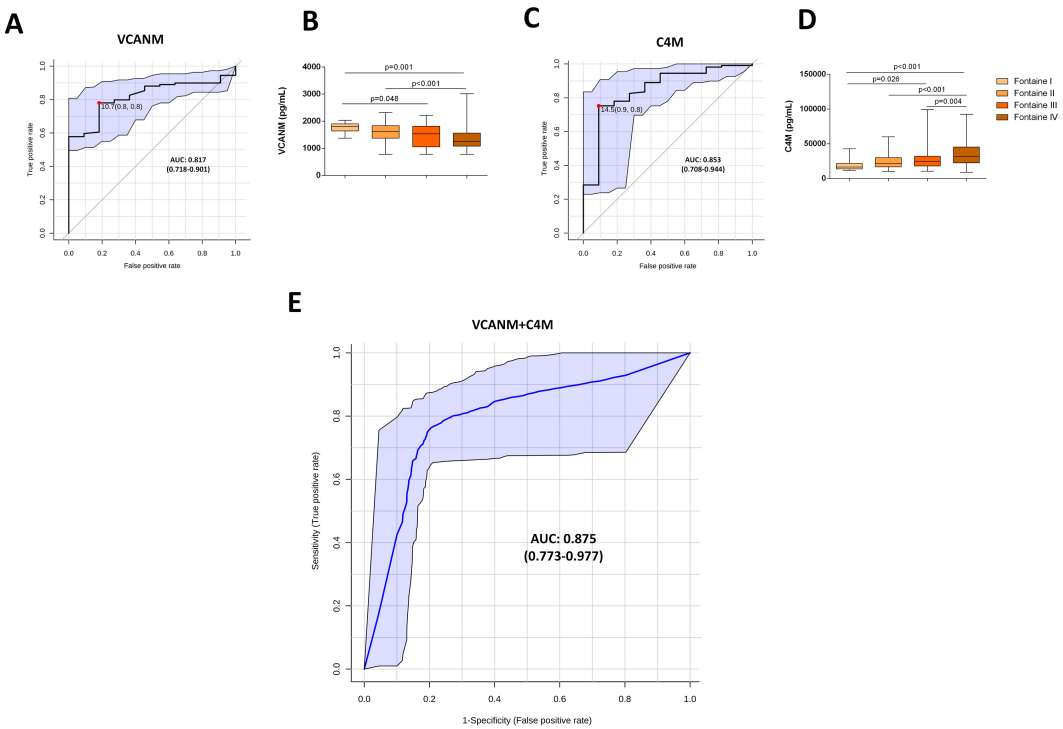


Figure 3: Candidate biomarkers for disease activity. (A) ROC curve for VCANM measurements between Fontaine I and Fontaine IV patients. (B) Graphical representation of VCANM concentrations among Fontaine grades. (C) ROC curve for C4M measurements between Fontaine I and Fontaine IV patients. (D) Graphical representation of C4M concentrations among Fontaine grades. (E) ROC curve for the combination of VCANM and C4M obtained by binary logistic regression between Fontaine I and Fontaine IV patients

4. DISCUSSION

Atherosclerosis is a systemic disease. Despite identical pathogenesis, the affected vascular territories define not only clinical relevance but also different responses to injuries. Detecting asymptomatic stages and to predict or monitor disease progression is currently an unmet need for vascular surgeons [20] that might be fulfilled by non-invasive blood biomarkers. In limb arteries, the lumen loss (i.e., progression to ischemia) is not due to neointima formation, as is the case in coronary arteries [21]. As confirmed by our histological assessment, an important contribution of the reparative response to promote vascular remodeling, which includes inflammatory mediators and ECM degradation, is likely [21-23]. Our proteomics data indicate that severe atherosclerotic lesions in peripheral arteries have a specific proteome in which proteins related to tissue modeling and remodeling are underrepresented and those associated with inflammation seem overregulated. Among those overexpressed proteins, alpha-2-macroglobulin and carboxypeptidase B2 largely contribute to the differences observed between diseased and healthy arteries. Alpha-2-macroglobulin has been recently associated with plaque vulnerability in carotid arteries using a similar iTRAQ-based analysis [24] and carboxypeptidase B2 may be a potential indicator of a high risk of premature peripheral artery disease [25]. Conversely, other proteins were significantly decreased in diseased arteries. For example, low levels of lactadherin may indicate advanced atherosclerosis and poor adhesion of smooth muscle cells to elastin fibers [26]. We also observed low levels of versican, a major chondroitin sulfate proteoglycan, which is highly influenced by the increased MMP activity in diseased arteries [27,28]. The relative amounts of laminin [29] and mimecan [30] with crucial roles in cardiovascular function and migration of smooth muscle

cells, respectively, were also decreased in diseased arteries. Taken together, these results indicate the coexistence of multiple mechanisms involved in the maintenance of artery function in response to atherosclerotic injury.

The results highlight the central role of connective tissue turnover in the structural and signaling properties of arterial cells in PAD [14]. Our methods included identifying specific cleavage products generated by MMPs or age-related processes of proteins involved in matrix turnover, the production of antibodies that recognize these neo-epitopes but not native proteins and to develop immunoassays searching for biomarkers of disease severity [31-34]. Based on histology and proteomic data, neo-epitopes generate from alpha-smooth muscle actin and laminin alpha-5 showed some potential value acting as surrogates for individual clinical endopoints. Further research may confirm this assumption but we focused our analyses in clinically separating patients with mild to moderate PAD (IC, Fontaine I-II) from severe PAD (CLI, Fontaine III-IV), and we observed that serum measurements of versican (KTFGKMKPRY; VCANM) and type IV collagen (CGG-GTPSVDHGFL; C4M) degradation products returned the best specificity and sensitivity levels. Interestingly, VCANM levels decreased and C4M levels increased according to disease severity, probably confirming that both age and the specific context regulate the activity of different MMPs as previously described [35,36]. This is important because type IV collagens are a major component of all basement membranes, and versican plays a central role in inflammation [37,38]. The combination of both indicators might integrate cellular pathways and processes reflecting PAD progression.

Our exploratory research has identified candidate biomarkers of PAD clinical severity, but their evaluation requires more detailed investigation. Our methods measured an objective, quantifiable characteristic successfully that apparently correlates with clinical endpoints. Therefore, these biomarkers may provide clinically relevant information. The next level of evaluation needs other designs to ascertain predictive power in other populations, especially in those with asymptomatic PAD, and to validate efficacy. Laboratory-measured biomarkers used as surrogate endpoints may have the potential to speed drug development in PAD, a prevalent condition in which the use of primary clinical endpoints, such as CLI, in clinical trials can be impractical or even unethical. Exploring and reevaluating the relationship between measurable biological processes and clinical outcomes is also crucial for deepening our knowledge on arterial pathophysiology.

5. CONCLUSION

Severe lesions in PAD are characterized by a specific proteome that significantly differs from that found in healthy arteries of persons of similar age. This proteome informs that both inflammation and ECM turnover (i.e., vascular remodeling) are quantitatively the most important processes in diseased arteries. Subsequent studies indicate that remodeling of arterial tissue releases protein fragments into the blood, where they may be detected. We propose versican and type IV collagen degradation products as laboratory-measured biomarkers of disease activity in peripheral artery disease.

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STUDY 3

Plasma metabolome in PAD patients unveils limitations derived from comorbidities in unhealthy aging

Translational Research, 2017

UNIVERSITAT ROVIRA I VIRGILI
PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
Anna Hernández Aguilera

ABSTRACT

Systemic atherosclerosis affecting lower extremities, also called peripheral artery disease (PAD) is a common disease affecting 20-25% of old population. An early diagnostic is still not possible because symptoms become evident in advanced stages. Inflammation, impaired metabolism and mitochondrial dysfunction may predispose to the disease, which normally is associated to other pathologies (type-2 diabetes, dyslipidemia or hypertension). By using a targeted metabolomics approach, we measured metabolite concentration in atherosclerotic arteries and plasma of PAD patients segregated by Fontaine classification and in plasma of healthy volunteers. Our results show that many of measured metabolites, specially branched chain amino acids, were associated not with the disease but with other comorbidities, age or body mass index. After removal, six potential candidates were considered. Among them, (iso)citrate and glutamate were the metabolites with the best discriminant capacity between control group and PAD patients. Moreover, both were also useful for an early detection of the disease, discriminating between control group and Fontaine I-II patients. The obtained metabolic fingerprint in PAD patients can be used as a source of novel biomarkers of diagnosis and progression.

1. INTRODUCTION

Peripheral artery disease (PAD) of the lower extremities is a serious global health problem with an increasing prevalence among atherosclerotic diseases and affecting 20-25% of population over 60 years[1]. There is a wide spectrum between signs of PAD classified in four stages attending the symptoms using the Fontaine scheme: from a non-symptomatic stage (Fontaine I), intermittent claudication (Fontaine II) to rest pain (Fontaine III) and tissue damage and necrosis (Fontaine IV).

Hypertension, hypercholesterolemia, diabetes and smoking are the principal risk factors to develop PAD. Undesirable lifestyle can leads to a pro-inflammatory situation inducing complications at the crossroads of metabolic stress and immunity[2,3]. Moreover, imbalance in energy metabolism, by which nutrients are transformed into ATP, can predispose to obesity, type-2 diabetes (T2D) and atherosclerosis[3-5]. Alterations in metabolic pathways like tricarboxylic acid (TCA) cycle may induce the production of reactive oxygen species (ROS) and oxygen deficiency (ischemia)[6,7]. Impaired bioenergetics in affected lower extremities can be probably due to abnormal mitochondria in ischemic skeletal muscles[8,9].

One of the main challenge that specialists and researches face is to improve the diagnose of PAD even in the asymptomatic stages[10,11]. Many plasma, serum and total blood biomarkers have been proposed and associated to a high cardiovascular risk, although none of them has been established[12]. The emerging metabolomics approaches are an essential tool to improve the interpretations of atherosclerotic pathologies[13–15]. These techniques are mainly focused on the quantification of metabolites to better understand the disease and propose new

therapeutic strategies[14,16,17]. In this sense, metabolites involved in TCA cycle have been proposed as cardiovascular biomarkers[8,9,18,19].

In the present work we assume that the combination of inflammation, disrupted metabolism and mitochondrial dysfunction may predispose to atherosclerosis[20–22]. The obtaining metabolic profile in atherosclerotic patients is a useful tool to discover new biomarkers and therapeutic targets and, for the first time, we propose potential metabolic circulating markers of initial stages of peripheral artery disease by using a targeted metabolomics approach.

2. MATERIALS AND METHODS

2.1 Participants and study design

This observational, cross-sectional study implicated 201 men with clinically diagnosed peripheral artery disease attending Vascular Surgery Service at *Hospital Universitari Joan XXIII* between 2010 and 2015. Patients were classified according Fontaine classification[23] from grade I to IV and obtained plasma and serum samples were stored at -80 ºC until use. Artery samples were obtained during surgical procedures for infrainguinal revascularization and stored at -80ºC.

Inclusion criteria were men, older than 18 and with a confirmed diagnose of peripheral artery disease. Diagnostic criteria involved ankle-brachial index (ABI), non-invasive imaging techniques (computerized tomography scan or magnetic resonance imaging) and arteriography when indicated. The exclusion criteria were presence of acute ischemia, signs of infection, renal failure, liver disease, cancer or autoimmune disease. Clinical data and laboratory variables were obtained from patients' clinical records. Local Ethics Committee of the Hospital

approved the study (epinols/12-03-09/3proj6, inflamet/15-04-30/4proj6). Written informed consent was obtained from the participants prior to entry the study.

For comparisons, we used bio-banked samples (n=48) from healthy, age-matched, men, whose details have been previously described[24].

2.2. Metabolomics analysis

To detect and quantify metabolites of energy metabolism, we followed the method developed by Riera-Borrull *et al*[7]. Briefly, 25 mg of tissue were homogenized in 1 mL of methanol:water (8:2, v/v) using a *Precellys 24 system* (Bertin Technologies, Montigny-le-Bretonneux, France) working at 5000rpm for 10 seconds three times. The homogenate was then centrifuged at 14000 rpm 10 min at 4ºC and supernatant was collected. Lipids were removed following Folch protocol, by using 9 mL of chloroform[25]. Samples were again centrifuged at 14000 rpm for 10min at 4ºC; the aqueous phase was collected and dried under N_2 flow. Metabolites from plasma (100 mL) were extracted using 400 mL of methanol/water (8:2, v/v) and proteins were precipitated for two hours at -20 ºC. After centrifugation at 14000 rpm for 10 minutes at 4 ºC, the supernatant was collected and dried under N_2 flow. Metabolites were then derivatized with methoxyamine in pyridine (40 mg/mL) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide and injected into a gas chromatograph coupled to a quadrupole time-of-flight mass spectrometer by an electron impact source. Metabolites were detected and quantified attending the standard calibration curves.

2.3. Statistical analysis

Statistical analyses were performed with SPSS 22.0 (IBM Corp, Chicago, IL, USA). MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) was used to generate scores/loading plots and random forest analyses.

3. RESULTS

3.1. Participants' characteristics

Clinical characteristics and biochemical variables of control group and PAD patients are shown in Table 1. We chose an age-matched control group with healthy volunteers without any cardiovascular disease and PAD patients, who had a higher BMI than control group ($p=0.021$). The incidence of atherosclerosis-related impairments (T2D, hypertension and dyslipidemia) was only present in PAD patients ($p<0.001$ in all cases). Consequently, cholesterol, triglycerides and glucose concentrations were altered in those patients.

3.2. Significant alterations in energy metabolism

We measured the concentration of energy metabolism intermediaries in plasma of both, control group and PAD patients. As shown in Figure 1A, most of analyzed metabolites were significantly increased in PAD patients, excluding fumarate, lactate and succinate, which were decreased in patients.

When displayed in a graphical pathway (Figure 1B), we observed that glutaminolysis was disrupted, as glutamate and glutamine were increased in PAD patients. Moreover, reactions involving amino acid catabolism seemed to be slowed down, as serine, valine, isoleucine and leucine concentrations were higher compared to control group.

Tricarboxylic acid cycle was disturbed in 2 ways: some metabolites were higher in PAD patients ((iso)citrate, aconitate, α-ketoglutarate, succinyl-CoA and malate and others were diminished in PAD patients (fumarate and succinate).

Table 1. Clinical characteristics, complete blood count and biochemical characteristics of Control group and PAD patients.

	Control (n = 48)	**PAD (n = 201)**	**P-value**
BMI (kg/m^2)	24 (22.5 – 25.3)	25 (22.5 – 28)	0.021
Diabetes (%)	-	64.1	<0.001
Hypertension (%)	-	69.2	<0.001
Dyslipidemia	-	37.9	<0.001
Red blood cells, x10^{12}/L	4.9 (4.4 – 5.2)	4.16 (3.57 – 4.66)	<0.001
Hemoglobin, mmol/L	8.94 (8.32 – 9.43)	13.30 (11.50 – 14.90)	0.001
Leukocytes, x10^9/L	6.8 (5.4 – 8.2)	8.17 (6.50 – 10.22)	0.003
Platelets, x10^9/L	233 (205 – 273)	253 (200 – 329)	ns
Total-cholesterol, mmol/L	4.85 (4.40 – 5.85)	3.90 (3.31 – 4.94)	<0.001
HDL-cholesterol, mmol/L	1.34 (1.14 – 1.61)	0.96 (0.78 – 1.19)	<0.001
LDL-cholesterol, mmol/L	2.82 (2.40 – 3.86)	2.26 (1.77 – 2.79)	<0.001
Triglycerides, mmol/L	0.90 (0.70 – 1.38)	1.99 (1.40 – 3.08)	<0.001
Glucose, mmol/L	4.70 (4.37 – 4.92)	5.61 (4.60 – 6.88)	<0.001
ALT, U/L	20 (13.5 – 24.9)	21.5 (15 – 34.8)	ns
AST, U/L	20 (17.7 – 24)	21 (16 – 32)	ns

BMI: Body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Non-parametric variables are shown as median (IQR). Qualitative variables are expressed as (%) of total participants. Kruskal-Wallis test has been used for multiple comparisons between groups.

A

Metabolite	Control (µM)	PAD (µM)	PAD/control ratio	
3-hydroxybutirate	0.12 (0.11 - 0.13)	0.22 (0.14-0.40)	1.83	*
Aconitate	0.47 (0.39-0.63)	4.42 (2.45-6.26)	9.40	*
α-ketoglutarate	2.91 (2.15-3.97)	4.67 (2.85-7.07)	1.60	*
Alanine	199.95 (163.83-252.32)	208.71 (147.51-274.36)	1.04	ns
Aspartate	132.46 (114.27-147.78)	181.61 (142.32-229.68)	1.37	*
(Iso)citrate	267.20 (200.34-329.55)	687.82 (561.31-921.93)	2.57	*
Fumarate	0.31 (0.23-0.41)	0.25 (0.19-0.39)	-1.24	*
Glucose	4718.45 (4407.71-5073.74)	4878.41 (4312.48-5658.30)	1.03	ns
Glutamate	168.57 (106.17-258.28)	1417.56 (711.82-2669-54)	8.41	*
Glutamine	1705.99 (990.44-2736.23)	5073.46 (2616.13-9189.28)	2.97	*
Isoleucine	48.28 (40.66-55.59)	61.55 (52.22-73.56)	1.27	*
Lactate	399.68 (348.17-429.08)	358.59 (305.02-440.07)	-1.11	ns
Leucine	71.25 (62.95-80.77)	88.41 (71.57-107.83)	1.24	*
Malate	1.43 (1.15-1.77)	2.51 (1.90-3.88)	1.76	*
Pyruvate	9.12 (6.47-13.49)	13.49 (3.79-23.04)	1.48	ns
Serine	103.49 (93.41-112.86)	135.86 (105.32-167.17)	1.31	*
Succinate	10.52 (10.25-11.09)	9.40 (8.44-15.07)	-1.12	*
Succinyl-CoA	7.27 (5.27-9.76)	10.45 (7.58-15.54)	1.44	*
Valine	92.81 (82.87-104.55)	105.44 (83.91-135.10)	1.14	*

B

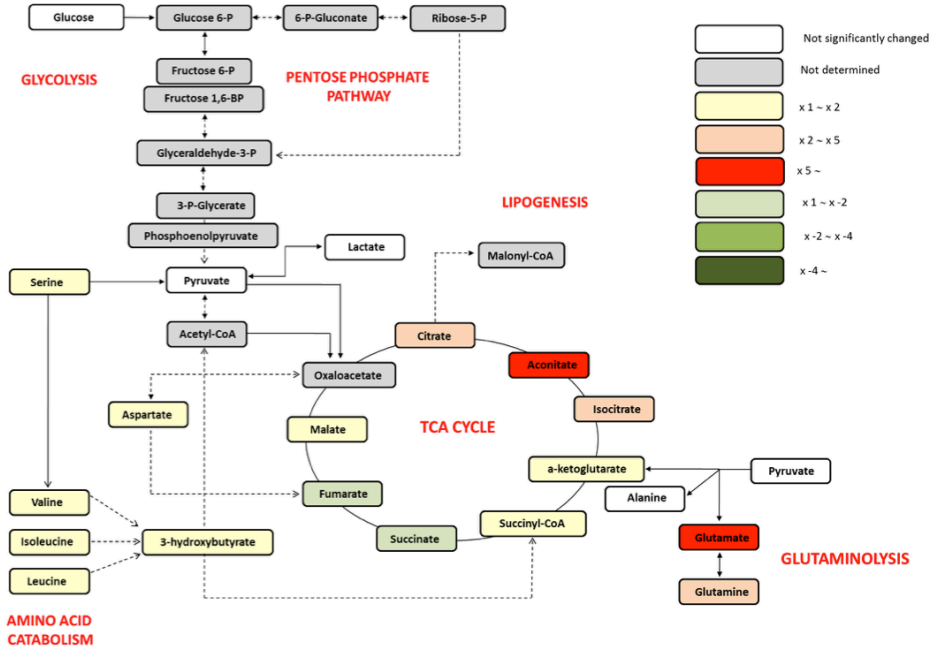

Figure 1. Energy metabolism in PAD patients. (A) Concentrations of measured metabolites in PAD patients and control group, expressed as median (IQR), fold-change ratio between PAD patients and control group, and p-value. * $p<0.05$. ns: no significant. (B) Graphical display of fold-change ratios in energy metabolism.

3.3. Metabolites are linked to comorbidities, age and BMI

Changes in those metabolites could be used for disease diagnose, but associated comorbidities may act as confounding factors in almost 80% of PAD patients as they presented some metabolic disturbances (hyperlipidemia, hypertension or T2D). For this reason, we segregated PAD patients according these disturbances to investigate whether metabolites were different among metabolically healthy or unhealthy patients.

Univariant analyses confirmed that many metabolites were associated to T2D, hypertension or dyslipidemia (Table 2) while multivariate analyses (principal component analyses) revealed that the combination of those metabolites were not able to separate groups regarding hypertension (Figure 2A) and dyslipidemia (Figure 2B). We discovered that glucose and isoleucine were associated to T2D in PAD patients and glucose had the highest discriminant capacity (Figure 2C). Hyperlipidemic and normolipidemic patients showed differences in alanine, aspartate, glucose, isoleucine, lactate, leucine, succinyl-CoA and valine concentrations, and among them, isoleucine had the higher discriminant capacity (Figure 2D). Fumarate, glucose, isoleucine, lactate, malate, serine and pyruvate were associated to hypertension in PAD patient and serine was the metabolite with the best discriminant capacity (Figure 2E). All of these metabolites were discarded for being a possible PAD biomarker.

Table 2. Metabolite concentration in PAD patients segregated according co-morbidities.

Metabolite	PAD patients								
	Normoglycemic	Type-2 diabetic	p-value	Normotensive	Hypertensive	p-value	Normolipidemic	Hyperlipidemic	p-value
3-hydroxybutyrate	0.27 (0.13 – 0-39)	0.27 (0.14 – 0.40)	ns	0.21 (0.14 – 0.38)	0.35 (0.14 – 0.42)	ns	0.31 (0.16 – 0.40)	0.19 (0.13 – 0.419	ns
Aconitate	3.75 (2.38 – 6.50)	4.60 (2.67 – 6.28)	ns	4.59 (2.54 – 6.65)	4.13 (2.60 – 6.60)	ns	4.64 (2.91 – 6.61)	4.22 (2.36 – 6.16)	ns
α-ketoglutarate	4.24 (2.56 – 7.22)	4.13 (2.76 – 6.50)	ns	4.71 (2.81 – 6.67)	3.70 (2.51 – 7.07)	ns	3.99 (2.44 – 6.55)	5.05 (3.05 – 6.90)	ns
Alanine	210.60 (147.51 – 266.30)	189.61 (139.04 – 271.47)	ns	213.52 (152.77 – 273.76)	184.35 (137.34 – 255.16)	ns	**180.54 (125.18 – 247.53)**	**233.62 (171.42 – 302.86)**	**0.001**
Aspartate	172.57 (133.37 – 215.81)	179.76 (143.01 – 225.10)	ns	181.61 (141.46 – 239.56)	173.17 (137.30 – 200.45)	ns	**168.65 (134.12 – 205.47)**	**191.72 (148.8 – 253.22)**	**0.016**
Isocitrate	721.78 (584.42 – 867.38)	665.64 (538.00 – 880.59)	ns	721.80 (566.64 – 934.03)	654.24 (476.56 – 815.96)	ns	678.64 (545.44 – 864.55)	712.36 (566.17 – 959.17)	ns
Fumarate	0.26(0.19 – 0.41)	0.24 (0.18 – 0.37)	ns	**0.27 (0.19 – 0.40)**	**0.22 (0.17 – 0.30)**	**0.035**	0.23 (0.18 – 0.36)	0.27 (0.19 – 0.40)	ns
Glucose	**4546.02 (4104.05 – 5115.56)**	**4959.36 (440.88 – 5804.93)**	**0.001**	**4955.95 (4459.14 – 5679.69)**	**4418.11 (4134.89 – 5191.71)**	**0.007**	**4663.92 (4172.31 – 5417.54)**	**4925.38 (4503.68 – 5684.91)**	**0.027**
Glutamate	1457.27 (743.06 – 2912.42)	1416.96 (671.78 – 2684.24)	ns	1335.82 (725.07 – 2628.52)	1786.69 (691.16 – 2891.74)	ns	1556.27 (679.84 – 2785.70)	1342.51 (763.74 – 2372.15)	ns
Glutamine	5073.46 (3054.24 – 7754.55)	4742.54 (1842.39 – 8881.28)	ns	5083.38 (2842.09 – 9072.22)	4742.54 (1700.95 – 7003.86)	ns	4742.54 (2007.90 – 7169.01)	5073.46 (2219.30 – 10427.79)	ns
Isoleucine	**57.14 (47.71 – 63.99)**	**63.68 (52.83 – 75.20)**	**0.002**	**63.52 (52.24 – 75.19)**	**57.88 (51.23 – 63.22)**	**0.050**	**57.90 (49.81 – 68.46)**	**65.02 (55.35 – 77.95)**	**0.002**
Lactate	367.23 (283.94 – 423.66)	341.07 (297.13 – 441.68)	ns	**373.57 (308.33 – 452.52)**	**332.36 (279.50 – 405.92)**	**0.047**	**334.77 (274.24 – 435.64)**	**393.34 (323.21 – 457.69)**	**0.004**
Leucine	85.07 (70.62 – 94.86)	86.68 (69.33 – 109.65)	ns	90.17 (71.33 – 109.35)	84.54 (69.77 – 93.84)	ns	**83.96 (66.70 – 98.71)**	**92.19 (76.10 – 109.92)**	**0.011**
Malate	2.38 (1.85 – 4.31)	2.45 (1.88 – 3.56)	ns	**2.79 (1.96 – 4.09)**	**2.23 (1.75 – 3.08)**	**0.043**	2.37 (1.83 – 3.66)	2.87 (2.12 – 3.80)	ns
Pyruvate	12.47 (3.64 – 22.87)	12.83 (3.55 – 21.89)	ns	**13.86 (3.85 – 24.11)**	**8.78 (2.72 – 18.15)**	**0.042**	9.50 (3.08 – 21.00)	13.58 (6.00 – 25.78)	ns
Serine	147.54 (101.70 – 167.45)	137.69 (109.40 – 169.59)	ns	**135.30 (106.14 – 162.12)**	**161.02 (103.62 – 172.90)**	**0.34**	150.04 (104.94 – 170.39)	131.92 (106.07 – 18.82)	ns
Succinate	9.57 (8.35 – 15.11)	9.70 (8.47 – 15.23)	ns	9.15 (8.42 – 15.04)	11.71 (8.49 – 15.47)	ns	10.82 (8.44 – 15.23)	9.11 (8.46 – 15.15)	ns
Succinyl-CoA	10.14 (7.74 – 15.12)	10.46 (7.37 – 17.62)	ns	11.21 (7.72 – 16.31)	9.94 (7.52 – 14.25)	ns	**9.79 (6.71 – 14.00)**	**13.36 (8.90 – 18.47)**	**0.001**
Valine	102.06 (83.06 – 129.40)	104.17 (80.70 – 138.39)	ns	107.33 (86.67 – 136.83)	96.63 (78.83 – 134.87)	ns	**99.36 (78.62 – 125.54)**	**113.33 (89.62 – 139.43)**	**0.010**

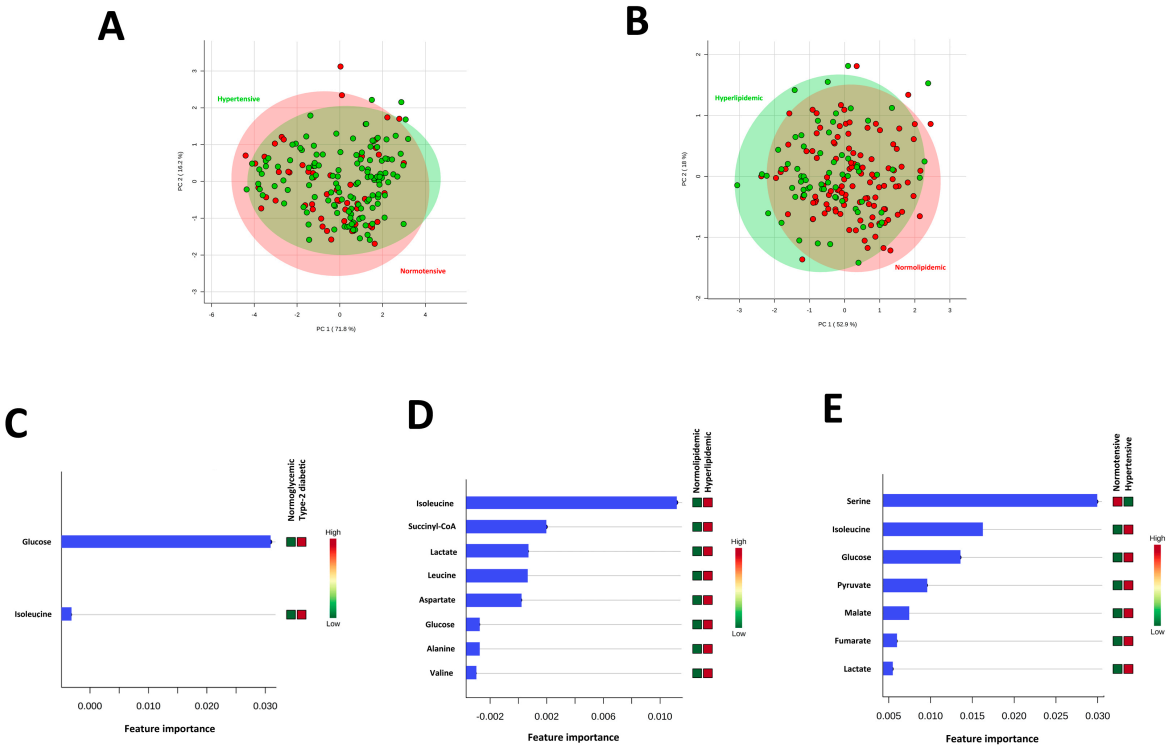

Figure 2. Principal component analysis (PCA) between hypertensive and normotensive (A) and between hyperlipidemic and normolipidemic (B) PAD patients. Random Forest analysis shows the metabolites with the best discriminant capacity between normoglycemic and diabetic patients (C), between normolipidemic and hyperlipidemic patients (D) and between normotensive and hypertensive patients (E).

Age and body mass index (BMI) were other two confounding variables. Subsequently, we analyzed whether any of the metabolites correlated with age or BMI between PAD patients. Aconitate, fumarate and malate were associated to age, and aconitate, alanine, aspartate, glucose, isoleucine, leucine and valine correlated with BMI (Table 3, Supplementary figure 2).

Table 3. Spearman correlation coefficients for age, body mass index and related metabolites.

	Age		**BMI**	
	Spearman's Rho	**p-value**	**Spearman's Rho**	**p-value**
3-hydroxybutirate	0.057	ns	0.022	ns
Aconitate	**0.205**	**0.003**	**-0.236**	**0.010**
α-ketoglutarate	0.054	ns	-0.031	ns
Alanine	-0.081	ns	**0.230**	**0.013**
Aspartate	0.083	ns	**0.256**	**0.005**
Citrate+Isocitrate	0.113	ns	0.147	ns
Fumarate	**0.220**	**0.002**	-0.085	ns
Glucose	-0.031	ns	**0.202**	**0.029**
Glutamate	0.063	ns	-0.032	ns
Glutamine	0.007	ns	0.157	ns
Isoleucine	0.124	ns	**0.194**	**0.036**
Lactate	-0.023	ns	0.034	ns
Leucine	-0.005	ns	**0.220**	**0.017**
Malate	**0.248**	**<0.001**	-0.079	ns
Pyruvate	-0.009	ns	0.103	ns
Serine	0.062	ns	-0.112	ns
Succinate	0.011	ns	-0.145	ns
Succinyl-CoA	0.038	ns	0.134	ns
Valine	-0.122	ns	**0.241**	**0.009**

BMI: Body mass index.

3.4. Metabolic biomarkers of PAD

PAD patients were segregated according Fontaine classification to perform a more accurate analysis (Table 4). Remaining metabolites significantly different between control group and PAD patients were considered possible biomarkers of PAD: 3-hydroxybutyrate, α-ketoglutarate, glutamate, glutamine, (iso)citrate and succinate (Figure 3). Moreover, glutamate and (iso)citrate concentrations were statistically different between PAD I-II, III and IV groups (Figure 3C and 3E).

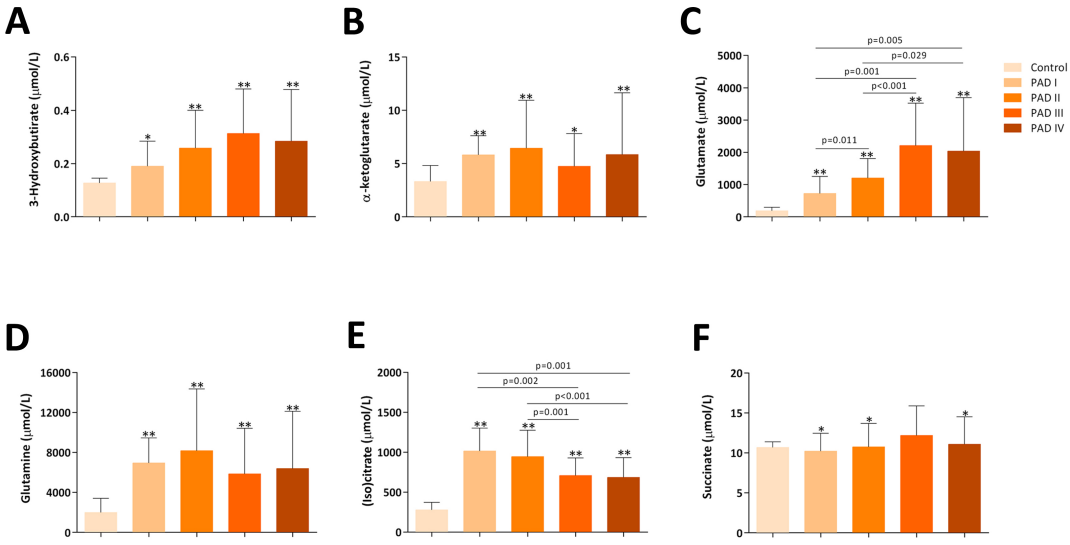

Figure 3. Candidate biomarkers for PAD patients. Graphical representation of candidate biomarkers concentration among groups: (A) 3-hydroxybutirate, (B) α-ketoglutarate, (C) glutamate, (D) glutamine, (E) (Iso)citrate and (F) succinate. *: $p < 0.05$; **: $p < 0.001$.

Table 4. Clinical characteristics, complete blood count and biochemical characteristics of PAD patients segregated by Fontaine classification.

	Fontaine I (n = 9)	Fontaine II (n = 30)	Fontaine III (n = 46)	Fontaine IV (n = 116)	P-value
Clinical characteristics					
Age (years)	55 (51 – 69)	73 (60 – 77)	65 (61 – 75)	71 (64 – 79)	0.003
BMI (kg/m^2)	28.9 (25.2 – 30.6)	27 (23.5 – 29.5)	26 (22.5 – 28)	24 (22-27.8)	ns
Diabetes (%)	12.5	60.9	38.1	78.7	<0.001
Hypertension (%)	50	74.1	60	73.3	<0.001
Dyslipidemia	42.9	42.3	37.8	36.4	<0.001
Complete Blood Count					
Red blood cells, x10^{12}/L	5.1 (4.5 – 5-4)	4.5 (4.1 – 4.8)	4.2 (3.8 – 4.6)	3.9 (3.3 – 4.4)	<0.001
Hemoglobin, mmol/L	9.12 (7.57 – 10.18)	8.56 (8.01 – 9.56)	8.87 (8.32 – 9.43)	7.63 (6.70 – 8.50)	<0.001
Leukocytes, x10^9/L	7.4 (6.8 – 10.4)	7.3 (6.3 – 8.9)	7.6 (6.4 – 10.4)	8.4 (6.6 – 10.7)	ns
Platelets, x10^9/L	205 (159 – 246)	216.5 (173.5 – 257.2)	251 (197 – 310)	277 (213 – 361)	0.001
Biochemical variables					
Total-cholesterol, mmol/L	4.06 (3.69 – 4.22)	4.23 (3.74 – 4.91)	3.94 (3.27 – 4.41)	3.80 (3.20 – 43.21)	ns
HDL-cholesterol, mmol/L	0.80 (0.58 – 1.22)	1.03 (0.80 – 1.27)	1.27 (0.81 – 1.25)	0.93 (0.78 – 1.16)	ns
LDL-cholesterol, mmol/L	2.04 (1.92 – 3.07)	2.43 (1.92 – 3.08)	2.22 (1.76 – 2.66)	2.24 (1.75 – 2.81)	ns
Triglycerides, mmol/L	1.57 (1.07 – 3.29)	1.81 (1.12 – 2.93)	2.34 (1.88 – 3.44)	1.85 (1.34 – 2.83)	ns
Glucose, mmol/L	6.99 (6.2 – 8.21)	6.09 (5.01 – 7.05)	5.29 (4.56 – 6.79)	5.55 (4.40 – 6.85)	ns
ALT, U/L	18 (13.3 – 41-5)	21 (16 – 28)	23.5 (18.8 – 46.5)	21 (13.7 – 35)	ns
AST, U/L	22.9 (16.2 – 32.4)	21 (17 – 25.6)	22.5 (18 – 44-7)	20 (15 – 32)	ns

BMI: Body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Non-parametric variables are shown as median and IQR (25-75%). Qualitative variables are expressed as (%) of total participants. Multiple comparisons between groups using Kruskal-Wallis test.

To evaluate discriminant capacity, we perform random forest analyses and found that (iso)citrate and glutamate were the most powerful metabolites to separate control individuals from PAD group (Figure 4A). ROC curve for these metabolites showed good area under the curve (AUC) values for both metabolites (Figure 4B). When testing discriminant capacity between control group and the early manifestation of PAD (Intermittent claudication, PAD I-II), (iso)citrate and glutamate were again the best discriminant metabolites (Figure 4C) and ROC curve confirmed this potential (Figure 4D). To follow disease progression in PAD patients, (iso)citrate and glutamate were also the best indicators (Figure 4E).

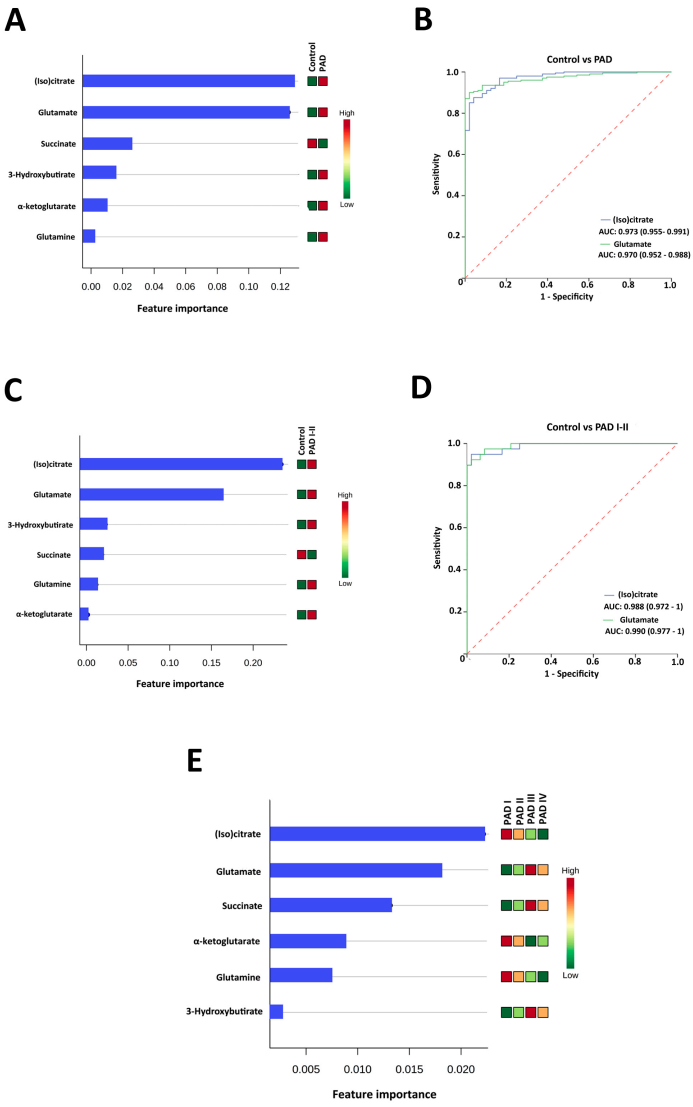

Figure 4. Validation of candidate biomarkers. Random Forest analysis showing the metabolites with the best discriminant capacity between control group and (A) PAD patients, (C) PAD I-II patients and (E) during disease progression. ROC curve for the best candidates. Discriminating between control group and (B) PAD and (D) PAD I-II patients. AUC, area under curve.

4. DISCUSSION

Dietary changes and lifestyle can modify our metabolome, and thus metabolomics gives feedback about the status of individuals and offers the opportunity to study pathologies and propose new interventions[5,13,26]. The metabolomic characterization of atherosclerotic peripheral artery disease is gaining interest, as its incidence has increased worldwide[1,27].

However, biological and technical limitations are present, and tissue and plasma metabolome does not provide the same information. In our case, and as expected, we were not able to quantify phosphate metabolites in plasma due to the impermeability of the cellular membrane to these compounds (Supplementary Figure 1).

Here, we found alterations in energy metabolism in PAD patients, compared to control group, especially impairments in the connection citrate-aconitate-isocitrate. The mitochondrial enzymes involved in those reactions are isocitrate dehydrogenase (IDH2) and aconitase 2 (ACO2). IDH2 has been related with a proper mitochondrial function, and mice lacking IDH2 exhibited mitochondrial dysfunction[28]. Moreover, 7-ketocholesterol is known to contribute to atherosclerosis progression by decreasing IDH2 expression and increasing oxidative stress thus modifying mitochondrial function[29]. Furthermore, an oxidative environment (mainly superoxides) can inactivate aconitase, which in turn undergo age-dependent oxidative modification[4]. Whether IDH2 and

ACO2 may be the cause or consequence of the well-described mitochondrial dysfunction in PAD is still unknown[8].

Preventive treatment could slow down the progression or even stop the disease. For this reason, a fast diagnostic is necessary. However, many of the current biomarkers are based on risk factors associated to co-morbidities like dyslipidemia, hypertension or T2D[10,12]. In fact, non-communicable diseases are mostly multi-factorial and, in our population, we found that approximately 80% of these patients had any of these impairments, which could be affecting metabolites concentration. Among those metabolites, branched-chain amino acids (BCAAs) were influenced by hypertension, T2D and dyslipidemia. Our results ratify the relationship between an impairment in branched chain amino acid (BCAA) catabolism and obesity and insulin resistance[30,31]. Moreover, increased serum concentration of BCAAs have been also associated to metabolic dyslipidemia[32] and BCAA supplementation during maternal food restriction has been related to a less hypertension incidence in adult offspring[33].

After discarding the influenced metabolites, six candidates remained with statistically significant differences in concentration between control group and PAD patients: 3-hydroxybutyrate, α-ketoglutarate, glutamate, glutamine, (iso)citrate and succinate. Those candidates were useful to distinguish between PAD patients and control group but also to discriminate between PAD grades. Isocitrate is an

intermediate in the TCA cycle. Its conversion to α-ketoglutarate is mediated by IDH2. It was found that higher concentrations of this metabolite are associated with a worse cardiovascular prognostic[34]. PAD patients showed higher concentrations of isocitrate, but PAD I patients showed higher levels than PAD IV patients. Glutamate, another metabolite with a good discriminant capacity, is the substrate for many enzymes located in the mitochondria[35]. Glutamate plays an important role in heart metabolism, as during ischemia, it improves the mechanical function of the ischemic myocardium[36]. Maybe this increased concentration of glutamate in PAD patients could be an attempt to improve biomechanical functions of the ischemic portions of the arteries. However, further research is needed to understand glutamate overproduction in blood of atherosclerotic patients.

To diagnose PAD in the asymptomatic or early symptomatic stages (PAD in Fontaine I and II; intermittent claudication) and to find a clinical biomarker for these stages is of great interest. In our case, (iso)citrate and glutamate were able to distinguish control group from PAD patients in stages I-II. The implementation of (iso)citrate and glutamate measurements in clinical practice (*bench-to-bed* approach) would allow an early detection of the disease and would permit vascular specialists apply better treatments to delay or even stop the disease.

Our study provides evidences that metabolic fingerprints can be used to differentiate PAD patients from control population. We propose

two potential biomarkers for the disease – (iso)citrate and glutamate – that can be used for an early diagnosis. Although our results are potentially translational and limited to little population, other metabolic pathways and the validation in other cohorts may be considered for future studies.

5. CONCLUSION

Our metabolomics approach served to propose candidate biomarkers for PAD diagnosis after interpretation of the metabolome fingerprint. (Iso)citrate and glutamate were not influenced by other comorbidities and their concentrations differ between control group and PAD patients, allowing a good discrimination between stages. More important, both can detect PAD I-II patients, the less symptomatic stages of the disease.

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DISCUSSION

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Discussion

PAD is a global pandemic disease affecting more than 202 million individuals [153]. Arteries of the extremities are affected, and there is a stiffening, thickening and loss of elasticity of the artery wall [24–26,154]. Despite its common occurrence, PAD is often underdiagnosed: first stages are asymptomatic and disease only becomes evident when associated pathologies come to light implying that preventing its derived consequences becomes practically impossible [57,155].

A good and correct diagnosis is generally difficult. Vascular specialists normally perform examinations supported by non-invasive or invasive techniques [59,155,156]. The ABI is one of the most used tests to perform the diagnostic of PAD regardless of its proven limitations [85,155]. Once diagnosed, patients are normally classified using the Fontaine classification and then, depending on disease severity, they undergo different treatments. Management of cardiovascular risk factors is the usual treatment for the initial stages of the disease. Exercise, rehabilitation, pharmacologic intervention and invasive surgical procedures are other treatments if the disease aggravates [59,156,157].

A deeper understanding of the disease would provide novel biomarkers and also novel therapeutic strategies. Finding a worthy biomarker for PAD is an unresolved question. Most of these markers are based on cardiovascular risk factors, inflammation or oxidative processes [55–57,88]. However, there is still controversy about them, as they indicate not only PAD but also atherosclerosis in general.

Discussion

Vascular remodeling is an important characteristic of atherosclerosis. Inflammation and oxidation are also involved in this process: previous studies of our group revealed that serum PON1 concentration was significantly lower and circulating PON3 and CCL2 concentration were higher in PAD patients compared to non-affected patients [158,159]. However, there were limited data about protein expression of these molecules in arteries from PAD patients.

In our exploratory ***Study 1***, we found that paraoxonases, CCL2 and CCL2 receptors were increased in atherosclerotic peripheral arteries, maybe due to increased cellular oxidative stress and inflammation. The increased PON1 staining in affected arteries suggested that this enzyme is reducing oxidative stress processes in macrophages and cell foam formation. Although the physiological role of PON3 is still unclear, our results support our previous findings in which serum PON3 concentration were increased in these patients [158]. The causal mechanisms by which paraoxonases may be increased in atherosclerotic arteries are not clear, but may involve PPARγ and NFκB-related pathways [160–162]. However, PON1 is also regulated by proinflammatory cytokines (like IL-6), epigenetic mechanisms (DNA or histone methylation) and nuclear receptors (PPAR, AhR) [163].

Contradictorily to the increased expression of PON1 in atherosclerotic arteries, our group had previously found that PON1 concentration was decreased in the sera of PAD patients [159]. A

possible explanation for this observation may be related to PPARδ expression. An increased PPARδ expression was associated to an inhibition of HDL synthesis and consequently decreased PON1 secretion to extracellular medium [164]. The diminished HDL-cholesterol concentration observed in PAD patients may supports the concept but this hypothesis has to be confirmed by further studies.

The decrease of oxidative stress and the modulation of PON1 activity could be possible therapeutic targets. Many polyphenolic compounds and phytoestrogens have been proved to reduce oxidative stress [165–167]. It has also been reported that curcumin, a natural polyphenol, improves HDL functionality, thus modulating the activities and levels of HDL markers (i.e. PON1) [168]. Moreover, fibrates and statins used for treating dyslipidemias exert effect on PON1 expression [163,169].

PON1 inhibits the production of oxidative stress-induced CCL2 and decreased PON1 activity is associated with an increased of CCL2 concentration in circulation [138,159,170]. However, we found that PON1 and CCL2 were expressed together in arterial tissue, maybe suggesting a coordinated role [171,172].

It is collectively accepted that chemokines play a crucial role in mediating the inflammatory and immune cell trafficking. CCL2, by inducing monocyte migration, is involved in atherosclerosis development [112,113,120,173]. We found that arteries from PAD patients had

increased expression of CCL2 and their receptors (CCR2, DARC and D6). DARC and D6 (also known as ACKRs) are atypical chemokine receptors with no signal transmission: they internalize ligands and are able to modulate chemokine concentration [123,124,174]. We found that, although increased, these receptors were not uniformly distributed among arterial tissue. The expression of ACKRs in atherosclerotic arteries of PAD patients has not been previously reported, and our data suggest that they may modify chemokine availability, and therefore indirectly modifying cell migration.

The link between chemokines and their receptor has been of great interest: novel therapeutic agents against these key inflammatory mediators are being tested in some prevalent diseases with promising results [29,118,175]. Our results point to encouraging therapeutic strategies based on the blockade of CCL2/CCR2 axis or in increasing the availability of ACKRs to modulate CCL2 concentration [29,113,120,125,174,176]. Nowadays, many CCR2 and CCR5 antagonists, such as the pharmacological drugs maraviroc or cenicriviroc, have been developed and many of them are in the last stages of clinical trials [177–181].

As confirmed in ***Study 1*** and in previous studies by our group, during atherosclerosis, the arterial tissue is governed by inflammatory and oxidative processes, [113]. Vascular remodeling (concretely in ECM) is also implicated in atherosclerotic plaque formation but detailed

information about ECM composition and turnover in PAD patients has not previously reported. In ***Study 2***, we performed a histologic and proteomics approach to evaluate arterial remodeling and ECM composition in PAD patients compared to non-atherosclerotic control arteries.

We confirmed an important vascular remodeling as a reparative response, characterized by the presence of inflammatory intermediaries and ECM turnover [23,96]. Proteomics results confirmed that proteins related to inflammatory process (alpha-2-macroglobulin and carboxypeptidase B2) were overregulated, and proteins related with extracellular matrix components (versican, lactadherin) were underexpressed in affected arteries from PAD patients compared to healthy non-atherosclerotic arteries. These decreased levels of structural proteins may be associated to a deregulation of the normal function of extracellular matrix. All results taken together suggest that there are multiple mechanisms involved in the maintenance of artery structure and function during the atherosclerotic process [182]. Recent data about a proteomics approach on carotid plaques also revealed a 4-biomarker signature of ECM related proteins that may be used to improve the risk prediction and diagnostics of cardiovascular disease [183], seconding the important role of ECM turnover in atherosclerotic diseases.

During ECM remodeling, there is a newly formed ECM scaffold constituted by collagen and proteoglycans. Macrophage derived

proteases are secreted to remove this excessive ECM components, and this degradation processes would create protein fingerprints detectable in bloodstream [102,182].

The underexpressed levels of ECM-related proteins in atherosclerotic arteries were representative of the subtle equilibrium between production and degradation of ECM proteins during turnover by MMP activity [103–105]. Consequently, we assumed that degradation of ECM proteins was increased and, for this reason, candidate circulating ECM-degradation fragments (neo-epitopes) were selected to be quantified in PAD patients in order to propose biomarkers for disease severity.

These neo-epitopes result from post-translational modifications of extracellular matrix proteins by proteinases, mainly MMPs. These cleavage products are tissue-specific and disease-specific, so they can be used as novel and potential biomarkers [184,185]. In fact, many studies have reported impaired levels of these neo-epitopes as novel biomarkers for different pathologies [186–192].

Based on the literature and on our previous histologic and proteomics results, we selected a panel of six neo-epitopes to be measured on PAD patients to assess their usefulness in separating patients with mild-moderate PAD (Fontaine I-II, IC) from severe PAD (Fontaine III-IV, CLI). We found that the combination of VCANM and C4M could be useful in indicating PAD clinical severity, as they showed the

best ability to discriminate between mild-moderate PAD and severe PAD, with high specificity and sensitivity. VCANM concentration tended to decrease during disease progression and C4M seemed to increase according disease severity, which may denote a difficult regulation of MMP activity generally depending on specific contexts [193,194]. Proposed biomarkers may have potential to benefit drug development for PAD. However, our findings need exhaustive evaluation and other designs considering predictive power in asymptomatic PAD need to be performed.

The imbalance in ECM turnover, inflammation and oxidative processes is also linked to metabolic stress and immunity [107,195–197]. An excessive energy intake can promote a disrupted energy metabolism, impaired bioenergetics and mitochondrial dysfunction and predispose to non-communicable diseases such as atherosclerosis (***Annex 2 and 3***) [130,197–199]. In ***Study 3***, we performed a targeted metabolomics analysis to find any metabolic disturbances in atherosclerotic patients and, again, propose novel biomarkers or new therapeutic strategies [200].

Metabolomics can provide a unique profile of metabolites, which reflects the outcomes of biochemical reactions in cellular physiology [200–202]. It is mainly used for disease diagnosis, to understand disease mechanisms, to identify new druggable targets and monitor therapeutic outcomes [203–205].

During our study, we found important technical and biological limitations to overcome. Firstly, isocitrate and citrate were not distinguished during the quantification. Secondly, and due to the lack of phosphate compounds in blood, there were some differences between using arterial tissue or plasma samples from individuals. Nevertheless, targeted metabolomics using mass spectrometry brings the necessary sensibility, reproducibility and accuracy for the unequivocal identification and quantification of metabolites.

PAD patients showed alterations in their energy metabolism, mainly in the connection between citrate-aconitate-isocitrate, as those metabolites were increased in PAD patients. Enzymes regulating these reactions have been related with mitochondrial function, thus the accumulation of the metabolites may be linked to an abnormal enzymatic activity and mitochondrial impairment [196,206–208].

However, we found out that 80% of our PAD patients had associated comorbidities (dyslipidemia, hypertension or type-2 diabetes) and this may lead to a significant change on metabolite concentrations. We then segregated PAD patients according comorbidities and observed that, among others, branched-chain amino acids were influenced by these comorbidities and also by age and BMI, confirming previous results [209–212]. Moreover, BCAA associations with diabetes and metabolic abnormalities are stronger in Caucasian and Hispanics [213].

We discarded these metabolites in our search for a PAD biomarker, and we selected six remaining candidates with significant differences between control group and PAD patients: 3-hydroxybutyrate, α-ketoglutarate, glutamate, glutamine, (iso)citrate and succinate. Glutamate and (iso)citrate had the best discriminant capacity between control group and PAD patients, between PAD grades and also between control group and IC (Fontaine I-II). Isocitrate has been recently associated with a worse cardiovascular prognostic. Glutamate has been related to biomechanical function in ischemic heart, and it is the required substrate for different enzymes in the mitochondria [214–216]. Although confirmation is needed, we suspect that the increased concentration of glutamate may be related with an effort to recover biomechanics of ischemic arteries.

We think that a possible implementation of (iso)citrate or glutamate measurements in clinical practice may facilitate an earlier diagnose of PAD. Despite metabolomics does not display real fluxes [200,217], it can reveal interesting metabolic fingerprints which in turn, are useful to propose novel biomarkers or therapeutic targets and strategies.

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CONCLUSIONS

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Conclusions

→ Inflammation, oxidation and vascular remodeling are present in atherosclerotic arteries and their effect may provide clinically useful circulating markers.

→ The relationship between ligands and receptors in chemokine system and response to oxidative stress play a role in atherosclerosis progression.

→ There is a specific proteome profile in PAD arteries.

→ Circulating ECM products of degradation VCANM and C4M may be useful in the assessment of disease progression.

→ Comorbidities are limitations to the interpretation of plasma metabolome.

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SUPPLEMENTARY MATERIAL

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PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
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Article

Immunohistochemical Analysis of Paraoxonases and Chemokines in Arteries of Patients with Peripheral Artery Disease

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Abstract: Oxidative damage to lipids and lipoproteins is implicated in the development of atherosclerotic vascular diseases, including peripheral artery disease (PAD). The paraoxonases (PON) are a group of antioxidant enzymes, termed PON1, PON2, and PON3 that protect lipoproteins and cells from peroxidation and, as such, may be involved in protection against the atherosclerosis process. PON1 inhibits the production of chemokine (C–C motif) ligand 2 (CCL2) in endothelial cells incubated with oxidized lipoproteins. PON1 and CCL2 are ubiquitously distributed in tissues, and this suggests a joint localization and combined systemic effect. The aim of the present study has been to analyze the quantitative immunohistochemical localization of PON1, PON3, CCL2 and CCL2 receptors in a series of patients with severe PAD. Portions of femoral and/or popliteal arteries from 66 patients with PAD were obtained during surgical procedures for

infra-inguinal limb revascularization. We used eight normal arteries from donors as controls. PON1 and PON3, CCL2 and the chemokine-binding protein 2, and Duffy antigen/chemokine receptor, were increased in PAD patients. There were no significant changes in C–C chemokine receptor type 2. Our findings suggest that paraoxonases and chemokines play an important role in the development and progression of atherosclerosis in peripheral artery disease.

Keywords: CCL2; chemokine receptors; chemokines; immunohistochemistry; paraoxonases

1. Introduction

Lower-extremity peripheral artery disease (PAD) is an important health problem that is associated with severe impairment of different arterial territories. Indeed, PAD is a predictor of substantial coronary and cerebral vascular risk [1,2]. The disease prevalence increases with age and, in people over the age of 55 years, it is estimated to be about 20% [3–6]. Atherosclerosis affects wide portions of numerous arteries in the lower extremities of PAD patients. This is the effect of a sustained and silent progression of the disease in which appropriate and effective prevention measures are applied too late, or not implemented at all [3–8].

Oxidative damage to lipids and lipoproteins is implicated in the development of atherosclerotic vascular diseases, including PAD [9,10]. The paraoxonases (PON) are a group of antioxidant enzymes that protect lipoproteins and cells from peroxidation and are involved in the atherosclerosis process and, consequently, in vascular diseases [11]. The PON family contains three enzymes: PON1, PON2 and PON3, the genes of which are located adjacent to each other on chromosome 7q21–22 [12,13]. PON1 and PON3 are found in many tissues, as well as in blood, where they are associated with high-density lipoproteins (HDL). Conversely, PON2 is exclusively intracellular [14–17]. Pioneer studies reported that oxidized low-density lipoprotein uptake by macrophages in tissue culture and *in vivo* increases the production of the inflammatory chemokine (C–C motif) ligand 2 (CCL2). The consequence is the stimulation of arterial fatty streak formation, which is the progenitor of atheroma. PON1 has been shown to inhibit these alterations [18–20]. Chemokines, CCL2 in particular, are central to the vascular inflammatory response in mediating monocyte recruitment into the arterial wall [21,22]. We have previously reported that PON1 and CCL2 are ubiquitously distributed in mouse tissues, suggesting a joint localization and combined systemic effects [23]. Clinical data suggest that circulating CCL2 concentrations or serum PON1 activity are important biomarkers of a variety of diseases involving inflammatory response to an increased oxidative stress [24–29].

Previous studies from our group found that serum PON1 activity and concentration were significantly lower, and CCL2 concentration higher, in PAD patients compared to controls, while the combination of plasma CCL2 and PON1-related variables, discriminated controls from patient almost completely [30]. In addition, we observed an increase in serum PON3 concentration in PAD patients, relative to the healthy population [31]. However, data on the protein expression of these molecules at the lesion level in patients with PAD are scarce. The aim of the present study was to quantify the

immunohistochemical localization of PON1, PON3, CCL2 and CCL2 receptors in a wide series of patients with severe PAD.

2. Results

Patients with PAD did not significantly differ from the control group in age and gender distribution. The patient group had a significantly higher percentage of smokers, and lower serum cholesterol and low-density lipoprotein (LDL) cholesterol concentrations. We did not observe significant difference in any of the other standard biochemical and hematological variables. The circulating levels of CCL2 and 8-isoprostanes (a marker of oxidative stress) were significantly increased in PAD, while serum PON1 concentrations and activities were decreased (Table 1). C-reactive protein (CRP) protein levels were not significantly increased in our patients, a finding probably related to that they were treated with salicylates and antiplatelet agents.

Table 1. Selected descriptive characteristics and laboratory variables in participants.

Parameter	Control (*n* = 8)	PAD (*n* = 66)	*p*-Value
Clinical characteristics			
Age, years	66 (30–76)	70 (62–77)	0.223
Male, *n* (%)	5 (62.5)	55 (85.9)	0.094
Smokers, *n* (%)	1 (14.3)	16 (31.4)	**0.048**
Complete blood count			
Red blood cells, $\times 10^{12}$/L	4.32 (3.18–4.47)	3.67 (3.14–4.24)	0.449
Hemoglobin, g/dL	12.46 (9.99–13.28)	10.85 (9.45–12.93)	0.468
Leukocytes, $\times 10^{9}$/L	9.22 (8.58–10.17)	9.89 (7.44–12.20)	0.668
Platelets, $\times 10^{9}$/L	227.5 (163.7–246.2)	312.5 (199.0–419.0)	0.080
Biochemical variables in serum or plasma			
Glucose, mmol/L	5.77 (5.11–6.77)	6.38 (5.11–8.83)	0.406
Total cholesterol, mmol/L	4.77 (3.87–6.39)	3.39 (2.90–4.47)	**0.030**
HDL cholesterol, mmol/L	1.24 (0.98–1.40)	0.93 (0.83–1.20)	0.074
LDL cholesterol, mmol/L	3.54 (3.11–4.42)	1.95 (1.68–2.69)	**0.001**
Triglycerides, mmol/L	1.47 (1.13–2.15)	1.31 (1.00–1.87)	0.449
Fibrinogen, g/L	5.51 (4.48–7.54)	6.96 (5.34–8.11)	0.237
C-reactive protein, mg/L	6.1 (0.6–7.2)	8.1 (2.7–16.0)	0.147
Total proteins, g/L	65 (55–68)	60 (55–69)	0.743
CCL2, ng/L	373.4 (255.2–431.8)	622.8 (472.7–898.4)	<**0.001**
PON1, mg/L	75.4 (56.7–143.8)	25.2 (18.4–35.8)	<**0.001**
PON3, mg/L	1.95 (1.51–2.50)	1.73 (1.43–2.27)	0.490
8-Isoprostanes, ng/L	14.2 (2.0–37.2)	100.8 (37.6–314.7)	<**0.001**
PON1 lactonase activity, U/L	5.69 (5.02–6.29)	3.04 (2.11–3.73)	<**0.001**

The bold numbers highlight the statistically significant differences.

The histological and immunohistochemical analyses of the peripheral arteries revealed that PAD patients had a significantly thicker tunica intima relative to the tunica media of the artery wall (termed the intima-media, or I/M ratio). There were significant increases in the percentage positive staining for PON1, PON3, CD68 antigen (a marker of macrophages), CCL2, and also in the CCL2 receptors

termed chemokine-binding protein 2 (CCBP2, also termed D6), and Duffy antigen/chemokine receptor (DARC). We did not observe any significant change in C–C chemokine receptor type 2 (CCR2) staining relative to controls (Table 2). Similar results were obtained when smokers were excluded from the PAD group (Table S1).

Table 2. Differences in selected variables between control individuals and PAD patients.

Parameter	Control (*n* = 8)	PAD (*n* = 66)	*p*-Value
IMT (mm)	1.00 (0.70–1.30)	1.29 (1.00–1.74)	0.150
I/M ratio	0.16 (0.13–0.65)	2.10 (1.33–3.22)	**<0.001**
% PON1 staining	1.70 (1.54–3.72)	11.19 (7.25–20.81)	**<0.001**
% PON3 staining	0.55 (0.22–0.73)	3.25 (2.01–4.37)	**<0.001**
% CCL2 staining	2.26 (0.36–3.65)	30.75 (9.63–44.41)	**<0.001**
% CCR2 staining	18.29 (7.02–27.56)	22.99 (13.21–42.71)	0.263
% CD68 staining	1.10 (0.65–2.88)	4.57 (2.40–9.24)	**0.007**
% D6 staining	0.83 (0.22–12.9)	41.21 (24.55–58.39)	**<0.001**
% DARC staining	3.29 (2.01–5.06)	37.26 (18.06–51.85)	**<0.001**

IMT: Intima-Media thickness. Results are shown as medians and interquartile ranges. Staining for chemokine (C–C motif) ligand 2 (CCL2), C–C chemokine receptor type 2 (CCR2), cluster of differentiation 68 (CD68), Duffy antigen/chemokine receptor (DARC), chemokine-binding protein 2 (D6), paraoxonase-1 (PON1) and paraoxonase-3 (PON3) were measured as the area of positive staining and expressed as percentage of the total area examined using the image analysis system (see text for details). The bold numbers highlight the statistically significant differences.

Affected arteries had severe alterations compared to the normal artery histology (Figure 1). The intima was thicker and had extensive deposits of cholesterol and inflammatory cells. Calcium deposits were clearly identified in the media. Masson's trichrome stain was used to evaluate the arteries' architecture which, in affected arteries, highlighted an infiltration of smooth muscle cells from the media into the intima, or perhaps a loss of muscle cells from the media and increase in connective tissue, and greater obstruction of the arterial lumen.

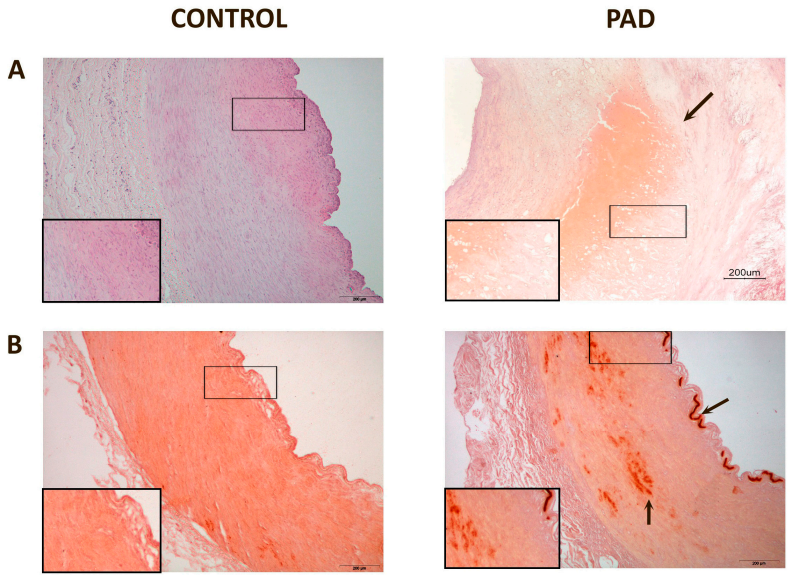

Figure 1. *Cont.*

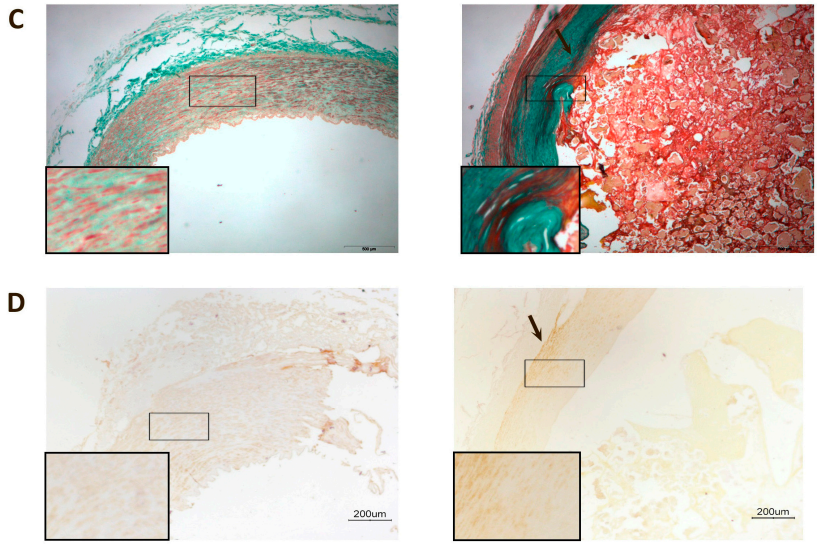

Figure 1. Representative histological images of peripheral arteries: (**A**) Arteries stained with Hematoxylin-Eosin. The intima in affected arteries was thicker and replete with cholesterol deposits and inflammatory cells (arrow). Magnification 20×; (**B**) Alizarin Red staining to detect the presence of calcium. There were calcium deposits in affected arteries located, mainly, in the media and, in some cases, calcium was observed in the internal elastic lamina (arrows). Magnification 20×; (**C**) Masson's Trichrome stain showing, in affected arteries, an infiltration of smooth muscle cells from the media to the intima (arrow). The lumen shows partial obstruction. Magnification 40×; (**D**) Actin staining to detect the presence of smooth muscle cells. The arrow shows the area of infiltration of these cells from the media to the intima. Magnification 20×. The inserts show higher magnification (100×) images of the indicated areas.

In normal arteries, PON1 expression was low and located in the intima and in the adventitia. PON3 expression was imperceptible. Conversely, in the arteries of PAD patients, PON1 and PON3 expression were higher. PON1 presented two types of localization: (1) when the intima was only moderately enlarged, PON1 was located in the adventitia vessels and the media; (2) when the intima was disorganized and with cholesterol deposits, PON1 was found surrounding the cholesterol crystals at the site of the lesion. In affected arteries, PON3 was found in the adventitia or in the injury sites of the intima (Figure 2). Areas of CD68 staining had a similar spatial distribution than those of paraoxonases and CCL2 (Figures S1 and S2).

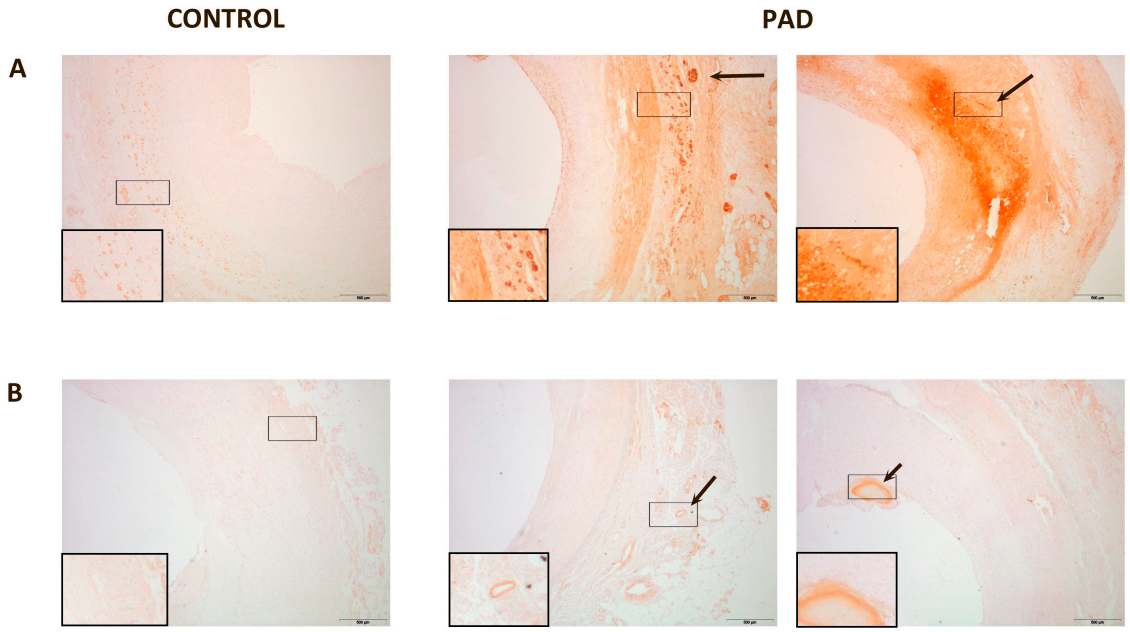

Figure 2. Representative immunohistochemical images for paraoxonase-1 (PON1) and paraoxonase-3 (PON3) staining of peripheral arteries: (**A**) PON1 expression in normal artery was almost undetectable, and located in the media and adventitia. PON1 had two types of localization in affected arteries: when the intima was not very thick, PON1 was located in the adventitia and media of the vessels (arrow). When the intima was disorganized and with cholesterol deposits, PON1 was expressed in the lesion site (arrow); (**B**) PON3 expression was undetectable in normal tissue whereas, in affected arteries, PON3 was located in the adventitia or in the injury sites of the intima (arrow). Magnification 20×. The inserts show higher magnification (100×) images of the indicated areas.

In normal arteries, CCL2 was mildly expressed in the adventitia, while CCR2 was found mostly in the media, with weaker expressions in the adventitia and intima. CD68, D6 and DARC expressions were mild. Conversely, the arteries of PAD patients had higher expressions of CCL2, CD68, D6 and DARC. CCL2 was found mostly in the adventitia while CCR2 was found mostly in the media, with weaker expressions in the adventitia and intima, as found in normal arteries. CD68 expression was observed mostly in the thickest areas of the intima. DARC was located mostly in the media, although it could also be found in the adventitia and/or intima of the vessels. D6 was found mostly in the adventitia (Figure 3).

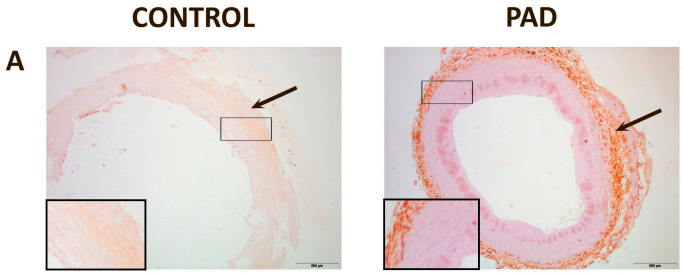

Figure 3. *Cont.*

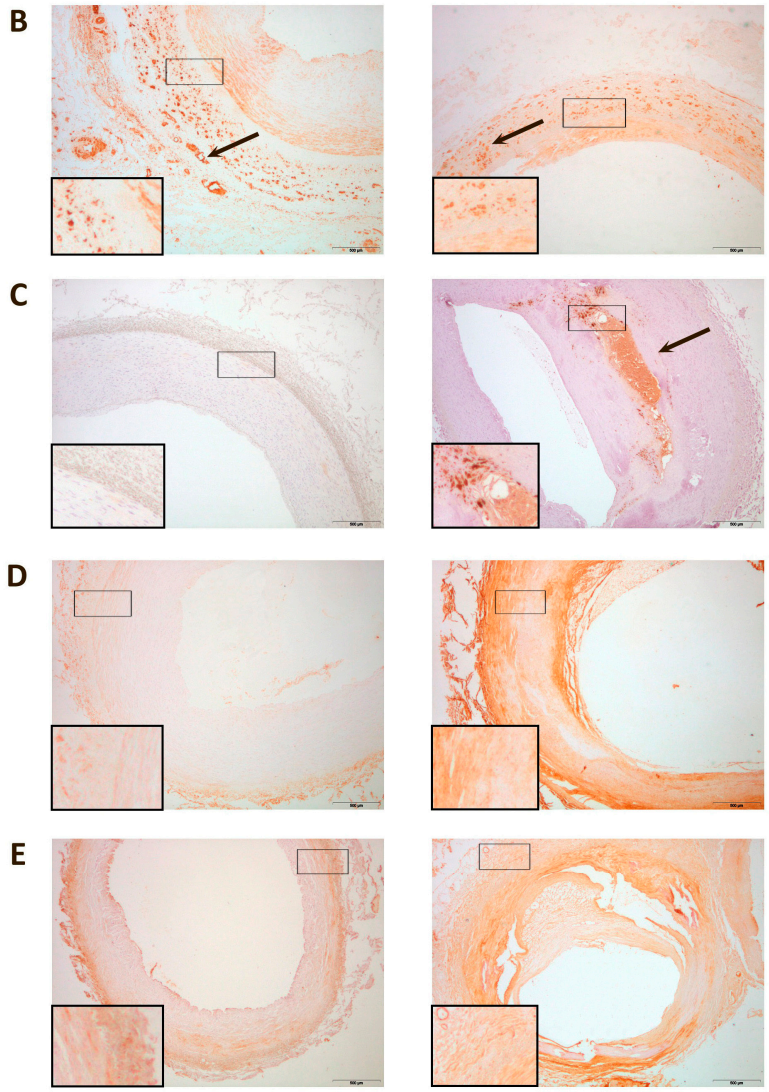

Figure 3. Representative immunohistochemical images for inflammatory markers in peripheral arteries: (**A**) Chemokine (C–C motif) ligand 2 (CCL2) was expressed in the adventitia in normal and affected arteries (arrow); (**B**) C–C chemokine receptor type 2 (CCR2) was expressed, mainly, in the media in normal and affected arteries. However, it can also be found in the intima and in adventitia of the vessels (arrow); (**C**) Cluster of differentiation 68 (CD68) was mildly expressed in control arteries while, in affected arteries, the expression was higher and located, mainly, in the intima (arrow); (**D**) Chemokine-binding protein 2 (D6) expression was found, mainly, in the adventitia; (**E**) Duffy antigen/chemokine receptor (DARC) was found, mainly, in the media, although it was observed as well in the adventitia and/or intima of some vessels. Magnification 20×. The inserts show higher magnification (100×) images of the indicated areas.

3. Discussion

The present study shows (by immunostaining) that paraoxonases, CCL2 and several CCL2 receptors are increased in peripheral arteries with indications of atherosclerosis. This could be a response to increased cellular oxidative stress as well as the migration of monocytes. In PAD patients,

we observed an increased CD68 staining which is a specific marker of macrophages. Macrophage mitochondrial oxidative stress plays a major role in atherosclerosis via mechanisms involving the NF-κB-CCL2 pathway [32]. Paraoxonases prevent oxidative stress by reducing the amount of oxidized LDL in the circulation as well as the vessel wall. This, in turn, reduces monocyte infiltration into the vessel wall and, as such, is anti-inflammatory [33,34]. The protein expression of this enzyme has been observed in many tissues in humans [35] and mice [23]. PON1 reduces macrophage oxidation of LDL as well as macrophage oxidative stress, and increases cholesterol efflux from macrophages to high-density lipoprotein (HDL), thus reducing foam cell formation and, as a consequence, the development or progression of atherosclerosis. Therefore, the increase in PON1 staining found in this study could indicate that a protective response to increased oxidative stress was occurring in the macrophages of the diseased arteries. For example, it is of considerable note that PON1 expression was found surrounding cholesterol deposits in severely diseased arteries, and which strongly supports the hypothesis of a protective role for this enzyme, *i.e.*, that PON1 infiltrates the arterial tissue to combat the deposition of the atherosclerosis-promoting cholesterol. The physiological role of PON3 is still unclear. Results from the present study support previous findings from our group showing increased serum PON3 concentrations in patients with PAD [31]. Studies on cellular expression of this enzyme and the elucidation of its athero-protective role are scarce and inconclusive. PON3 has lactonase but not paraoxonase activity [36,37]. Previous studies reported that PON3 attenuates the oxidation of LDL *in vitro* [38] and that the overexpression of human PON3 decreased atherosclerosis and adiposity in mice [39]. Although the increase in PON3 protein expression in the arteries of PAD patients is quantitatively small, it needs to be taken into account that PON3 is about 100 times more potent per mg of protein than PON1, in protecting LDL against lipid peroxidation [36]. Hence, the increase in the enzyme's expression in these patients could be of clinical relevance.

In the peripheral circulation, decreased PON1 activities are associated with increased concentration of CCL2 [30], and *in vitro* studies found that PON1 inhibits the production of CCL2 induced by oxidative stress in endothelial cells [20]. However, this inverse relationship is not confirmed at tissue level. Indeed, both molecules are ubiquitously expressed in most tissues and are located in close proximity to one another, suggesting some manner of coordinated function [23,40]. Results of the present study, and previous others, show that the expression of both proteins is increased in the arteries of patients with atherosclerosis [40]. This observation would suggest that the variations in PON1 and CCL2 concentrations in plasma do not necessarily correlate with their roles at the cellular level. Perhaps PON1 protein expression is increased in diseased arteries to counteract oxidative stress and CCL2-induced inflammation. However, this hypothesis has to be confirmed by further studies.

CCL2 is likely to have considerable impact on PAD since the biological function of this chemokine is to induce monocyte migration and, as well, because the arteries with moderate atherosclerosis appear to accumulate CCL2 in response to a variety of pro-inflammatory stimuli [24,30,41–44]. Atherosclerosis is an inflammatory disease, and the consensus is that CCL2 is involved in its pathogenesis [45]. In the present study, we found increased CCL2 expression in the arteries of PAD patients, together with an increased expression of two of the CCL2 receptors *i.e.*, D6 and DARC. D6 and DARC belong to the poorly-understood chemokine receptors collectively known as atypical or silent. These are G-protein coupled receptors that do not activate conventional signaling events. Conversely, they may internalize, degrade or transport ligands (*i.e.*, they have the potential to create

clinically relevant chemokine patterns in tissues) [46]. Their levels of expression have not been explored previously in diseased arteries of PAD patients. The availability of CCL2 may be complicated by potential effects induced by differential expression of the specific receptor CCR2 and the presence of these atypical chemokine receptors. We observed that the expression of these receptors was increased in diseased arteries, and that their histological distributions are not uniform. A pathogenic role is likely, and data suggests that atypical chemokine receptors modify chemokine availability in PAD. Although these receptors have no involvement in cell migration, their modulatory effect on inflammatory response is likely.

Previous studies from our group also reported increased PON1 and PON3 expressions in aortas from patients undergoing coronary or aortic artery bypass grafting [40]. This is relevant, because it suggests that, despite the atherosclerosis burden being higher in PAD, changes inside the tissue are similar at a molecular level. The mechanisms underlying the increased PON1 and PON3 immunohistochemical staining in the arteries of PAD patients cannot be ascertained from the present investigation, but these patients had oxidative stress, as indicated by the elevated serum 8-isoprostanes concentration. Oxidative stress stimulates PPARγ and NF-κB-related pathways [47], and these molecules have been reported to stimulate the expression of paraoxonases [48,49]. However, this increase is in an apparent contradiction with the decrease in the serum levels of the enzymes, and a possible explanation could be an increase in PPARδ expression and decreased PON1 proteolysis. This is the case in a rat model of liver fibrosis that our group published a few years ago [50]. Rats with carbon tetrachloride-induced liver fibrosis had oxidative stress and increased PPARδ gene expression. These alterations were associated to an inhibition of the HDL synthesis and, consequently, a decreased PON1 secretion to the extracellular medium. In addition, the hepatic levels of the protease cathepsin B were decreased, leading to an inhibition of protein degradation. Thus, hepatic PON1 levels were elevated as a consequence of the combination of a decreased HDL secretion, and to an inhibition of lysosomal protein degradation. To ascertain if the same phenomena occur in the arteries of PAD patients requires further studies, but the strong decrease in HDL-cholesterol concentrations observed in our patients is in agreement with this hypothesis.

A caveat of the present study is that we could not analyze PON2 in the arteries of PAD patients. This enzyme plays an important role in the intracellular protection against oxidative stress [14], and new investigations focused in PON2 and PAD should be further pursued.

4. Experimental Section

4.1. Study Population

Patients with clinically diagnosed PAD were recruited from the outpatient clinics of Hospital Universitari Joan XXIII. Diagnosis was with standard clinical assessments including measurement of the ankle-brachial index (ABI), non-invasive imaging, and angiography when indicated. Symptoms of chronic ischemia were detected using the Fontaine classification, the standardized physician-administered questionnaire that seeks to identify the presence of calf discomfort on exertion, such as walking uphill or walking rapidly [51]. Exclusion criteria from our study were the presence of acute ischemia, signs of infection, renal failure, liver disease, cancer, or autoimmune disease. Portions of femoral and/or

popliteal arteries from patients were obtained during surgical procedures for infra-inguinal limb revascularization (n = 66). All patients were at Stages III and IV of the Fontaine classification. Eight normal arteries obtained from accident victims and stored at the Blood and Tissue Bank of Catalonia (Banc de Sang i Teixits, www.bancsang.net/es/donants/donacio_teixits.html, Barcelona, Spain) were used as controls. All tissues (patients and controls) were kept at −80 °C until thawed for processing. After thawing, the tissues were rinsed in phosphate buffer to remove residual blood and placed in at least 10 volumes of buffered formalin using a standard protocol for embedding tissue in paraffin wax for subsequent histology slide preparation. Three sections per slide were used for histological and immunohistochemical analyses. A peripheral blood sample was also obtained from each patient (and control individual) at the time of the surgery for biochemical and hematological measurements. The hospital's Ethics Committee (Institutional Review Board) approved the procedures of the study protocol on 31 July 2014, and written informed consent was obtained from the participants prior to entry into the study (OBESPAD 14-07-31/7proj3).

4.2. Biochemical Analyses

Serum concentrations of glucose, cholesterol, HDL cholesterol, triglycerides, fibrinogen, C-reactive protein, total proteins, and complete blood cell counts were performed by standard methods in the Hospital Universitari Joan XXIII. LDL cholesterol concentrations were estimated using the Friedewald formula. Serum concentrations of PON1 and PON3, and EDTA-plasma concentrations of CCL2 were determined by ELISA as previously reported [30,31]. Serum concentrations of 8-isoprostanes were analyzed by Enzyme Immunoassay (Cayman Chemical Co., Ann Arbor, MI, USA). Serum PON1 lactonase activity was analyzed by measuring the hydrolysis of 5-thiobutyl butyrolactone [27]. Inter-assay coefficients of variation were as follows: Glucose, 1.8%; cholesterol, 1.5%; HDL cholesterol, 2.0%; triglycerides, 2.2%; fibrinogen, 7.5%; C-reactive protein, 4.8%; total proteins, 1.3%; LDL cholesterol, 3.5%; PON1, 10.5%; PON3, 12.2%; CCL2, 7.3%; 8-isoprostanes, 10.2%; lactonase, 11.5% (n = 20 for each variable).

4.3. Histological and Immunohistochemical Analyses

Sections, of 4-µm thickness, were stained with hematoxylin-eosin for arterial histology. Masson's trichrome stain (Masson's Trichrome Goldner with light green, Bio Optica, Milano, Italy) was used to assess the structure and extent of fibrosis. Alizarin Red staining (Sigma-Aldrich, Steinheim, Germany) was used to identify the sites of micro-crystalline, or non-crystalline, calcium phosphate salts. The intima and media thicknesses were measured in all histological sections as an estimate of the extent of atherosclerosis. Antibodies against PON1 and PON3 were raised in rabbits using peptides derived from specific sequences of mature PONs, as previously reported [52–54]. PON1 and PON3 antibodies were used at a dilution of 1/50 and 1/300, respectively. A previous study already demonstrated that these antibodies were highly specific for PON1 and PON3 [54]. Commercial primary antibodies were purchased: CCL2 (dilution 1/200), CCR2 (dilution 1/100), and D6 (dilution 1/500) from Abcam plc (Cambridge, UK); antibodies against DARC (dilution 1/200) from Abnova (Taipei, Taiwan); and antibodies against CD68 from Dako (Glostrup, Denmark). The appropriate biotinylated secondary antibodies (anti-rabbit, anti-mouse or anti-goat; purchased from Vector Laboratories Inc., Burlingame,

CA, USA) were used at a dilution of 1:200. Detection was performed with the ABC peroxidase system (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine (DAB) peroxidase substrate (Dako). The times of the detection reactions were 4 min for PON1 and PON3, 1 min for DARC, 1.5 min for CCR2 and D6, 10 min for CCL2, and 5 min for CD68. All immunohistochemical sections were counterstained with Mayer's hematoxylin. Negative control samples were processed identically to the test samples except that the primary antibodies were omitted from the incubation. Representative immunohistochemical images of negative controls in control arteries and arteries from patients with PAD are shown in Figures S3 and S4. The positively-stained area was quantified automatically for each antibody using an image analysis system (AnalySIS®, Soft Image System GmbH, Olympus Corp., Munster, Germany), and expressed as percentage of the total area. Initially the colors of the images that have been stained to the molecule of interest were defined. Once these colors were defined, they were automatically detected in all samples. The software analyzed the stained area in relation to the total image area, which is termed phase analysis. The rationale for this method is described in more detail in the Supplementary Methods, and is also available on the Internet [55]. This is a semi-quantitative analysis that measures areas and not intensities. This method is commonly accepted and has been employed previously in several immunohistochemical studies by our group and other authors [23,28,40,54,56–59]. Inter-assay coefficients of variation were as follows: PON1, 9.6%; PON3, 7.3%; CCL2, 4.5%; CCR2, 5.3%; D6, 6.4%; DARC, 7.1% (n = 20 for each variable).

4.4. Statistical Analyses

Significance of difference between groups was assessed by the Mann–Whitney *U*-test. Results are expressed as medians and IQR (Interquartile Range). All statistical analyses were performed with the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc., IBM Corp., Chicago, IL, USA).

5. Conclusions

In conclusion, PON1 and PON3, CCL2 together with the D6 and DARC receptors are increased in the arteries of patients with PAD. The findings suggest that these molecules may be involved in the development and progression of atherosclerosis in peripheral artery disease.

Supplementary Materials

Supplementary materials can be found at http://www.mdpi.com/1422-0067/16/05/11323/s1.

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Author Contributions

Julio Sepúlveda, and Vicente Martín-Paredero were responsible for patient management and clinical data collection; Anna Hernández-Aguilera and Esther Rodríguez-Gallego took responsibility for the

biological samples and prepared the database; Maria Guirro, Anabel García-Heredia, Noemí Cabré, Fedra Luciano-Mateo, and Isabel Fort-Gallifa performed the biochemical and histological analyses; Anna Hernández-Aguilera, Jorge Joven and Jordi Camps performed the statistical analyses and interpreted the results; Jordi Camps wrote the manuscript, which was subsequently discussed and modified by the rest of the team. All the authors have read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Information

Table S1. Differences in selected variables between control individuals and PAD patients, excluding smokers from the PAD group.

Parameter	Control (*n* = 8)	PAD (*n* = 50)	*p*-Value
IMT (mm)	1.00 (0.70–1.298)	1.35 (0.99–1.83)	0.133
I/M ratio	0.16 (0.13–0.65)	2.14 (1.42–3.22)	<**0.001**
% PON1 staining	1.70 (1.54–3.72)	11.17 (6.16–15.37)	<**0.001**
% PON3 staining	0.55 (0.22–0.73)	3.34 (2.05–4.55)	<**0.001**
% CCL2 staining	2.26 (0.36–3.65)	30.75 (9.63–43.86)	<**0.001**
% CCR2 staining	18.29 (7.02–27.56)	20.42 (12.79–30.50)	0.650
% CD68 staining	1.10 (0.65–2.88)	4.84 (1.43–9.23)	**0.027**
% D6 staining	0.83 (0.22–12.9)	41.91 (27.85–56.15)	<**0.001**
% DARC staining	3.29 (2.01–5.06)	30.62 (17.79–46.76)	<**0.001**

IMT: Intima-Media thickness. Results are shown as medians (IQR). Staining for CCL2, CCR2, CD68, DARC, D6, PON1 and PON3 were measured as the area of positive staining and expressed as percentage of the total area examined using the image analysis system (see text for details). The bold numbers highlight the statistically significant differences.

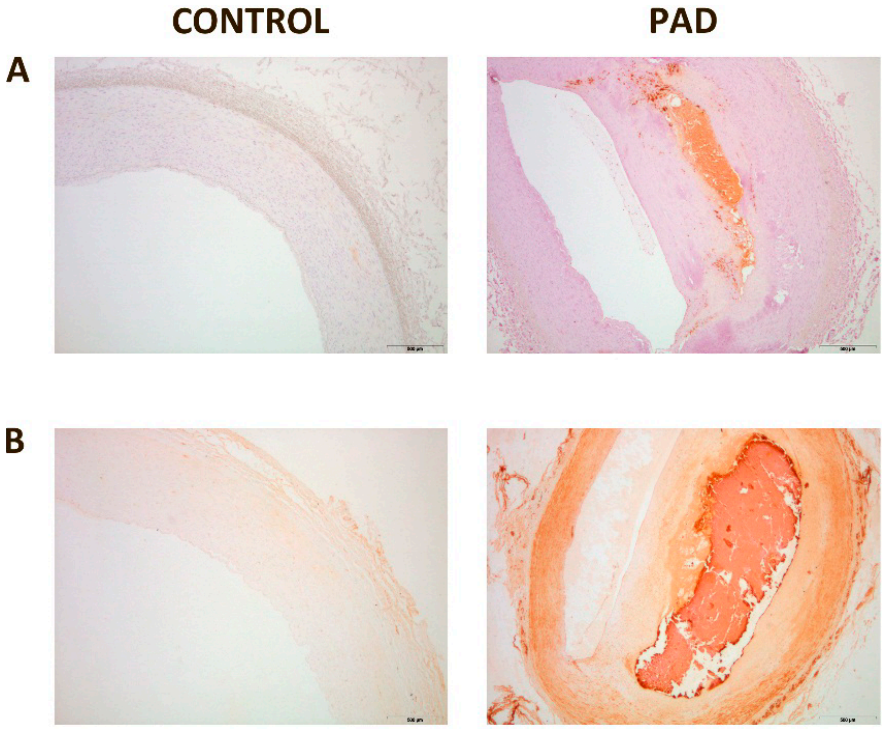

Figure S1. Representative immunohistochemical images of serial sections for CD68 and PON1 staining of peripheral arteries: (**A**) CD68; (**B**) PON1. Both stainings are observed at the lesion site. Magnification 20×.

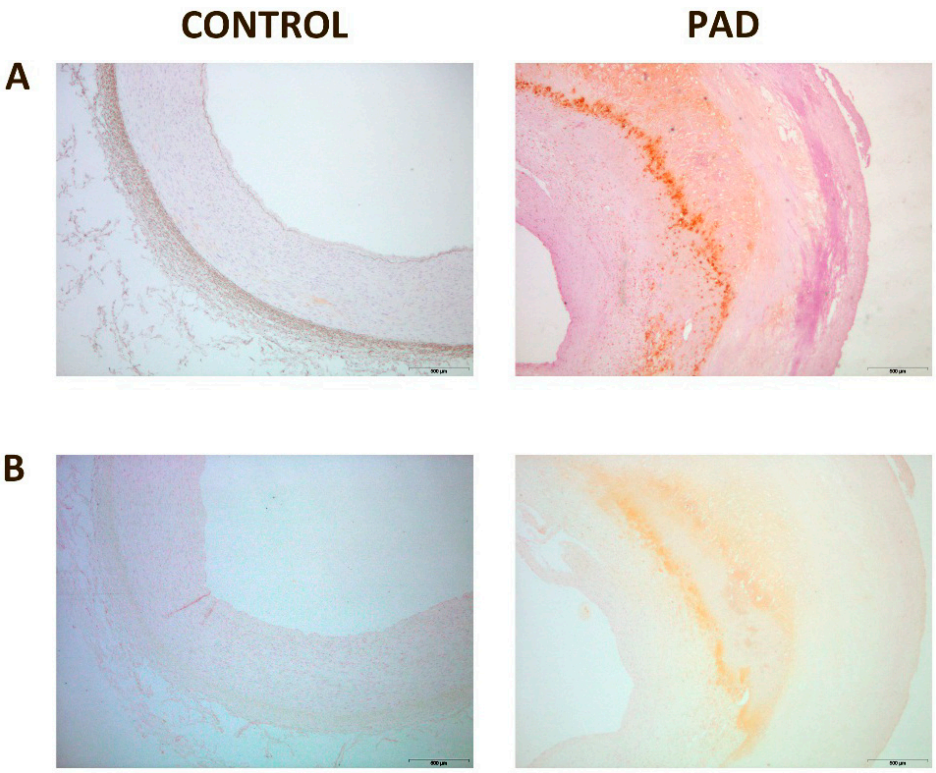

Figure S2. Representative immunohistochemical images of sections from the same tissue block for CD68 and CCL2 staining of peripheral arteries: (**A**) CD68; (**B**) CCL2. Magnification 20×.

Figure S3. Representative immunohistochemical images of negative controls in control arteries and arteries from patients with peripheral artery disease: (**A**) PON1; (**B**) PON3.

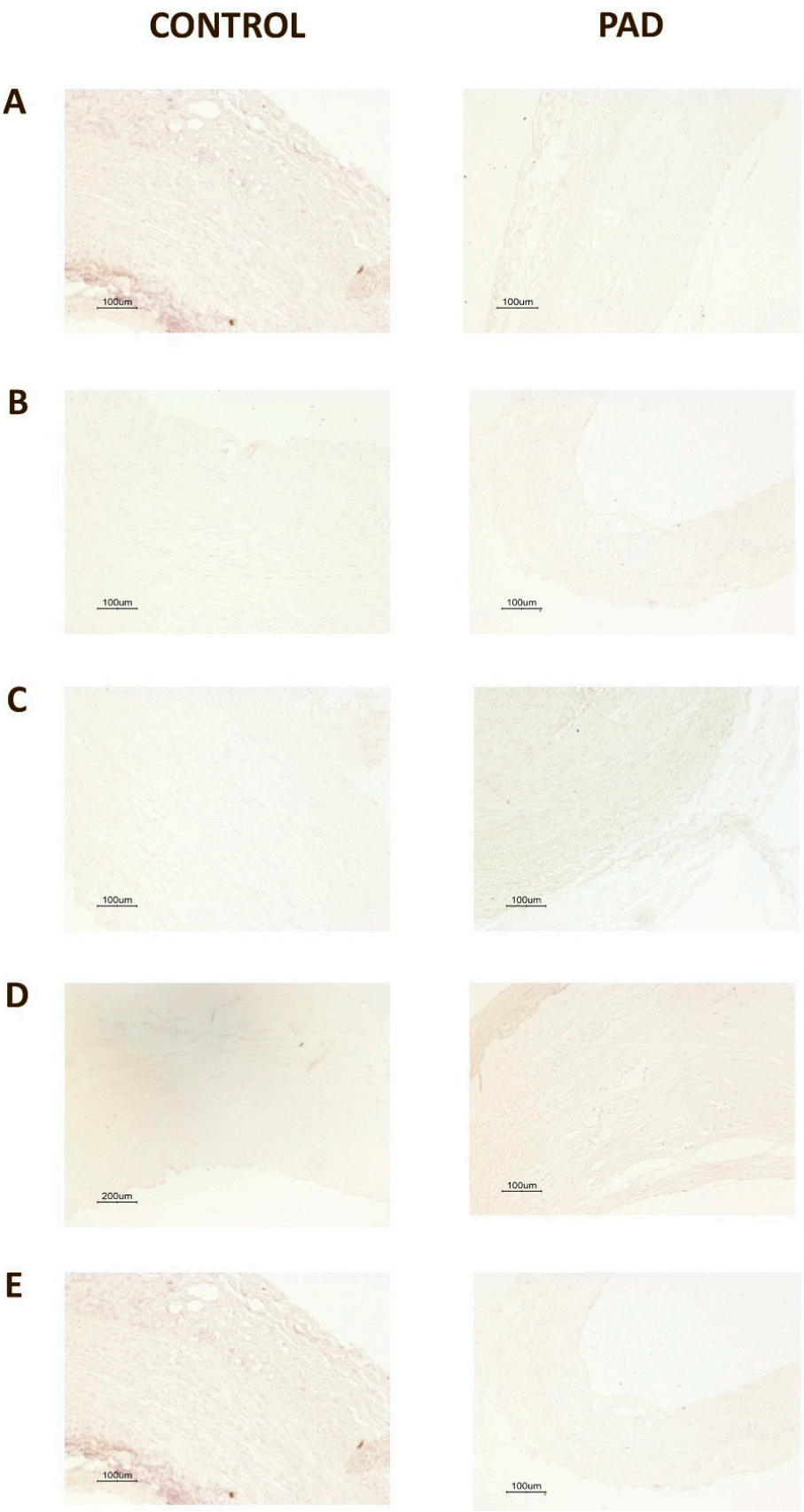

Figure S4. Representative immunohistochemical images of negative controls in control arteries and arteries from patients with peripheral artery disease: (**A**) CCL2; (**B**) CCR2; (**C**) CD68; (**D**) D6; (**E**) DARC.

Supplementary Methods: Phase Analysis with the AnalySIS® System

Objects in true-color images to be displayed in false color are defined with the Set color threshold command. Low and up threshold for the three basic color parameters (red, green and blue) are set and assigned a phase. Thresholds can also be interactively set in the image. To do this, a set of pixels is selected. For each phase, a false color for the respective object is selected. Areas of color ranges not assigned to a phase will not be calculated. This command generates an 8-bit false-color image in the destination image buffer. All gray-value areas assigned to a phase will be colored according to that phase. A measurement sheet is generated containing the absolute areas of the gray-value phases, as well as the percentage area of each phase relative to either the total image area or the area within the active frame. Sheet column headers contain phase name and lower and upper thresholds. The column header's color corresponds to its respective phase. Phase analysis of various gray-value ranges enables to determine the percentage surface area of a particular material on a background. Surface area can be calculated in true-color images using selected color ranges. Summarized from the AnalySIS® User's Guide, freely available at: ftp://ftp.ccmr.cornell.edu/utility/FEI%20temp/AnalySIS%20docs/Getting%20Started.pdf).

Identification of candidate biomarkers of disease activity in peripheral artery disease by targeting the extracellular matrix

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Background and objective: The prevalence of peripheral artery disease (PAD) is high (20-25%) in the population older than 65 years, and patients frequently do not present for treatment until the disease is advanced. Circulating markers of disease activity might provide patients with a key opportunity to be treated. The established role of matrix metalloproteinases (MMPs) in vascular remodeling and their association with atherosclerosis progression is the basis on which to explore the feasibility of detecting blood-specific peptides generated during the degradation of the extracellular matrix (ECM).

Methods: A combined histological and non-targeted proteomic approach using liquid chromatography and tandem mass spectrometry was used to assess the protein profile in arterial specimens from patients undergoing elective surgery. We then selected a panel of neo-epitopes, likely indicating ECM turnover, and measured them by enzyme-linked immunosorbent assays in serum samples from a cohort of 195 PAD patients who were in a stable state and exhibited different disease activity.

Results: Histological and proteomic analyses confirmed the structural disorganization of affected arteries. Several proteins (14 out of 81) were identified as differentially expressed in diseased arteries; most of them were related to ECM-components and the difference in expression was likely due to an imbalance in vascular remodeling. Multivariate analyses suggest that severe lesions in PAD patients may have a specific proteome. Targeting selected neo-epitope fragments in the serum revealed that some but not all fragments had potential value in the clinical management of PAD. Notably, the detection of neo-epitopes from fragments of MMP-mediated degradation of versican and collagen type IV segregated patients with mild/moderate PAD (intermittent claudication, Fontaine I-II) from those with severe PAD (critical limb ischemia, Fontaine III-IV).

Conclusion: We propose novel non-invasive candidate biomarkers that may be clinically useful across the PAD spectrum.

Keywords: Atherosclerosis; Biomarker; Collagen; Extracellular matrix; Laminin; Neo-epitopes; Versican.

INTRODUCTION

Systemic atherosclerosis is a progressive, age-related disease underlying the most common causes of death. The prevalence of peripheral artery disease (PAD) is high, with over 200 million afflicted patients in industrialized countries, and is further increasing due to the convergent epidemics of diabetes and obesity.[1, 2] Lesions in the arteries of the lower extremities represent a clinical spectrum encompassing asymptomatic and underdiagnosed illnesses as well as symptomatic disorders in which the initial manifestations are either intermittent claudication (IC) or critical limb ischemia (CLI).[3, 4] The challenge is to establish non-invasive biomarkers for predicting patients likely to progress to CLI and for improving success in offering preventive medical management.

Changes in lumen caliber are major determinants of the course of PAD symptoms, and wall remodeling in peripheral arteries of a certain size and length seems to be a crucial process to understand the reaction of old and damaged tissue to atherosclerotic injuries.[5] Mechanistic knowledge is incomplete, but the major role of the extracellular matrix (ECM) in providing a mechanical scaffold and support to cell migration is undisputed.[6] The dynamic regulation of the ECM is governed by the balance between synthesis and degradation of ECM components, which is context-specific and involves the correct functioning of cytokines, enzymes such as matrix metalloproteinases (MMPs) and growth factors.[7-9] We now know that atherosclerosis-associated remodeling is the complex response to inflammatory cells, lipid deposition and mechanic or shear-dependent stimuli, which are responsible for changes in ECM composition and for disrupted cytoskeletal architecture.[10-14] Here we

provide an insight into the proteome composition and the relative expression of ECM components in severely affected peripheral arteries and we test the hypothesis that a directed choice of neo-epitopes may provide clinically useful non-invasive biomarkers in PAD patients.

MATERIALS AND METHODS

Participants and study design. The local Ethics Committee and Institutional Review Board approved the procedures involved in this study (Epinols/12-03-09/3proj6). Written informed consent was obtained from all participants before inclusion. Patients (n=195) were consecutively enrolled men selected from among those attending our Department of Vascular Surgery and with an established diagnosis of PAD according to Fontaine classification[15]. Patients with infected lesions, evidence of neoplastic disease, chronic kidney disease, liver disease or inflammatory disease (or receiving anti-inflammatory drugs) were not included. Ankle-brachial index (ABI) was measured per standard technique in both lower limbs and non-invasive imaging techniques or arteriographies were performed according to the standard of care. Serum was collected at the time of inclusion and stored at -80ºC until analyses. Patients were followed up every 3 months for 1 year and there was no mortality during this period. No patient was included postoperatively or lost to follow-up but some patients needed infrainguinal limb revascularization (n=18). These patients were invited to participate in a case-control study (Inflamet/15-04-30/4proj6) that required donating portions of diseased artery for proteomic analyses that were compared with healthy artery samples obtained from road accident victims of a similar age range (66-

70 years). New informed consent was obtained from either the participants or a next of kin.

Histological examination. To examine tissue morphological features, serial sections of tissue were obtained from samples fixed in 10% neutral-buffered formalin and embedded in paraffin. Hematoxylin and eosin staining (Sigma-Aldrich, Steinheim, Germany) was used to identify different cellular structures. Masson's trichrome staining (Bio Optica, Milano, Italy) was used to assess collagen fibers, smooth muscle cells, nucleus and cytoplasm and Sirius red staining (Direct Red 80, Sigma-Aldrich, Steinheim, Germany) was used to identify collagen fibers. Images were obtained at x200 magnification and the Intima/Media ratio (IMR) was obtained by dividing the thickness of the intima by the thickness of the media measured using an optical microscope (Nikon, Eclipse E600, Madrid, Spain) equipped with image analysis.

Proteomics. We have previously used these methods to analyze the protein secretion profile of carotid atherosclerotic plaques[16]; ancillary methods and specific details may be found in supplementary material and methods, S1. Briefly, sample arteries were cut into pieces and homogenized in the presence of type 1 collagenase (Sigma-Aldrich, Steinheim, Germany). Following different rounds of centrifugation and chemical treatment, precipitated proteins were vacuum-dried and dissolved in 0.5 M triethylammonium bicarbonate, pH= 7.2, to be sequentially denatured, reduced and alkylated. For digestion, samples were incubated with sequencing-grade trypsin overnight at 37 °C. We used a mass spectrometry approach for quantification by performing isobaric tag for relative and absolute quantitation (iTRAQ) labeling using iTRAQ 8-plex reagent kits (SCIEX,

Madrid, Spain), as previously described.[17] Labeled peptides were then purified using a SCX column (Strata® SCX 55um, 70Å, Phenomenex), desalted and concentrated through a C18 Sep-Pak column (Waters, Bedford, MA, USA) and analyzed by using a C-18 reversed phase nano-column coupled to a trap nano-column for real time ionization and peptide fragmentation on a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA). To identify proteins, information was obtained from tandem mass spectra with the aid of Proteome Discoverer, version 1.4.0.288, from Thermo Fisher Scientific. All MS and MS/MS samples were analyzed using Mascot (Thermo Fisher Scientific; version 2.4.1.0). Protein quantification was performed by comparing the peak intensity of the reporter ions in the MS/MS spectra to that of the selected peptides to assess the relative abundance of the peptides. Normalized concentrations of selected proteins were used to assess the increased or decreased expression of proteins in PAD arteries.

Enzyme-linked immunosorbent assays (ELISA). Methods involved in monoclonal antibody development and technical evaluation of the assays were essentially similar to those recently described.[18] Specific details may be found in supplementary material and methods, S2. The list of selected neo-epitopes from the MMP-degraded proteins is shown in Table S1, and also in the supplementary information and the references therein. This selection was made after confirming that these neo-epitopes were consistent with proteomic data and pathway analysis according to the ConsensusPathDB-human platform.[19]

Statistical analysis. The Kolmogorov-Smirnov test was used to assess

normal distribution of the variables. We used the Mann-Whitney U test to compare non-parametric variables, Student's t-test for parametric variables and contingency tables and the chi-square test for categorical variables. For multiple comparisons, the Kruskal-Wallis test or Analyses of Variance (one-way ANOVA) was used. The results were expressed as median and interquartile range or percentage of the total participants. For proteomic analyses, principal component analysis (PCA) and hierarchical clustering analysis were performed using the Mass Profiler Professional software v.12.1 (Agilent Technologies). Only proteins that appeared in more than 70% of the samples were considered, and the PANTHER system (www.pantherdb.org) was used for functional classification. We used the Benjamini−Hochberg method to avoid false positives in differences due to multiple testing. Analyses with receiving operating characteristics (ROC) curves and binary logistic regression were performed using the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc., IBM Corp, Chicago, IL, USA). MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) was used to generate scores/loading plots, heatmaps and random forest analysis.

RESULTS

The clinical characteristics and laboratory measurements (Table I) suggest that the cohort of patients used for this study is representative of the clinical spectrum of PAD patients seeking attention in our facilities. The high prevalence of cardiovascular risk factors and associated treatment, including the fact that most were smokers, did not significantly affect severity of disease, with the possible exception of a lower prevalence of diabetes in

Fontaine I patients. However, age was a significant factor in establishing disease severity and a major consideration in further analyses.

In a case-control study combining histology and proteomics, we first evaluated the differences in the integrity of arterial tissue and signs of vascular remodeling, in severely lesioned and normal arteries (Figure I). Atherosclerosis was evident in all samples from the PAD patients. The *tunica* intima was disorganized and thicker, and the presence of lipid vacuoles and cholesterol crystals and other histologic features (Figure 1A) were consistent with the higher ($p<0.0001$) intima/media ratio observed in PAD patients (2.10 [1.33-3.22]) with respect to that of similarly aged donors of healthy arteries (0.16 [0.13-0.65]). Furthermore, smooth muscle cells normally located in the media were also present in the intima of atherosclerotic arteries (Figure 1B) and the distribution of collagen fibers was disrupted (Figure 1C).

Our untargeted proteomics approach identified and quantified 81 proteins present in both control and diseased arteries and in more than 70% of the samples (Table S2). However, once filtered, corrected and normalized, we identified a unique subset of proteins (n=14) with statistically significant differences between diseased and healthy arteries and therefore with the potential to represent specific biomarkers (Figure 2 A). Putative functions of these proteins in atherosclerosis, according to the literature, are listed in Table S2. Notably, most of these proteins were ECM or cytoskeletal components (Figure 2B) suggesting that vascular remodeling provides a specific target that might be used to explore progression of atherosclerosis. Hierarchical clustering analyses and principal component analysis strongly suggest that severe disease in PAD patients may have a specific proteome,

as illustrated in Figure 2C-E. Normalized concentrations identified some proteins that were either underexpressed or overexpressed in atherosclerotic arteries indicating the delicate balance between production and degradation or removal of proteins in ECM turnover. Nevertheless, we assumed an imbalance favoring degradation of ECM proteins to select candidate neo-epitopes (Table S1) to be measured in the serum of PAD patients with validated ELISA tests.

Median and IQR values (Table II) indicated that measurements of specific fragments of MMP-8- and MMP-12-mediated degradation of versican (VCANM), MMP-9-mediated degradation of alpha 5 chain of laminin (Lam-a5) and MMP-mediated degradation of type IV collagen (C4M) had discriminative value in the clinical presentation of PAD patients. This was further confirmed by using random forest analyses, but Lam-a5 levels failed to discriminate patients with IC from those with CLI and were not considered in further analyses. Serum VCANM concentration decreased progressively, was correlated with clinical severity, and the analysis of ROC curves displayed a high sensitivity and specificity to distinguish between Types I and IV patients (Figure 3A, B). A similar discriminative value was obtained for C4M concentrations, but this variable increased according to disease severity (Figure 3C, D). The combination of both potential biomarkers provided specificity higher than 90% to discriminate between patients with mild IC and those with CLI (Figure 3E).

DISCUSSION

Atherosclerosis is a systemic disease. Despite identical pathogenesis, the affected vascular territories define not only clinical relevance but also different responses to injuries. Detecting asymptomatic stages and to predict or monitor disease progression is currently an unmet need for vascular surgeons[20] that might be fulfilled by non-invasive blood biomarkers. In limb arteries, the lumen loss (i.e., progression to ischemia) is not due to neointima formation, as is the case in coronary arteries.[21] As confirmed by our histological assessment, an important contribution of the reparative response to promote vascular remodeling, which includes inflammatory mediators and ECM degradation, is likely.[21-23] Our proteomics data indicate that severe atherosclerotic lesions in peripheral arteries have a specific proteome in which proteins related to tissue modeling and remodeling are underrepresented and those associated with inflammation seem overregulated. Among those overexpressed proteins, alpha-2-macroglobulin and carboxypeptidase B2 largely contribute to the differences observed between diseased and healthy arteries. Alpha-2-macroglobulin has been recently associated with plaque vulnerability in carotid arteries using a similar iTRAQ-based analysis[24] and carboxypeptidase B2 may be a potential indicator of a high risk of premature peripheral artery disease.[25] Conversely, other proteins were significantly decreased in diseased arteries. For example, low levels of lactadherin may indicate advanced atherosclerosis and poor adhesion of smooth muscle cells to elastin fibers.[26] We observed low levels of versican, a major chondroitin sulfate proteoglycan, which is highly influenced by the increased MMP activity in diseased arteries. [27,28] The relative amounts of laminin[29] and mimecan[30]

with crucial roles in cardiovascular function and migration of smooth muscle cells, respectively, were also decreased in diseased arteries. Taken together, these results indicate the coexistence of multiple mechanisms involved in the maintenance of artery function in response to atherosclerotic injury.

The results highlight the central role of connective tissue turnover in the structural and signaling properties of arterial cells in PAD.[14] Our methods included identifying specific cleavage products generated by MMPs or age-related processes of proteins involved in matrix turnover, the production of antibodies that recognize these neo-epitopes but not native proteins and to develop immunoassays searching for biomarkers of disease severity.[31-34] Based on histology and proteomic data, neo-epitopes generated from alpha-smooth muscle actin and laminin alpha-5 showed some potential value acting as surrogates for individual clinical endpoints. Further research may confirm this assumption but we focused our analyses in clinically separating patients with mild to moderate PAD (IC, Fontaine I-II) from severe PAD (CLI, Fontaine III-IV), and we observed that serum measurements of versican (KTFGKMKPRY; VCANM) and type IV collagen (CGG-GTPSVDHGFL; C4M) degradation products returned the best specificity and sensitivity levels. Interestingly, VCANM levels decreased and C4M levels increased according to disease severity, probably confirming that both age and the specific context regulate the activity of different MMPs as previously described.[35, 36] This is important because type IV collagens are a major component of all basement membranes, and versican plays a central role in inflammation.[37, 38] The combination of both indicators might integrate cellular pathways and processes reflecting PAD progression.

Our exploratory research has identified candidate biomarkers of PAD clinical severity, but their evaluation requires more detailed investigation. Our methods measured an objective, quantifiable characteristic successfully that apparently correlates with clinical endpoints. Therefore, these biomarkers may provide clinically relevant information. The next level of evaluation needs other designs to ascertain predictive power in other populations, especially in those with asymptomatic PAD, and to validate efficacy. Laboratory-measured biomarkers used as surrogate endpoints may have the potential to speed drug development in PAD, a prevalent condition in which the use of primary clinical endpoints, such as CLI, in clinical trials can be impractical or even unethical. Exploring and reevaluating the relationship between measurable biological processes and clinical outcomes is also crucial for deepening our knowledge on arterial pathophysiology.

CONCLUSION

Severe lesions in PAD are characterized by a specific proteome that significantly differs from that found in healthy arteries of persons of similar age. This proteome informs that both inflammation and ECM turnover (i.e., vascular remodeling) are quantitatively the most important processes in diseased arteries. Subsequent studies indicate that remodeling of arterial tissue releases protein fragments into the blood, where they may be detected. We propose versican and type IV collagen degradation products as laboratory-measured biomarkers of disease activity in peripheral artery disease.

Author contributions

Conceptualization: VMP, JJ.
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Formal analysis: AHA, MF, CB, SHN
Methodology: MF, SFA, AHA, SHN.
Project administration: JJ, VMP.
Resources: VMP, MAK, FG, JC, JJ
Supervision: JC, SFA, FG, MAK.
Validation: SFA, JC, FG.
Visualization: AHA, MF, CB.
Writing – original draft: JJ, AHA.
Writing – review & editing: AHA, SHN, CB, SFA, VMP, MF, MAK, FG, JC, JJ.

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Conflicts of Interest

Signe Holm Nielsen, Morten Karsdal and Federica Genovese are full-time employees at Nordic Bioscience, and Morten Karsdal holds stock in Nordic Bioscience. All other authors have no conflicts of interest.

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Table I. Clinical characteristics, complete blood count and biochemical characteristics of PAD patients segregated by Fontaine classification.

	Fontaine I N=11	**Fontaine II N= 41**	**Fontaine III N=34**	**Fontaine IV N=109**	**p-value**
Clinical characteristics					
Age (years)	55 (50 – 69)	70 (59.25-75)	63 (55-69.25)	71 (64-77)	<0.001
BMI (kg/m^2)	28.9 (23.05-31.16)	27.3 (23-29.4)	25.5 (22.25-27.9)	24 (22-27.8)	ns
Diabetes (%)	10	69.4	45.5	79.8	<0.001
Hypertension, (%)	50	63.2	57.6	75	ns
Dyslipidaemia, (%)	55.6	41.7	24.2	36.7	ns
Complete blood count					
Red blood cells, x10^{12}/L	5.11 (4.41-5.4)	4.48 (3.95-4.79)	4.29 (3.74-4.53)	4.00 (3.34-4.59)	<0.001
Hemoglobin, g/dL	14.6 (13.23-16.35)	13.1 (11.5-15.2)	13.57 (12.02-14.07)	11.5 (10.5-13.5)	0.02
Leukocytes, x10^9/L	7.44 (6.85-10.23)	7.51 (6.3-9.42)	7.61 (6.39-9.56)	8.35 (6.4-10.1)	ns
Platelets, x10$^{9/L}$	217.25 (186-243.5)	219 (183-268)	252 (200.5-333.65)	270 (209.5-343)	0.011
Biochemical variables					
Total-cholesterol, mmol/L	4.06 (2.84-5.65)	4.04 (3.72-4.74)	3.95 (3.37-4.47)	3.77 (3.1-4.51)	ns
HDL-cholesterol, mmol/L	0.8 (0.72-1.14)	1.1 (0.86-1.29)	1.1 (0.87-1.26)	0.92 (0.74-1.14)	ns
LDL-cholesterol, mmol/L	2.04 (1.4-3.32)	2.41 (1.94-3.4)	2.2 (1.73-2.81)	2.18 (1.72-2.83)	ns
Triglycerides, mmol/L	1.56 (1.18-4.53)	1.51 (1.14-2.56)	2.35 (1.87-3.47)	1.97 (1.37-2.86)	ns
Glucose, mmol/L	6.69 (4.1-7.64)	5.93 (4.96-8.82)	5.59 (4.62-7.49)	5.77 (4.59-7.6)	ns
ALT, U/L	19 (12.14-35)	21 (16-26)	22 (16-40)	21 (13-32)	ns
Gamma-GT, U/L	27.5 (16.94-39.8)	28 (18-47)	24 (17.25-43)	31.5 (17-48.8)	ns
AST, U/L	21 (12-27)	19 (16-22)	20 (14.75-31)	19 (15-30)	ns
Fibrinogen, g/L	4.07 (3.6-5.48)	4.84 (4.12-6.43)	5.39 (4.27-6.11)	5.82 (4.44-7.78)	ns

BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ALT: Alanine Aminotransferase; AST: Aspartate aminotransferase. Non-parametric variables are shown as median and IQR (25-75%). Qualitative variables are expressed as (%) of total participants. Multiple comparisons between groups using Kruskal-Wallis test.

Table II. Differences in selected neo-epitopes between PAD patients segregated by Fontaine classification.

	Fontaine I N = 11	**Fontaine II N = 41**	**Fontaine III N = 34**	**Fontaine IV N= 109**	**p-value**
VCANM	1800 (1640 – 1900)	1610 (1375 – 1830)	1530 (1055 – 1810)	1250 (1080 – 1560)	<0.001
C4M	16530 (13720 -21710)	21480 (16860 – 30120)	24790 (18095 – 31940)	31730 (22415 – 45165)	<0.001
Lam-a5	5610 (4630 – 8490)	6660 (4855 – 9810)	6130 (3928 – 8278)	8710 (6755 – 11960)	<0.001
CRPM	7620 (5690 – 11520)	9380 (6780 – 14100)	8645 (6615 – 11590)	9970 (7775 – 12430)	ns
α-SMA	3870 (2830 – 4900)	3620 (2770 – 5570)	3355 (2283 – 4513)	3600 (2430 – 5100)	ns
MIM	7600 (3600 – 20370)	7430 (3770 – 12090)	6770 (2795 – 13635)	8070 (3885 – 13290)	ns

Results are expressed in pg/mL and as median (IQR range) for non-parametric variables. VCANM: Specific fragment of MMP-8 and -12-mediated degradation of versican; C4M: MMP-mediated type IV (alpha 1) collagen degradation; Lam-a5: Specific fragment of MMP-9 mediated degradation of alpha 5 chain of laminin; CRPM: Specific fragment of MMP-1, -3, -8, -9, CatS/K, ADAMTS1-mediated degradation of C-reactive protein; α-SMA: Alpha-smooth muscle actin, acetylated N-terminal; MIM: Specific fragment of MMP-9 and -12-mediated degradation of mimecan. Multiple comparisons between groups using Kruskal-Wallis test.

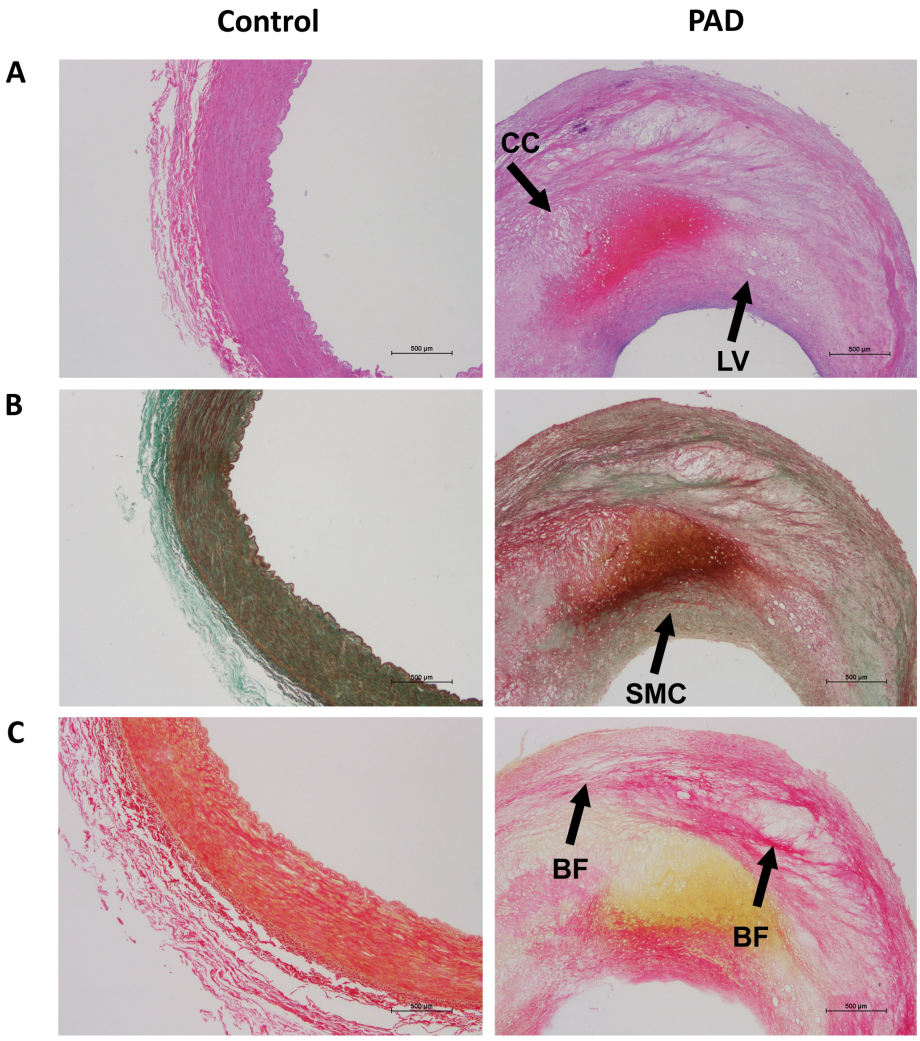
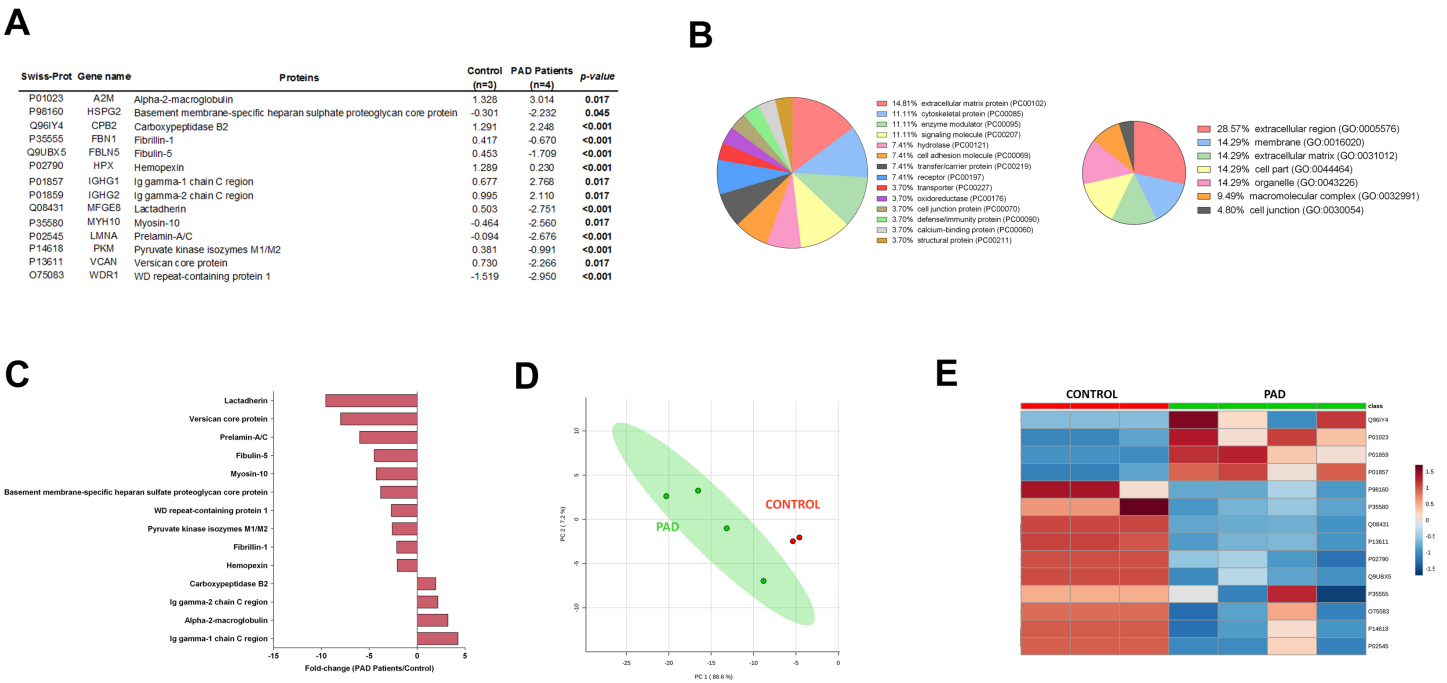


Figure 1

Representative micrographies of peripheral arteries from control group and PAD patients (x20). Hematoxylin & Eosin (A), Masson's Trichrome staining (B), Sirius Red staining (C) were performed in arteries from both groups. CC: Cholesterol crystals; LV: Lipid vacuoles; SMC: Smooth muscle cells; BF: Broken fibers of collagen.



Swiss-Prot	Gene name	Proteins	Control (n=3)	PAD Patients (n=4)	*p-value*
P01023	A2M	Alpha-2-macroglobulin	1.328	3.014	**0.017**
P98160	HSPG2	Basement membrane-specific heparan sulphate proteoglycan core protein	-0.301	-2.232	**0.045**
Q96IY4	CPB2	Carboxypeptidase B2	1.291	2.248	**<0.001**
P35555	FBN1	Fibrillin-1	0.417	-0.670	**<0.001**
Q9UBX5	FBLN5	Fibulin-5	0.453	-1.709	**<0.001**
P02790	HPX	Hemopexin	1.289	0.230	**<0.001**
P01857	IGHG1	Ig gamma-1 chain C region	0.677	2.768	**0.017**
P01859	IGHG2	Ig gamma-2 chain C region	0.995	2.110	**0.017**
Q08431	MFGE8	Lactadherin	0.503	-2.751	**<0.001**
P35580	MYH10	Myosin-10	-0.464	-2.560	**0.017**
P02545	LMNA	Prelamin-A/C	-0.094	-2.676	**<0.001**
P14618	PKM	Pyruvate kinase isozymes M1/M2	0.381	-0.991	**<0.001**
P13611	VCAN	Versican core protein	0.730	-2.266	**0.017**
O75083	WDR1	WD repeat-containing protein 1	-1.519	-2.950	**<0.001**

Figure 2

(A) Proteins showing statistically significant differences between control group and PAD patients. (B) Protein class (Left) and cellular component (Right) percentage of selected proteins obtained by PANTHER system. (C) Representation of the fold-changes obtained using normalized concentrations of selected proteins in both groups. D) Principal Component Analysis and E) Heatmap diagram of proteomics results. Q96IY4: Carboxypeptidase B2; P01023: Alpha-2-macroglobulin; P01859: Ig gamma-2 chain C region; P01857: Ig gamma-1 chain C region; P98160: Basement membrane-specific heparan sulfate proteoglycan core protein; P35580: Myosin-10; Q08431: Lactadherin; P13611: Versican core protein; P02790: Hemopexin; Q9UBX5: Fibulin-5; P35555: Fibrillin-1; O75083: WD repeat-containing protein 1; P14618: Pyruvate kinase isozymes M1/M2; P02545: Prelamin-A/C.

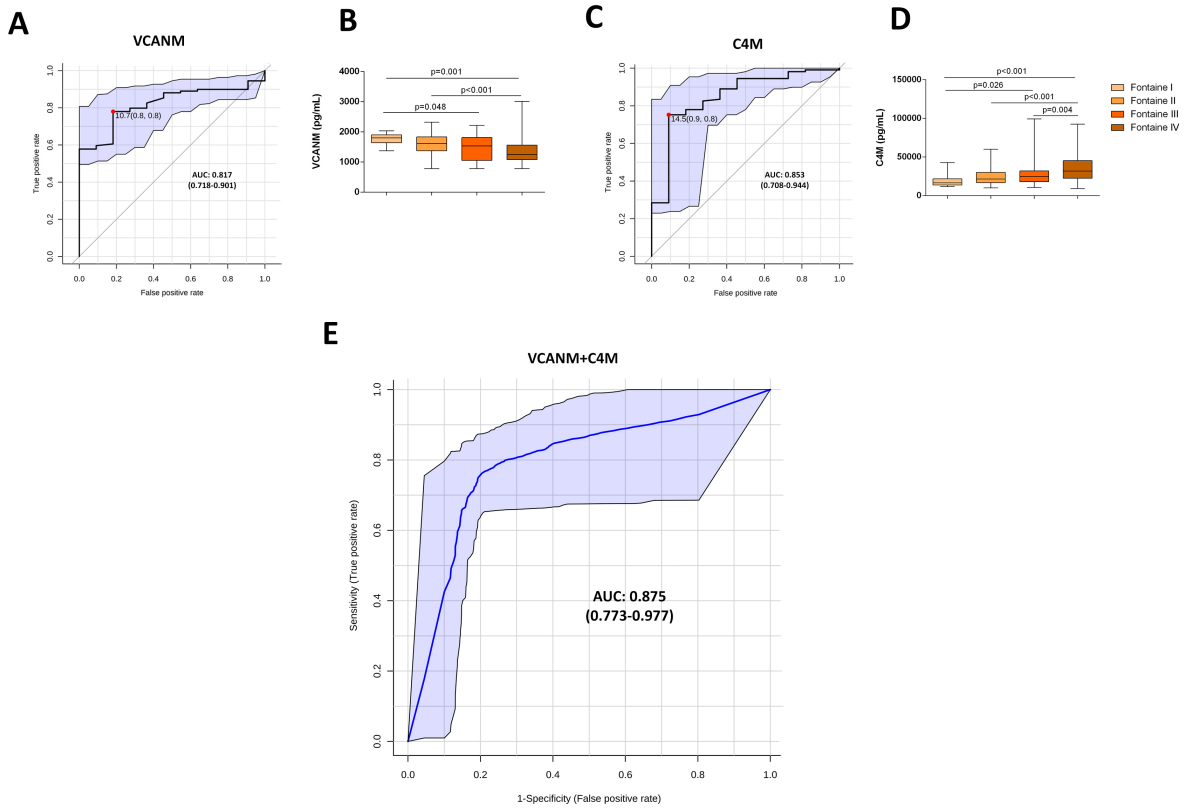


Figure 3

Candidate biomarkers for disease activity. (A) ROC curve for VCANM measurements between Fontaine I and Fontaine IV patients. (B) Graphical representation of VCANM concentrations among Fontaine grades. (C) ROC curve for C4M measurements between Fontaine I and Fontaine IV patients. (D) Graphical representation of C4M concentrations among Fontaine grades. (E) ROC curve for the combination of VCANM and C4M obtained by binary logistic regression between Fontaine I and Fontaine IV patients.

Supplementary material corresponding to the manuscript:

Identification of candidate biomarkers of disease activity in peripheral artery disease by targeting the extracellular matrix

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Supplementary material and methods S1: Proteomics protocol

Tissue processing and protein preparation. Stored frozen pieces of arteries were cut in small pieces and placed into tubes with 8mg type 1 collagenase in 2mL of Tris-$CaCl_2$ buffer. Samples were incubated at 37ºC for 30 minutes with shaking. They were then centrifuged at 5000rpm at 4ºC, supernatants were stored at -80ºC and pellets were resuspended in 1.5 mL of urea lysis buffer + 0.1% SDS. Samples were homogenized using *Precellys 24* (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000rpm for 10 seconds. Immediately, they were sonicated and then centrifuged again at 2100 rpm for 10 minutes at 4ºC. Pellets were discarded and supernatants were transferred into new tubes, centrifuged at 14000 rpm for 1 hour at 4ºC and proteins precipitated with trichloroacetic acid. Samples were placed at 4ºC for 24 h and then centrifuged at 5000 rpm for 10 minutes at 4ºC. Supernatants were rejected and pellets resuspended in 1 mL of cold acetone. Samples were again centrifuged at 5000 rpm for 10 minutes at 4ºC, supernatants were rejected and pellets were resuspended in 0.5M triethylammonium bicarbonate pH=8.5 (TEAB). Protein quantification was performed and samples were stored at -80ºC. **Protein digestion and validation.** After being vacuum-dried, samples were

resuspended in 0.5M TEAB pH=7 and 2% sodium dodecyl sulfate (SDS) was added to denature proteins. Samples were reduced using 5mM tris(2-carboxyethyl)phosphine (TCEP) in 50mM TEAB pH=7.9 for 1h at 60ºC and then alkylated with 3.65mM iodoacetamide during 30min at room temperature in the dark. For the digestion, samples were incubated with 1µg/µl trypsin sequencing-grade in 500 mM TEAB at pH=7.9 overnight at 37ºC. Digestions were checked by analyzing a small aliquot using MALDI-TOF MS or nano-LC. Peptides were separated onto a C-18 reversed phase nano-column (75um I.D.; 15cm length; 3um particle diameter, Nikkyo Technos Co. LTD, Japan) coupled to a trap nano-column (100 um I.D.; 2cm length; 5um particle diameter, Thermo Fisher Scientific, San Jose, CA, USA). Digested samples were analyzed by injecting 18uL sample, using a continuous acetonitrile gradient of 0-35% in 13min, 35-80% in 7min and 80-100% in 5min. In all the analysis, a flow rate of 300nL/min was used to elute peptides for real time ionization and peptide fragmentation on a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution=30,000 FHMW) followed by data dependent MS/MS scan (R=15,000 FHMW) from most intense ten parent ions with a charge state rejection of one were analyzed along the chromatographic run. The MS/MS scan was acquired in the FT analyzer using a HCD collision cell with a normalized collision energy of 45 and dynamic exclusion of 0.5min.

iTRAQ labeling. iTRAQ-8plex labeling reagents were added to samples according to manufacturer's instructions and incubated at room temperature for 2 hours. Quantification results are expressed as ratios of the different labeling tags versus a control tag, and these ratios were used for statistical purposes.

Labeled samples were purified using a SCX column (Strata® SCX 55um, 70Å, Phenomenex). Then, they were desalted and concentrated through C18 Sep-Pak column (Waters, Bedford, MA, USA). Eluted peptides were dried and resuspended in 0.1% (v/v) formic acid for nanoLC-MS/MS detection. Labeled iTRAQ peptides were separated onto a C-18 reversed phase (RP) nano-column (75um I.D.; 15cm length; 3um particle diameter, Nikkyo Technos Co. LTD, Japan) coupled to a trap nano-column (100um I.D.; 2cm length; 5um particle diameter, Thermo Fisher Scientific, San José, CA, USA). All samples were analyzed by triplicate. For each analysis, 2 µg of sample was injected using a continuous acetonitrile gradient consisting of 0−
5% B in 4 min, 5−15% B in 60 min, 15−35% B in 60 min, and 35−95% B in 10 min, which was maintained for 20 min (A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid). In all the analysis a flow rate of 300nl/min was used to elute peptides for real time ionization and peptide fragmentation on an LTQ-ObritrapVelosPro mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution = 30,000 FHMW) followed by data dependent MS/MS scan (R=15,000 FHMW) from most intense parent ions was analyzed throughout the chromatographic run. The MS/MS scan was acquired in the FT analyzer using an HCD collision cell with normalized collision energy of 45%, a precursor mass window selection of 2 m/z, a charge state rejection of +1, and a dynamic exclusion of 0.5 min. **Protein identification analysis.** Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific). All MS and MS/MS samples were analyzed using Mascot (Thermo Fisher Scientific; version 2.4.1.0). Mascot was set up to search SwissProt_2012_03.fasta database (535248 entries),

restricting for human taxonomy (26944 sequences) and assuming trypsin digestion. Two missed cleavages were allowed and an error of 0.02 Da for fragment ion mass and 10.0 ppm for a parent ion were allowed. Oxidation of methionine, acetylation of N-termini and iTRAQ 8-plex were specified as variable modifications, whereas carbamidomethylation of cysteine was set as static modification. The false discovery rate (FDR) and protein probabilities were calculated by Target Decoy PSM Validator working between 0.01 and 0.05 for Strict and relaxed respectively. For proteins identified with only one peptide, visual verification of fragmentation spectra was done. **Quantitative proteome analysis.** In tandem MS mode, which isolates and fragments peptides, each tag generates a unique reporter ion used for a relative quantification. Protein quantification compares the peak intensity of the reporter ions in the MS/MS spectra to assess the relative abundance of the peptides and the proteins they are derived from. The quantification method allows normalization using filters to measure the abundance of proteins in the sample using unique peptides of each protein.

Supplementary material and methods S2: ELISA protocol.

The ELISAs were technically validated according to the Nordic Bioscience standard operating procedures. Linearity, lower detection limit (LDL), intra- and inter-variation, spiking recovery and assay stability were assessed. Protocols and buffers differ among assays. Generally, a 96-well streptavidin pre-coated plate was coated with the selected biotinylated synthetic peptide dissolved in specific buffer and incubated. The peptide calibrator or sample was added to appropriate wells, followed by the HRP-conjugated mAb, and again incubated. Finally, tetramethyl benzidine (TMB) developer (Kem-En-Tec cat.438OH, Taastrup, Denmark) was added, and the plate was incubated in the dark. All the above incubation steps included shaking. After each incubation step the plates were washed in washing buffer. TMB reaction was stopped by adding stopping solution and measured at an indicated wavelength. A standard calibration curve was also plotted.

Table S1: Overview of measured biomarkers to assess ECM degradation in serum.

Biomarker	Measurement	Peptide	Reference	Upper normal level in general population (pg/mL)
VCANM	Specific fragment of MMP-8 and -12-mediated degradation of Versican	KTFGKMKPRY	[1]	1500
CRPM	Specific fragment of MMP-1, -3, -8, -9, CatS/K ADAMTS-mediated degradation of C-reactive protein	KAFVFPKESDK	[2]	7500
C4M	MMP-mediated type IV (alpha 1) collagen degradation in plasma	CGG-GTPSVDHGFL	[3]	21500
Lam-a5	Specific fragment of MMP-9 mediated degradation od alpha 5 chain of laminin	DLELADAYYL	Unpublished	10166
a-SMA	Acetylated N-terminal fragment of alpha-smooth muscle actin	EEEDSTALV	[4]	1480
MIM	Specific fragment of MMP-9 and -12-mediated degradation of mimecan	EDIEDGTF-SKL	[5]	5050

[1] Barascuk N, Genovese F, Larsen L, Byrjalsen I, Zheng Q, Sun S *et al*. A MMP derived versican neo-epitope is elevated in plasma from patients with atherosclerotic heart disease. *Int J Clin Exp Med* 2013;6:174-84. [2] Skjøt-Arkil H, Schett G, Zhang C, Larsen DV, Wang Y, Zheng Q *et al*. Investigation of two novel biochemical markers of inflammation, matrix metalloproteinase and cathepsin generated fragments of C-reactive protein, in patients with ankylosing spondylitis. *Clin Exp Rheumatol* 2012;30:371-9. [3] Sand JM, Larsen L, Hogaboam C, Martinez F, Han M, Røssel Larsen M *et al*. MMP mediated degradation of type IV collagen alpha 1 and alpha 3 chains reflects basement membrane remodeling in experimental and clinical fibrosis--validation of two novel biomarker assays. *PLoS One* 2013;8:e84934. [4] Papasotiriou M, Genovese F, Klinkhammer BM, Kunter U, Nielsen SH, Karsdal MA *et al*. Serum and urine markers of collagen degradation reflect renal fibrosis in experimental kidney diseases. *Nephrol Dial Transplant* 2015;30:1112-21. [5] Barascuk N, Vassiliadis E, Zheng Q, Wang Y, Wang W, Larsen L *et al*. Levels of Circulating MMCN-151, a Degradation Product of Mimecan, Reflect Pathological Extracellular Matrix Remodeling in Apolipoprotein E Knockout Mice. *Biomark Insights* 2011;6:97-106.

Table S2: Proteins identified in control and PAD human arteries by untargeted proteomics (in alphabetic order).

Swiss-prot ID	Compound Name	Biological function
P60709	Actin, cytoplasmic 1	ATP binding
P63267	Actin, gamma-enteric smooth muscle	ATP binding
P01009	Alpha-1-antitrypsin	Glycoprotein, protease and binding
P01023	Alpha-2-macroglobulin	Enzyme, growth factor and protease binding.
O43707	Alpha-actinin-4	Involved in vesicular trafficking via its association with the CART complex
P04114	Apolipoprotein B-100	Cholesterol transporter activity
P02649	Apolipoprotein E	Mediates the binding, internalization, and catabolism of lipoprotein particles
P25705	ATP synthase subunit alpha, mitochondrial	Transmembrane transporter activity
P02730	Band 3 anion transport protein	Transporter mediates electroneutral anion exchange across the cell membrane
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	Metal ion and protein C-terminus binding
P51911	Calponin-1	Regulation of smooth muscle contraction
P00915	Carbonic anhydrase 1	Arylesterase activity
Q96IY4	Carboxypeptidase B2	Metallocarboxypeptidase activity
P07339	Cathepsin D	Aspartic-type endopeptidase activity
P00450	Ceruloplasmin	Chaperone and copper ion binding
P10909	Clusterin	Chaperone, misfolded protein and ubiquitin protein ligase binding
P00488	Coagulation factor XIII A chain	Metal ion binding.
P12109	Collagen alpha-1(VI) chain	Platelet-derived growth factor binding
Q99715	Collagen alpha-1(XII) chain	Extracellular matrix structural constituent conferring tensile strength
Q05707	Collagen alpha-1(XIV) chain	Extracellular matrix structural constituent
P08123	Collagen alpha-2(I) chain	Extracellular matrix structural constituent
P12110	Collagen alpha-2(VI) chain	Collagen VI acts as a cell-binding protein.
P12111	Collagen alpha-3(VI) chain	Serine-type endopeptidase inhibitor activity
P01024	Complement C3	C5L2 anaphylatoxin chemotactic receptor, cofactor, endopeptidase inhibitor and lipid binding

P07360	Complement component C8 gamma chain	Retinol binding
P02748	Complement component C9	Constituent of the membrane attack complex (MAC)
P00403	Cytochrome c oxidase subunit 2	Cytochrome-c oxidase activity
P60981	Destrin	Actin polymerization or depolymerization
P35555	Fibrillin-1	Extracellular matrix constituent conferring elasticity
P02671	Fibrinogen alpha chain	Metal ion binding
P02675	Fibrinogen beta chain	Chaperone binding
P02679	Fibrinogen gamma chain	Cell adhesion molecule binding
P02751	Fibronectin	Heparin, integrin, mercury ion, protease and collagen binding
Q9UBX5	Fibulin-5	Calcium, integrin, protein C-terminus binding.
P21333	Filamin-A	Promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins
P06396	Gelsolin	Actin, calcium, miosin II and protein domain specific binding
P04406	Glyceraldehyde-3-phosphate dehydrogenase	Microtubule binding
P00738	Haptoglobin	Hemoglobin binding
P69905	Hemoglobin subunit alpha	Oxygen transporter activity
P68871	Hemoglobin subunit beta	Oxygen transporter activity
P02790	Hemopexin	Metal ion binding
O60814	Histone H2B type 1-K	DNA binding
P01876	Ig alpha-1 chain C region	Antigen binding
P01857	Ig gamma-1 chain C region	Antigen binding
P01859	Ig gamma-2 chain C region	Antigen binding
P01765	Ig heavy chain V-III region TIL	Antigen binding
P01834	Ig kappa chain C region	Antigen binding
P01617	Ig kappa chain V-II region TEW	Antigen binding
P01619	Ig kappa chain V-III region B6	Antigen binding
P04433	Ig kappa chain V-III region VG (Fragment)	Antigen binding
P01717	Ig lambda chain V-IV region Hil	Antigen binding

P0CG04	Ig lambda-1 chain C regions	Antigen binding
A0M8Q6	Ig lambda-7 chain C region	Antigen binding
P01871	Ig mu chain C region	Antigen binding
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	Serine-type endopeptidase inhibitor and endopeptidase inhibitor activity
Q08431	Lactadherin	Phosphatidylethanolamine and phosphatidylserine binding
O15230	Laminin subunit alpha-5	Integrin binding
P11047	Laminin subunit gamma-1	Extracellular matrix structural constituent
Q16853	Membrane primary amine oxidase	Cell adhesion protein
P35580	Myosin-10	Actin filament binding
P35749	Myosin-11	DNA binding
P59665	Neutrophil defensin 1	Has antimicrobial activity against Gram-negative and Gram-positive bacteria
Q15063	Periostin	Metal, cell adhesion molecule and heparin binding
P32119	Peroxiredoxin-2	Anotioxidant and thioredoxin peroxidase activity
P00747	Plasminogen	Apolipoprotein binding
P02545	Prelamin-A/C	Structural molecule activity
Q9NQH7	Probable Xaa-Pro aminopeptidase 3	Aminopeptidase and metallopeptidase activity
P02760	Protein AMBP	Inhibits calcium oxalate crystallization.
P14618	Pyruvate kinase isozymes M1/M2	Glycolytic enzyme
Q13228	Selenium-binding protein 1	Selenium binding
P02787	Serotransferrin	Transport of iron from sites of absorption and heme degradation to those of storage and utilization
P02743	Serum amyloid P-component	Calcium ion, virion, unfolded protein and carbohydrate binding
P24821	Tenascin	Syndecan binding
P07996	Thrombospondin-1	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions
P29401	Transketolase	Cofactor and metal binding
P68366	Tubulin alpha-4A chain	Structural constituent of cytoskeleton
O00159	Unconventional myosin-Ic	ATP binding
P13611	Versican core protein	Calcium, carbohydrate, glycosaminoglycan and

		hyaluronic acid binding
P08670	Vimentin	Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria
P04004	Vitronectin	Vitronectin interact with glycosaminoglycans and proteoglycans
O75083	WD repeat-containing protein 1	Induces disassembly of actin filaments in conjunction with ADF/cofilin family proteins

Plasma metabolome in PAD patients unveils limitations derived from comorbidities in unhealthy aging

Running head: Metabolomics, peripheral artery disease and associated pathologies.

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ABSTRACT

Systemic atherosclerosis affecting lower extremities, also called peripheral artery disease (PAD) is a common disease affecting 20-25% of old population. An early diagnostic is still not possible because symptoms become evident in advanced stages. Inflammation, impaired metabolism and mitochondrial dysfunction may predispose to the disease, which normally is associated to other pathologies (type-2 diabetes, dyslipidemia or hypertension). By using a targeted metabolomics approach, we measured metabolite concentration in atherosclerotic arteries and plasma of PAD patients segregated by Fontaine classification and in plasma of healthy volunteers. Our results show that many of measured metabolites, specially branched chain amino acids, were associated not with the disease but with other comorbidities, age or body mass index. After removal, six potential candidates were considered. Among them, (iso)citrate and glutamate were the metabolites with the best discriminant capacity between control group and PAD patients. Moreover, both were also useful for an early detection of the disease, discriminating between control group and Fontaine I-II patients. The obtained metabolic fingerprint in PAD patients can be used as a source of novel biomarkers of diagnosis and progression.

Keywords: Peripheral artery disease; metabolomics; biomarkers; comorbidies; isocitrate; glutamate

1. INTRODUCTION

Peripheral artery disease (PAD) of the lower extremities is a serious global health problem with an increasing prevalence among atherosclerotic diseases and affecting 20-25% of population over 60 years[1]. There is a wide spectrum between signs of PAD classified in four stages attending the symptoms using the Fontaine scheme: from a non-symptomatic stage (Fontaine I), intermittent claudication (Fontaine II) to rest pain (Fontaine III) and tissue damage and necrosis (Fontaine IV).

Hypertension, hypercholesterolemia, diabetes and smoking are the principal risk factors to develop PAD. Undesirable lifestyle can leads to a pro-inflammatory situation inducing complications at the crossroads of metabolic stress and immunity[2,3]. Moreover, imbalance in energy metabolism, by which nutrients are transformed into ATP, can predispose to obesity, type-2 diabetes (T2D) and atherosclerosis[3-5]. Alterations in metabolic pathways like tricarboxylic acid (TCA) cycle may induce the production of reactive oxygen species (ROS) and oxygen deficiency (ischemia)[6,7]. Impaired bioenergetics in

affected lower extremities can be probably due to abnormal mitochondria in ischemic skeletal muscles[8,9].

One of the main challenge that specialists and researches face is to improve the diagnose of PAD even in the asymptomatic stages[10,11]. Many plasma, serum and total blood biomarkers have been proposed and associated to a high cardiovascular risk, although none of them has been established[12]. The emerging metabolomics approaches are an essential tool to improve the interpretations of atherosclerotic pathologies[13–15]. These techniques are mainly focused on the quantification of metabolites to better understand the disease and propose new therapeutic strategies[14,16,17]. In this sense, metabolites involved in TCA cycle have been proposed as cardiovascular biomarkers[8,9,18,19].

In the present work we assume that the combination of inflammation, disrupted metabolism and mitochondrial dysfunction may predispose to atherosclerosis[20–22]. The obtaining metabolic profile in atherosclerotic patients is a useful tool to discover new biomarkers and therapeutic targets and, for the first time, we propose potential metabolic circulating markers of initial stages of peripheral artery disease by using a targeted metabolomics approach.

2. MATERIALS AND METHODS

2.1 Participants and study design

This observational, cross-sectional study implicated 201 men with clinically diagnosed peripheral artery disease attending Vascular Surgery Service at *Hospital Universitari Joan XXIII* between 2010 and 2015. Patients were classified according Fontaine classification[23] from grade I to IV and obtained plasma and serum samples were stored at -80 ºC until use. Artery samples were obtained during surgical procedures for infrainguinal revascularization and stored at -80ºC.

Inclusion criteria were men, older than 18 and with a confirmed diagnose of peripheral artery disease. Diagnostic criteria involved ankle-brachial index (ABI), non-invasive imaging techniques (computerized tomography scan or magnetic resonance imaging) and arteriography when indicated. The exclusion criteria were presence of acute ischemia, signs of infection, renal failure, liver disease, cancer or autoimmune disease. Clinical data and laboratory variables were obtained from patients' clinical records. Local Ethics Committee of the Hospital approved the study (epinols/12-03-09/3proj6, inflamet/15-04-30/4proj6). Written informed consent was obtained from the participants prior to entry the study.

For comparisons, we used bio-banked samples (n=48) from healthy, age-matched, men, whose details have been previously described[24].

2.2. Metabolomics analysis

To detect and quantify metabolites of energy metabolism, we followed the method developed by Riera-Borrull *et al*[7]. Briefly, 25 mg of tissue were homogenized in 1 mL of methanol:water (8:2, v/v) using a *Precellys 24 system* (Bertin Technologies, Montigny-le-Bretonneux, France) working at 5000rpm for 10 seconds three times. The homogenate was then centrifuged at 14000 rpm 10 min at 4ºC and supernatant was collected. Lipids were removed following Folch protocol, by using 9 mL of chloroform[25]. Samples were again centrifuged at 14000 rpm for 10min at 4ºC; the aqueous phase was collected and dried under N_2 flow. Metabolites from plasma (100 μL) were extracted using 400 μL of methanol/water (8:2, v/v) and proteins were precipitated for two hours at -20 ºC. After centrifugation at 14000 rpm for 10 minutes at 4 ºC, the supernatant was collected and dried under N_2 flow. Metabolites were then derivatized with methoxyamine in pyridine (40 mg/mL) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide and injected into a gas chromatograph coupled to a quadrupole time-of-flight mass spectrometer by an electron impact source. Metabolites were detected and quantified attending the standard calibration curves.

2.3. Statistical analysis

Statistical analyses were performed with SPSS 22.0 (IBM Corp, Chicago, IL, USA). MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) was used to generate scores/loading plots and random forest analyses.

3. RESULTS

3.1. Participants' characteristics

Clinical characteristics and biochemical variables of control group and PAD patients are shown in Table 1. We chose an age-matched control group with healthy volunteers without any cardiovascular disease and PAD patients, who had a higher BMI than control group ($p=0.021$). The incidence of atherosclerosis-related impairments (T2D, hypertension and dyslipidemia) was only present in PAD patients ($p<0.001$ in all cases). Consequently, cholesterol, triglycerides and glucose concentrations were altered in those patients.

3.2. Significant alterations in energy metabolism

We measured the concentration of energy metabolism intermediaries in plasma of both, control group and PAD patients. As shown in Figure 1A, most of analyzed metabolites were significantly increased in PAD patients, excluding fumarate, lactate and succinate, which were decreased in patients.

When displayed in a graphical pathway (Figure 1B), we observed that glutaminolysis was disrupted, as glutamate and glutamine were increased in PAD patients. Moreover, reactions involving amino acid catabolism seemed to be slowed down, as serine, valine, isoleucine and leucine concentrations were higher compared to control group. Tricarboxylic acid cycle was disturbed in 2 ways: some metabolites were higher in PAD patients ((iso)citrate, aconitate, α-ketoglutarate, succinyl-CoA and malate and others were diminished in PAD patients (fumarate and succinate).

3.3. Metabolites are linked to comorbidities, age and BMI

Changes in those metabolites could be used for disease diagnose, but associated comorbidities may act as confounding factors in almost 80% of PAD patients as they presented some metabolic disturbances (hyperlipidemia, hypertension or T2D). For this reason, we segregated PAD patients according these disturbances to investigate whether metabolites were different among metabolically healthy or unhealthy patients.

Univariant analyses confirmed that many metabolites were associated to T2D, hypertension or dyslipidemia (Table 2) while multivariate analyses (principal component analyses) revealed that the combination of those metabolites were not able to separate groups regarding hypertension (Figure 2A) and dyslipidemia (Figure 2B). We discovered that glucose and isoleucine were associated to T2D in PAD patients and glucose had the highest discriminant capacity (Figure 2C). Hyperlipidemic and normolipidemic patients showed differences in alanine, aspartate, glucose, isoleucine, lactate, leucine, succinyl-CoA and valine concentrations, and among them, isoleucine had the higher discriminant capacity (Figure 2D). Fumarate, glucose, isoleucine, lactate, malate, serine and pyruvate were associated to hypertension in PAD patient and serine was the metabolite with the best discriminant capacity (Figure 2E). All of these metabolites were discarded for being a possible PAD biomarker.

Age and body mass index (BMI) were other two confounding variables. Subsequently, we analyzed whether any of the metabolites correlated with age or BMI between PAD patients. Aconitate, fumarate and malate were associated to age, and aconitate, alanine, aspartate, glucose, isoleucine, leucine and valine correlated with BMI (Table 3, Supplementary figure 2).

3.4. Metabolic biomarkers of PAD

PAD patients were segregated according Fontaine classification to perform a more accurate analysis (Table 4). Remaining metabolites significantly different between control group and PAD patients were considered possible

biomarkers of PAD: 3-hydroxybutyrate, α-ketoglutarate, glutamate, glutamine, (iso)citrate and succinate (Figure 3). Moreover, glutamate and (iso)citrate concentrations were statistically different between PAD I-II, III and IV groups (Figure 3C and 3E).

To evaluate discriminant capacity, we perform random forest analyses and found that (iso)citrate and glutamate were the most powerful metabolites to separate control individuals from PAD group (Figure 4A). ROC curve for these metabolites showed good area under the curve (AUC) values for both metabolites (Figure 4B). When testing discriminant capacity between control group and the early manifestation of PAD (Intermittent claudication, PAD I-II), (iso)citrate and glutamate were again the best discriminant metabolites (Figure 4C) and ROC curve confirmed this potential (Figure 4D). To follow disease progression in PAD patients, (iso)citrate and glutamate were also the best indicators (Figure 4E).

4. DISCUSSION

Dietary changes and lifestyle can modify our metabolome, and thus metabolomics gives feedback about the status of individuals and offers the opportunity to study pathologies and propose new interventions[5,13,26]. The metabolomic characterization of atherosclerotic peripheral artery disease is gaining interest, as its incidence has increased worldwide[1,27].

However, biological and technical limitations are present, and tissue and plasma metabolome does not provide the same information. In our case, and as expected, we were not able to quantify phosphate metabolites in plasma due to the impermeability of the cellular membrane to these compounds (Supplementary Figure 1).

Here, we found alterations in energy metabolism in PAD patients, compared to control group, especially impairments in the connection citrate-aconitate-isocitrate. The mitochondrial enzymes involved in those reactions are isocitrate dehydrogenase (IDH2) and aconitase 2 (ACO2). IDH2 has been related with a proper mitochondrial function, and mice lacking IDH2 exhibited mitochondrial dysfunction[28]. Moreover, 7-ketocholesterol is known to contribute to atherosclerosis progression by decreasing IDH2 expression and increasing oxidative stress thus modifying mitochondrial function[29]. Furthermore, an oxidative environment (mainly superoxides) can inactivate aconitase, which in turn undergo age-dependent oxidative modification[4]. Whether IDH2 and ACO2 may be the cause or consequence of the well-described mitochondrial dysfunction in PAD is still unknown[8].

Preventive treatment could slow down the progression or even stop the disease. For this reason, a fast diagnostic is necessary. However, many of the current biomarkers are based on risk factors associated to co-morbidities like dyslipidemia, hypertension or T2D[10,12]. In fact, non-communicable diseases are mostly multi-factorial and, in our population, we found that approximately 80% of these patients had any of these impairments, which could be affecting metabolites concentration. Among those metabolites, branched-chain amino acids (BCAAs) were influenced by hypertension, T2D and dyslipidemia. Our results ratify the relationship between an impairment in branched chain amino acid (BCAA) catabolism and obesity and insulin resistance[30,31]. Moreover, increased serum concentration of BCAAs have been also associated to metabolic dyslipidemia[32] and BCAA supplementation during maternal food restriction has been related to a less hypertension incidence in adult offspring[33].

After discarding the influenced metabolites, six candidates remained with statistically significant differences in concentration between control group and PAD patients: 3-hydroxybutyrate, α-ketoglutarate, glutamate, glutamine, (iso)citrate and succinate. Those candidates were useful to distinguish between PAD patients and control group but also to discriminate between PAD grades. Isocitrate is an intermediate in the TCA cycle. Its conversion to α-ketoglutarate is mediated by IDH2. It was found that higher concentrations of this

metabolite are associated with a worse cardiovascular prognostic[34]. PAD patients showed higher concentrations of isocitrate, but PAD I patients showed higher levels than PAD IV patients. Glutamate, another metabolite with a good discriminant capacity, is the substrate for many enzymes located in the mitochondria[35]. Glutamate plays an important role in heart metabolism, as during ischemia, it improves the mechanical function of the ischemic myocardium[36]. Maybe this increased concentration of glutamate in PAD patients could be an attempt to improve biomechanical functions of the ischemic portions of the arteries. However, further research is needed to understand glutamate overproduction in blood of atherosclerotic patients.

To diagnose PAD in the asymptomatic or early symptomatic stages (PAD in Fontaine I and II; intermittent claudication) and to find a clinical biomarker for these stages is of great interest. In our case, (iso)citrate and glutamate were able to distinguish control group from PAD patients in stages I-II. The implementation of (iso)citrate and glutamate measurements in clinical practice (*bench-to-bed* approach) would allow an early detection of the disease and would permit vascular specialists apply better treatments to delay or even stop the disease.

Our study provides evidences that metabolic fingerprints can be used to differentiate PAD patients from control population. We propose two potential biomarkers for the disease – (iso)citrate and glutamate – that can be used for an early diagnosis. Although our results are potentially translational and limited to little population, other metabolic pathways and the validation in other cohorts may be considered for future studies.

5. CONCLUSION

Our metabolomics approach served to propose candidate biomarkers for PAD diagnosis after interpretation of the metabolome fingerprint. (Iso)citrate and glutamate were not influenced by other comorbidities and their concentrations differ between control group and PAD patients, allowing a good discrimination between stages. More important, both can detect PAD I-II patients, the less symptomatic stages of the disease.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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Tables

Table 1. Clinical characteristics, complete blood count and biochemical characteristics of Control group and PAD patients.

	Control (n = 48)	**PAD (n = 201)**	**P-value**
BMI (kg/m^2)	24 (22.5 – 25.3)	25 (22.5 – 28)	0.021
Diabetes (%)	-	64.1	<0.001
Hypertension (%)	-	69.2	<0.001
Dyslipidemia	-	37.9	<0.001
Red blood cells, x10^{12}/L	4.9 (4.4 – 5.2)	4.16 (3.57 – 4.66)	<0.001
Hemoglobin, mmol/L	8.94 (8.32 – 9.43)	13.30 (11.50 – 14.90)	0.001
Leukocytes, x10^9/L	6.8 (5.4 – 8.2)	8.17 (6.50 – 10.22)	0.003
Platelets, x10^9/L	233 (205 – 273)	253 (200 – 329)	ns
Total-cholesterol, mmol/L	4.85 (4.40 – 5.85)	3.90 (3.31 – 4.94)	<0.001
HDL-cholesterol, mmol/L	1.34 (1.14 – 1.61)	0.96 (0.78 – 1.19)	<0.001
LDL-cholesterol, mmol/L	2.82 (2.40 – 3.86)	2.26 (1.77 – 2.79)	<0.001
Triglycerides, mmol/L	0.90 (0.70 – 1.38)	1.99 (1.40 – 3.08)	<0.001
Glucose, mmol/L	4.70 (4.37 – 4.92)	5.61 (4.60 – 6.88)	<0.001
ALT, U/L	20 (13.5 – 24.9)	21.5 (15 – 34.8)	ns
AST, U/L	20 (17.7 – 24)	21 (16 – 32)	ns

BMI: Body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Non-parametric variables are shown as median (IQR). Qualitative variables are expressed as (%) of total participants. Kruskal-Wallis test has been used for multiple comparisons between groups.

Table 2. Metabolite concentration in PAD patients segregated according co-morbidities.

Metabolite	PAD patients								
	Normoglycemic	**Type-2 diabetic**	**p-value**	**Normotensive**	**Hypertensive**	**p-value**	**Normolipidemic**	**Hyperlipidemic**	**p-value**
3-hydroxybutirate	0.27 (0.13 – 0-39)	0.27 (0.14 – 0.40)	ns	0.21 (0.14 – 0.38)	0.35 (0.14 – 0.42)	ns	0.31 (0.16 – 0.40)	0.19 (0.13 – 0.419	ns
Aconitate	3.75 (2.38 – 6.50)	4.60 (2.67 – 6.28)	ns	4.59 (2.54 – 6.65)	4.13 (2.60 – 6.60)	ns	4.64 (2.91 – 6.61)	4.22 (2.36 – 6.16)	ns
α-ketoglutarate	4.24 (2.56 – 7.22)	4.13 (2.76 – 6.50)	ns	4.71 (2.81 – 6.67)	3.70 (2.51 – 7.07)	ns	3.99 (2.44 – 6.55)	5.05 (3.05 – 6.90)	ns
Alanine	210.60 (147.51 – 266.30)	189.61 (139.04 – 271.47)	ns	213.52 (152.77 – 273.76)	184.35 (137.34 – 255.16)	ns	**180.54 (125.18 – 247.53)**	**233.62 (171.42 – 302.86)**	**0.001**
Aspartate	172.57 (133.37 – 215.81)	179.76 (143.01 – 225.10)	ns	181.61 (141.46 – 239.56)	173.17 (137.30 – 200.45)	ns	**168.65 (134.12 – 205.47)**	**191.72 (148.8 – 253.22)**	**0.016**
Isocitrate	721.78 (584.42 – 867.38)	665.64 (538.00 – 880.59)	ns	721.80 (566.64 – 934.03)	654.24 (476.56 – 815.96)	ns	678.64 (545.44 – 864.55)	712.36 (566.17 – 959.17)	ns
Fumarate	0.26(0.19 – 0.41)	0.24 (0.18 – 0.37)	ns	**0.27 (0.19 – 0.40)**	**0.22 (0.17 – 0.30)**	**0.035**	0.23 (0.18 – 0.36)	0.27 (0.19 – 0.40)	ns
Glucose	**4546.02 (4104.05 – 5115.56)**	**4959.36 (440.88 – 5804.93)**	**0.001**	**4955.95 (4459.14 – 5679.69)**	**4418.11 (4134.89 – 5191.71)**	**0.007**	**4663.92 (4172.31 – 5417.54)**	**4925.38 (4503.68 – 5684.91)**	**0.027**
Glutamate	1457.27 (743.06 – 2912.42)	1416.96 (671.78 – 2684.24)	ns	1335.82 (725.07 – 2628.52)	1786.69 (691.16 – 2891.74)	ns	1556.27 (679.84 – 2785.70)	1342.51 (763.74 – 2372.15)	ns
Glutamine	5073.46 (3054.24 – 7754.55)	4742.54 (1842.39 – 8881.28)	ns	5083.38 (2842.09 – 9072.22)	4742.54 (1700.95 – 7003.86)	ns	4742.54 (2007.90 – 7169.01)	5073.46 (2219.30 – 10427.79)	ns
Isoleucine	**57.14 (47.71 – 63.99)**	**63.68 (52.83 – 75.20)**	**0.002**	**63.52 (52.24 – 75.19)**	**57.88 (51.23 – 63.22)**	**0.050**	**57.90 (49.81 – 68.46)**	**65.02 (55.35 – 77.95)**	**0.002**
Lactate	367.23 (283.94 – 423.66)	341.07 (297.13 – 441.68)	ns	**373.57 (308.33 – 452.52)**	**332.36 (279.50 – 405.92)**	**0.047**	**334.77 (274.24 – 435.64)**	**393.34 (323.21 – 457.69)**	**0.004**
Leucine	85.07 (70.62 – 94.86)	86.68 (69.33 – 109.65)	ns	90.17 (71.33 – 109.35)	84.54 (69.77 – 93.84)	ns	**83.96 (66.70 – 98.71)**	**92.19 (76.10 – 109.92)**	**0.011**
Malate	2.38 (1.85 – 4.31)	2.45 (1.88 – 3.56)	ns	**2.79 (1.96 – 4.09)**	**2.23 (1.75 – 3.08)**	**0.043**	2.37 (1.83 – 3.66)	2.87 (2.12 – 3.80)	ns
Pyruvate	12.47 (3.64 – 22.87)	12.83 (3.55 – 21.89)	ns	**13.86 (3.85 – 24.11)**	**8.78 (2.72 – 18.15)**	**0.042**	9.50 (3.08 – 21.00)	13.58 (6.00 – 25.78)	ns
Serine	147.54 (101.70 – 167.45)	137.69 (109.40 – 169.59)	ns	**135.30 (106.14 – 162.12)**	**161.02 (103.62 – 172.90)**	**0.34**	150.04 (104.94 – 170.39)	131.92 (106.07 – 18.82)	ns
Succinate	9.57 (8.35 – 15.11)	9.70 (8.47 – 15.23)	ns	9.15 (8.42 – 15.04)	11.71 (8.49 – 15.47)	ns	10.82 (8.44 – 15.23)	9.11 (8.46 – 15.15)	ns
Succinyl-CoA	10.14 (7.74 – 15.12)	10.46 (7.37 – 17.62)	ns	11.21 (7.72 – 16.31)	9.94 (7.52 – 14.25)	ns	**9.79 (6.71 – 14.00)**	**13.36 (8.90 – 18.47)**	**0.001**
Valine	102.06 (83.06 – 129.40)	104.17 (80.70 – 138.39)	ns	107.33 (86.67 – 136.83)	96.63 (78.83 – 134.87)	ns	**99.36 (78.62 – 125.54)**	**113.33 (89.62 – 139.43)**	**0.010**

Table 3. Spearman correlation coefficients for age, body mass index and related metabolites.

	Age		**BMI**	
	Spearman's Rho	**p-value**	**Spearman's Rho**	**p-value**
3-hydroxybutirate	0.057	ns	0.022	ns
Aconitate	**0.205**	**0.003**	**-0.236**	**0.010**
α-ketoglutarate	0.054	ns	-0.031	ns
Alanine	-0.081	ns	**0.230**	**0.013**
Aspartate	0.083	ns	**0.256**	**0.005**
Citrate+Isocitrate	0.113	ns	0.147	ns
Fumarate	**0.220**	**0.002**	-0.085	ns
Glucose	-0.031	ns	**0.202**	**0.029**
Glutamate	0.063	ns	-0.032	ns
Glutamine	0.007	ns	0.157	ns
Isoleucine	0.124	ns	**0.194**	**0.036**
Lactate	-0.023	ns	0.034	ns
Leucine	-0.005	ns	**0.220**	**0.017**
Malate	**0.248**	**<0.001**	-0.079	ns
Pyruvate	-0.009	ns	0.103	ns
Serine	0.062	ns	-0.112	ns
Succinate	0.011	ns	-0.145	ns
Succinyl-CoA	0.038	ns	0.134	ns
Valine	-0.122	ns	**0.241**	**0.009**

BMI: Body mass index.

Table 4. Clinical characteristics, complete blood count and biochemical characteristics of PAD patients segregated by Fontaine classification.

	Fontaine I (n = 9)	**Fontaine II (n = 30)**	**Fontaine III (n = 46)**	**Fontaine IV (n = 116)**	**P-value**
Clinical characteristics					
Age (years)	55 (51 – 69)	73 (60 – 77)	65 (61 – 75)	71 (64 – 79)	0.003
BMI (kg/m^2)	28.9 (25.2 – 30.6)	27 (23.5 – 29.5)	26 (22.5 – 28)	24 (22-27.8)	ns
Diabetes (%)	12.5	60.9	38.1	78.7	<0.001
Hypertension (%)	50	74.1	60	73.3	<0.001
Dyslipidemia	42.9	42.3	37.8	36.4	<0.001
Complete Blood Count					
Red blood cells, x10^{12}/L	5.1 (4.5 – 5-4)	4.5 (4.1 – 4.8)	4.2 (3.8 – 4.6)	3.9 (3.3 – 4.4)	<0.001
Hemoglobin, mmol/L	9.12 (7.57 – 10.18)	8.56 (8.01 – 9.56)	8.87 (8.32 – 9.43)	7.63 (6.70 – 8.50)	<0.001
Leukocytes, x10^9/L	7.4 (6.8 – 10.4)	7.3 (6.3 – 8.9)	7.6 (6.4 – 10.4)	8.4 (6.6 – 10.7)	ns
Platelets, x10^9/L	205 (159 – 246)	216.5 (173.5 – 257.2)	251 (197 – 310)	277 (213 – 361)	0.001
Biochemical variables					
Total-cholesterol, mmol/L	4.06 (3.69 – 4.22)	4.23 (3.74 – 4.91)	3.94 (3.27 – 4.41)	3.80 (3.20 – 43.21)	ns
HDL-cholesterol, mmol/L	0.80 (0.58 – 1.22)	1.03 (0.80 – 1.27)	1.27 (0.81 – 1.25)	0.93 (0.78 – 1.16)	ns
LDL-cholesterol, mmol/L	2.04 (1.92 – 3.07)	2.43 (1.92 – 3.08)	2.22 (1.76 – 2.66)	2.24 (1.75 – 2.81)	ns
Triglycerides, mmol/L	1.57 (1.07 – 3.29)	1.81 (1.12 – 2.93)	2.34 (1.88 – 3.44)	1.85 (1.34 – 2.83)	ns
Glucose, mmol/L	6.99 (6.2 – 8.21)	6.09 (5.01 – 7.05)	5.29 (4.56 – 6.79)	5.55 (4.40 – 6.85)	ns
ALT, U/L	18 (13.3 – 41-5)	21 (16 – 28)	23.5 (18.8 – 46.5)	21 (13.7 – 35)	ns
AST, U/L	22.9 (16.2 – 32.4)	21 (17 – 25.6)	22.5 (18 – 44-7)	20 (15 – 32)	ns

BMI: Body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Non-parametric variables are shown as median and IQR (25-75%). Qualitative variables are expressed as (%) of total participants. Multiple comparisons between groups using Kruskal-Wallis test.

Figures

A

Metabolite	Control (µM)	PAD (µM)	PAD/control ratio	
3-hydroxybutirate	0.12 (0.11 - 0.13)	0.22 (0.14-0.40)	1.83	*
Aconitate	0.47 (0.39-0.63)	4.42 (2.45-6.26)	9.40	*
α-ketoglutarate	2.91 (2.15-3.97)	4.67 (2.85-7.07)	1.60	*
Alanine	199.95 (163.83-252.32)	208.71 (147.51-274.36)	1.04	ns
Aspartate	132.46 (114.27-147.78)	181.61 (142.32-229.68)	1.37	*
(Iso)citrate	267.20 (200.34-329.55)	687.82 (561.31-921.93)	2.57	*
Fumarate	0.31 (0.23-0.41)	0.25 (0.19-0.39)	-1.24	*
Glucose	4718.45 (4407.71-5073.74)	4878.41 (4312.48-5658.30)	1.03	ns
Glutamate	168.57 (106.17-258.28)	1417.56 (711.82-2669-54)	8.41	*
Glutamine	1705.99 (990.44-2736.23)	5073.46 (2616.13-9189.28)	2.97	*
Isoleucine	48.28 (40.66-55.59)	61.55 (52.22-73.56)	1.27	*
Lactate	399.68 (348.17-429.08)	358.59 (305.02-440.07)	-1.11	ns
Leucine	71.25 (62.95-80.77)	88.41 (71.57-107.83)	1.24	*
Malate	1.43 (1.15-1.77)	2.51 (1.90-3.88)	1.76	*
Pyruvate	9.12 (6.47-13.49)	13.49 (3.79-23.04)	1.48	ns
Serine	103.49 (93.41-112.86)	135.86 (105.32-167.17)	1.31	*
Succinate	10.52 (10.25-11.09)	9.40 (8.44-15.07)	-1.12	*
Succinyl-CoA	7.27 (5.27-9.76)	10.45 (7.58-15.54)	1.44	*
Valine	92.81 (82.87-104.55)	105.44 (83.91-135.10)	1.14	*

B

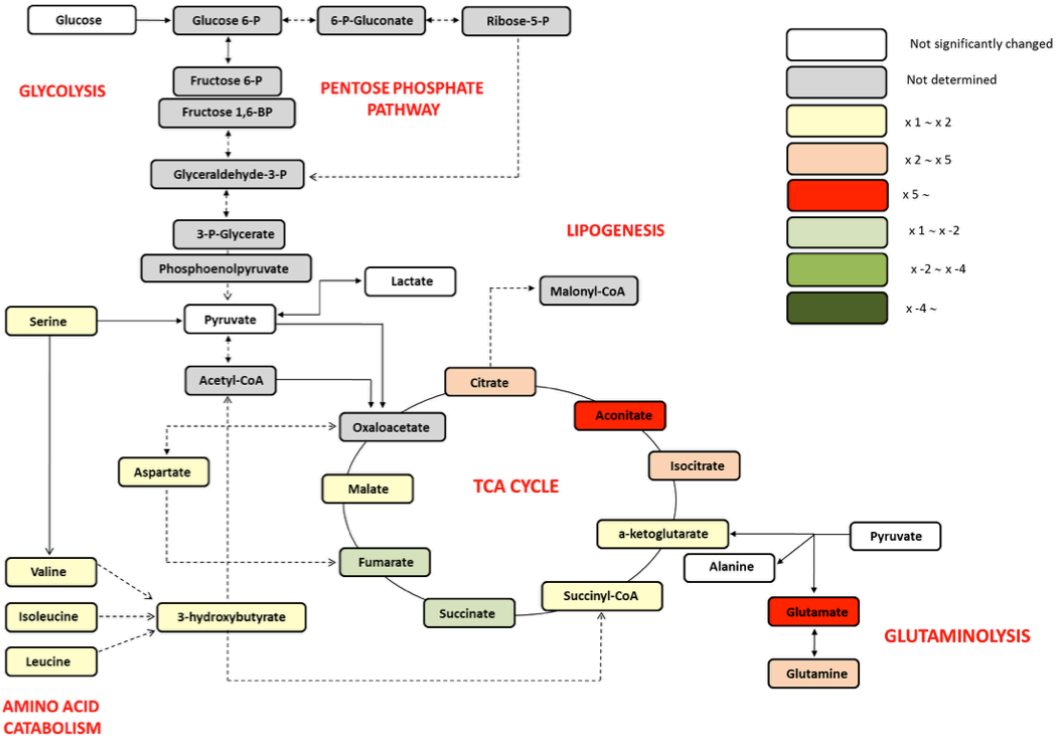


Figure 1. Energy metabolism in PAD patients. (A) Concentrations of measured metabolites in PAD patients and control group, expressed as median (IQR), fold-change ratio between PAD patients and control group, and p-value. * $p<0.05$. ns: no significant. (B) Graphical display of fold-change ratios in energy metabolism.

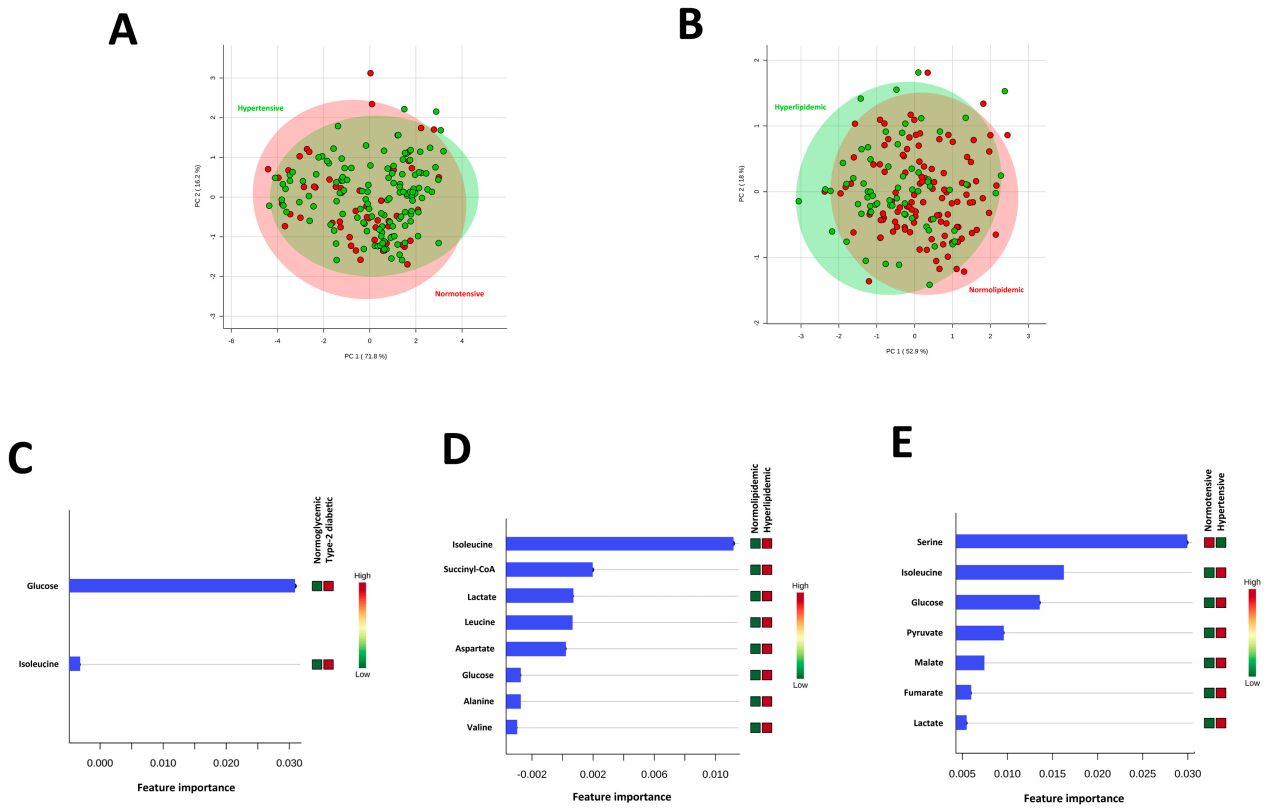


Figure 2. Principal component analysis (PCA) between hypertensive and normotensive (A) and between hyperlipidemic and normolipidemic (B) PAD patients. Random Forest analysis shows the metabolites with the best discriminant capacity between normoglycemic and diabetic patients (C), between normolipidemic and hyperlipidemic patients (D) and between normotensive and hypertensive patients (E).

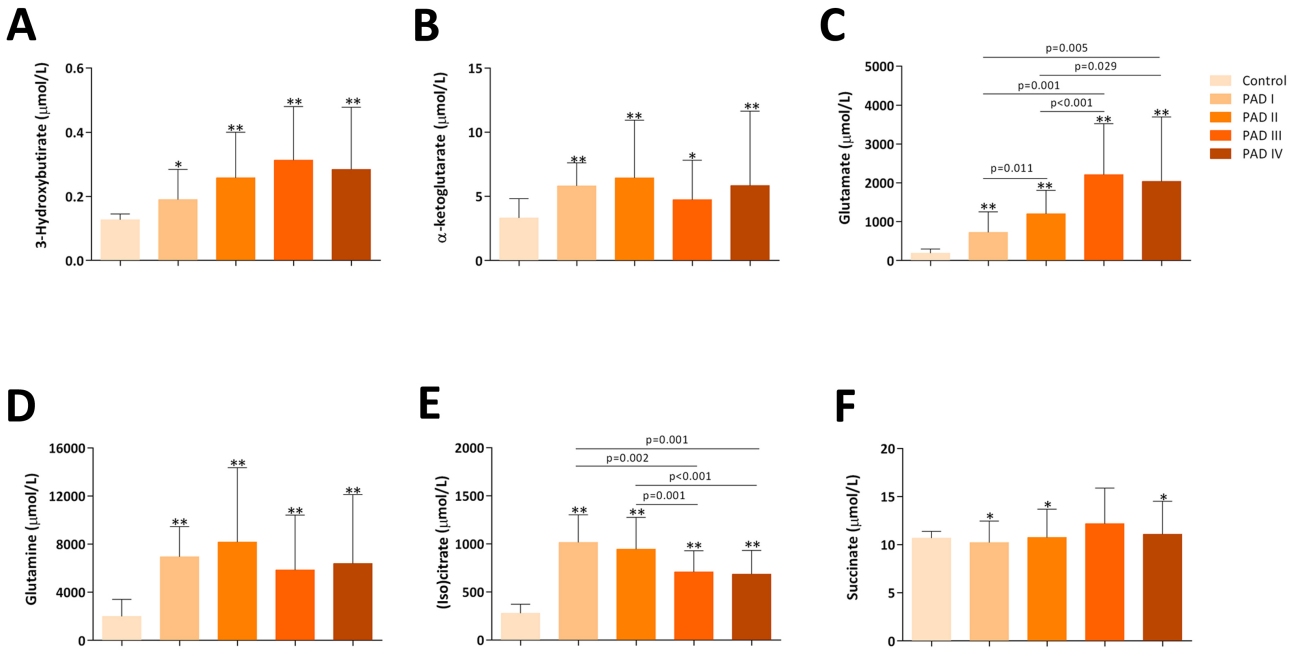


Figure 3. Candidate biomarkers for PAD patients. Graphical representation of candidate biomarkers concentration among groups: (A) 3-hydroxybutirate, (B) α-ketoglutarate, (C) glutamate, (D) glutamine, (E) (Iso)citrate and (F) succinate. *: $p < 0.05$; **: $p < 0.001$.

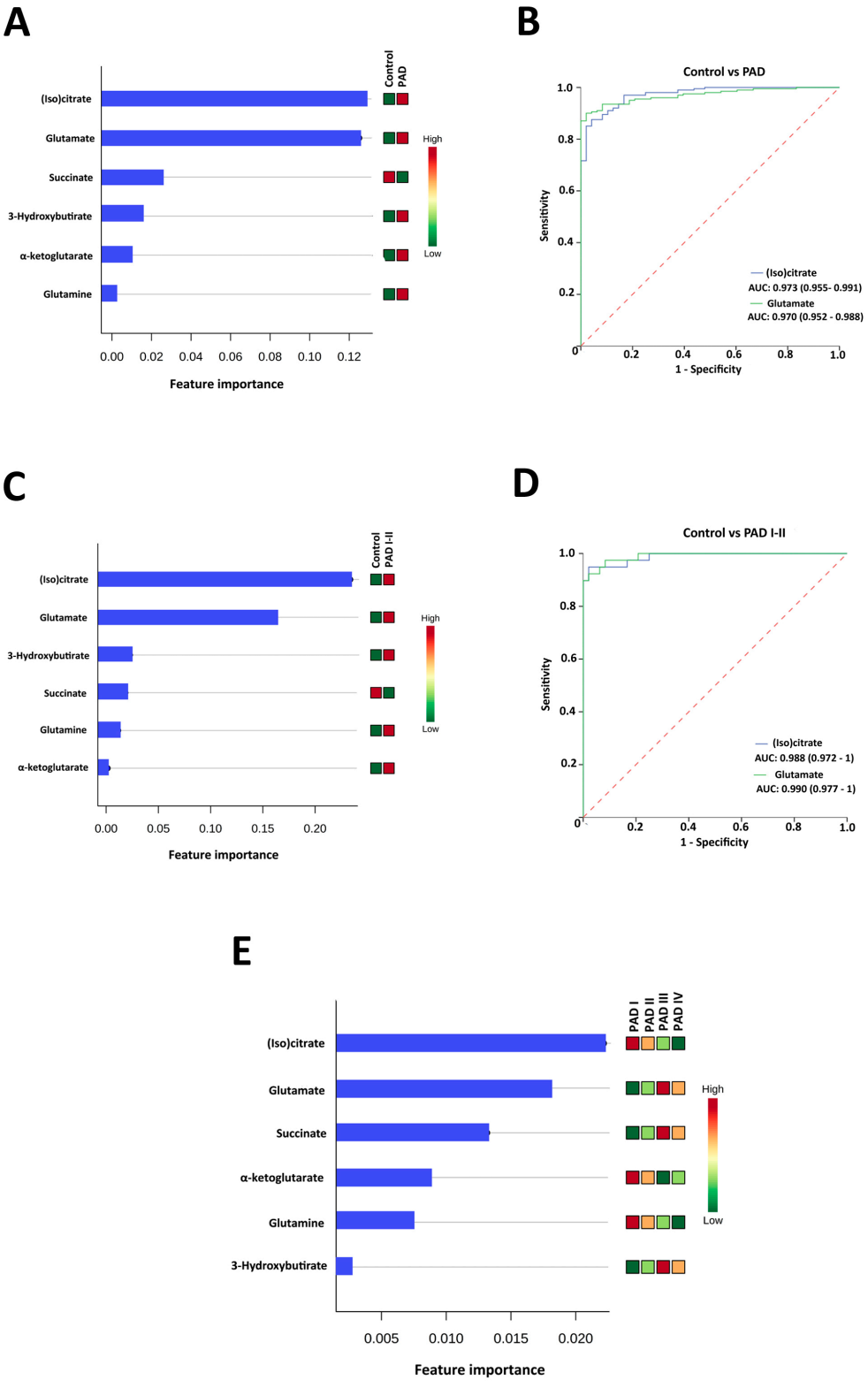


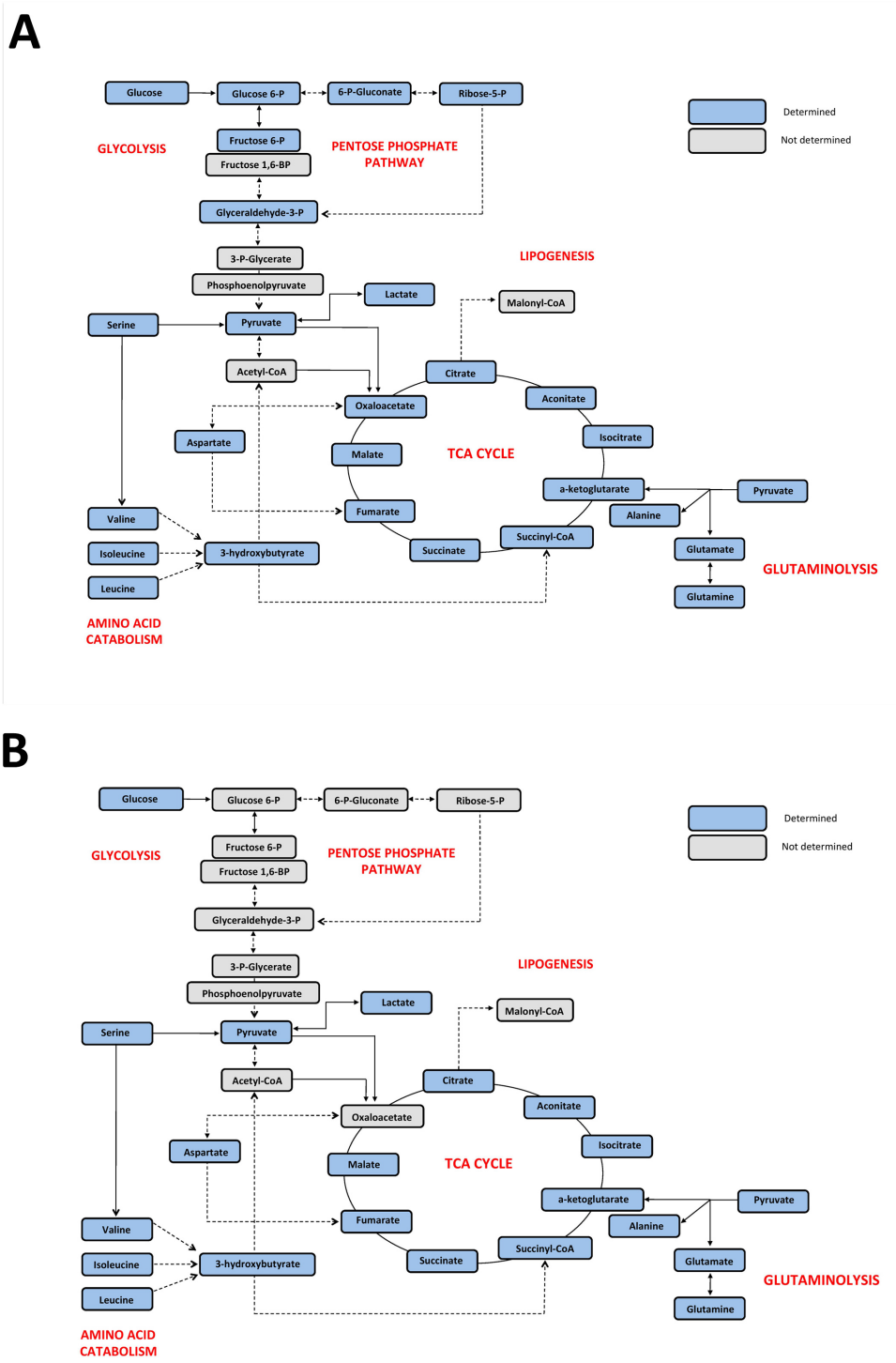
Figure 4. Validation of candidate biomarkers. Random Forest analysis showing the metabolites with the best discriminant capacity between control group and (A) PAD patients, (C) PAD I-II patients and (E) during disease progression. ROC curve for the best candidates. Discriminating between control group and (B) PAD and (D) PAD I-II patients. AUC, area under curve.

Supplementary Information for the manuscript:

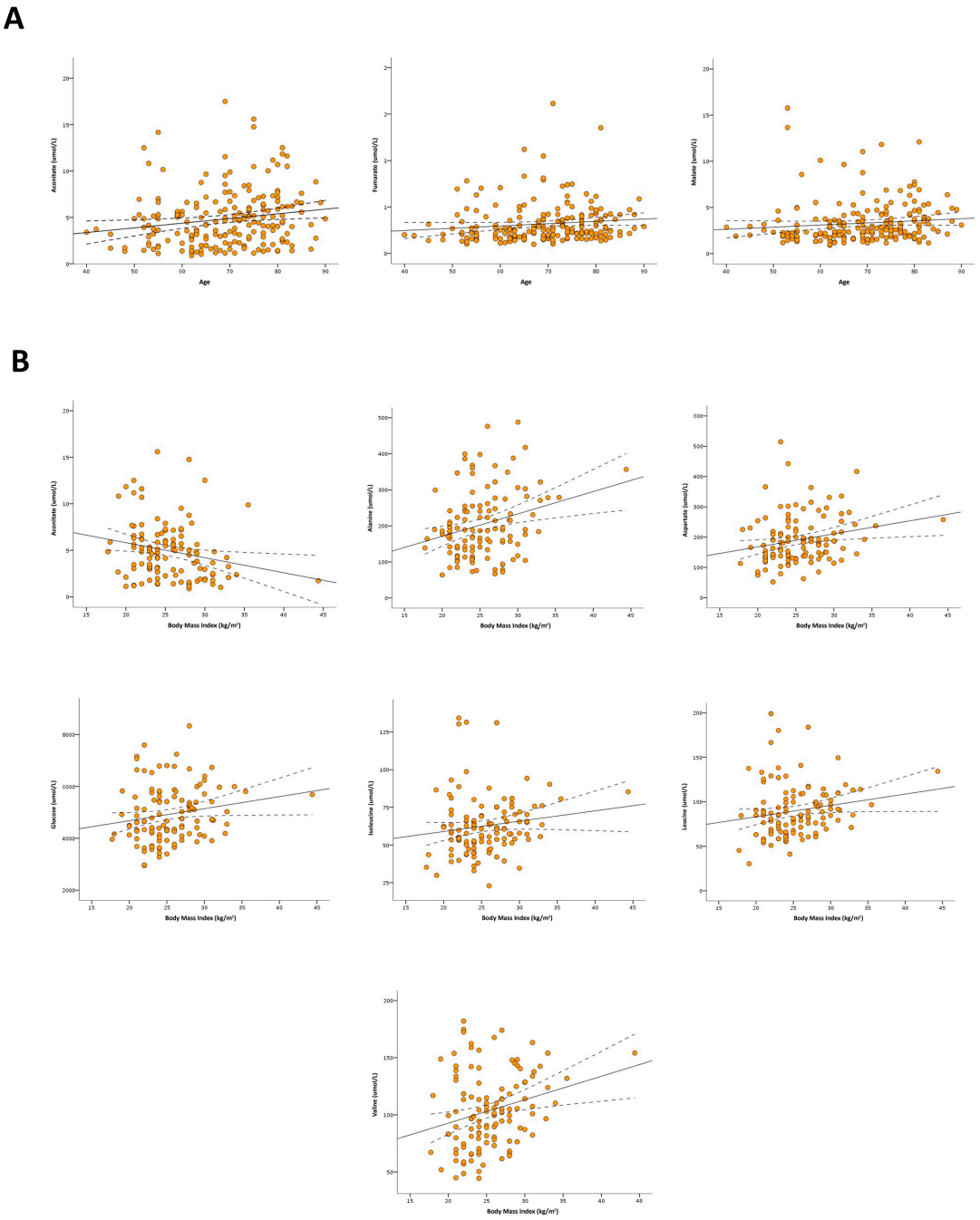
Plasma metabolome in PAD patients unveils limitations derived from comorbidities in unhealthy aging

Running head: Metabolomics, peripheral artery disease and associated pathologies.

Anna Hernández-Aguilera[a,1], Salvador Fernández-Arroyo[a,1,*], Noemí Cabre[a], Fedra Luciano-Mateo[a], Anabel García-Heredia[a], Montserrat Fibla[a], Vicente Martín-Paredero[b], Jordi Camps[a], Jorge Joven[a,c*].



Supplementary Figure 1. Determined metabolites in artery (A) and plasma samples (B).



Supplementary figure 2. (A) Correlations between aconitate, fumarate and malate with age. (B) Correlations between aconitate, alanine, aspartate, glucose, isoleucine, leucine and valine with body mass index.

Annex 1

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Relationships between metformin, paraoxonase-1 and the chemokine (C-C motif) ligand 2

Jordi Camps*, Anna Hernández-Aguilera, Anabel García-Heredia, Noemí Cabré, Fedra Luciano-Mateo, Meritxell Arenas, Jorge Joven

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Abstract: Metformin is a biguanide that is widely employed in the treatment of type 2 diabetes mellitus and obesity. The main mechanism of action is to decrease glucose absorption by the intestine and glucose production in the liver. It does not stimulate insulin secretion. Metformin also increases the affinity of the insulin receptor for insulin, reduces hyperinsulinemia and improves insulin resistance. Additionally, it promotes weight loss. Metformin is a pleiotropic compound but acts, largely, by activating 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK). Several lines of evidence suggest that the therapeutic effects of this compound are mediated, at least in part, through an upregulation of paraoxonase-1 (PON1) synthesis. PON1 is a thiolactonase that degrades lipid peroxides, and downregulates the chemokine (C-C motif) ligand 2 (CCL2) which is a pro-inflammatory chemokine that stimulates the migration of monocytes to areas of inflammation where they differentiate into macrophages. Studies in PON1-deficient mice suggest that PON1 is essential for the successful activation of AMPK in the liver and for facilitating metformin's therapeutic function.

Keywords: Chemokine (C-C motif) ligand 2; Diabetes mellitus; Inflammation; Metabolic syndrome; Metformin; Paraoxonase-1

PARAOXONASES CONSTITUTE AN ENDOGENOUS ENZYME SYSTEM AGAINST OXIDATIVE STRESS

Independently of intake of exogenous antioxidants, the organism has several endogenous mechanisms to protect against oxidative stress. Most recent research has focused on a family of enzymes, the paraoxonases (PON) that play a determinant role in protecting cells against oxidative stress. The PON enzyme family comprises three members (PON1, PON2, and PON3) the genes for which are located adjacent to each other on chromosome 7q21–22 [1,2]. PON1 and PON3 are almost ubiquitously expressed in tissues and, as well, bound to high-density lipoprotein (HDL) in the circulation [3-7]. Conversely, PON2 is an intracellular enzyme also expressed by most cells but, unlike PON1 and PON3, is not found in plasma [8]. PON1 has esterase and lactonase activities [9]. It hydrolyzes thiolactones and active metabolites of several organophosphate insecticides (paraoxon, chlorpyrifos oxon, and diazoxon) as well as the nerve agents sarin and soman [10]. PON2 and PON3 are not active against xenobiotics, but have lactonase activity [11]. All the three PON enzymes degrade lipid peroxides in low-density lipoproteins (LDL) [12], while PON2 reduces cellular oxidative stress and prevents apoptosis in several cell types [13].

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Extensive data indicate that the PON family plays a protective role in several diseases involving oxidative stress. These include cardiovascular disease, diabetes, Alzheimer's disease, metabolic syndrome, and liver diseases [14, 15].

The first evidence demonstrating the role of PON1 in protecting cells against lipid peroxidation was reported by Mackness *et al.* [16]. These authors investigated the protection against LDL oxidation provided by HDL, in the course of which PON1 was isolated and purified. They observed that PON1 and HDL prevented lipoperoxide generation during the process of LDL oxidation, and concluded that this enzyme may be involved in the anti-oxidative protective function of HDL. Further studies from this group, and others, demonstrated that PON1 protects LDL and HDL from lipid peroxidation by degrading specific oxidized cholesteryl esters and specific oxidized phospholipids contained in oxidized lipoproteins [17-23]. Of further note is that the PON1 free sulfhydryl group in cysteine 284 appears to be required for the enzyme's activity against lipid peroxides [20], but not for its activity against paraoxon or other xenobiotics. This suggests that the mechanisms of hydrolysis and detoxification are somewhat different i.e. different binding sites in the enzyme that could explain the different activities of the enzyme. Studies in genetically modified mice provide further support for the *in vitro* experiments that indicated that the physiological function of PON1 was to hydrolyze oxidized lipids i.e. to function as an antioxidant enzyme. Decreased serum PON1 activity and increased oxidative stress have been observed in

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apolipoprotein E-deficient mice, as well as in dyslipidemic obese mice [12,24]. The most conclusive data have been generated in mouse models [25-27] in which PON1-deficient plus apolipoprotein E-deficient mice showed greater lipoprotein oxidation (and atherosclerosis) than the apolipoprotein E-deficient mice alone [26]. Corroborating these observations, HDL isolated from PON1-deficient mice is unable to prevent LDL oxidation in cultured arterial tissue, in contrast to HDL obtained from control mice [27,28]. Moreover, transgenic mice overexpressing human PON1 have decreased HDL lipid peroxide formation and a preserved LDL structure and function [29].

PARAOXONASES, ATHEROSCLEROSIS, DIABETES AND OBESITY: THE ROLE OF THE CHEMOKINE (C-C MOTIF) LIGAND 2

Mackness *et al.* [30] first demonstrated that PON1 inhibits the production of the chemokine (C-C motif) ligand 2 (CCL2, also termed monocyte chemoattractant protein-1, MCP-1) in endothelial cells incubated with oxidized LDL. These authors found that HDL, and recombinant PON1, abolished CCL2 production (Figure 1). PON1 inhibition of CCL2 appeared to be due to its capacity to inhibit LDL oxidation. CCL2 is a chemokine that regulates the migration of monocytes into tissues and their differentiation into macrophages; these responses playing a major role in the inflammatory reaction. It seems, then, than PON1 and CCL2 play key roles linking oxidation and inflammation processes.

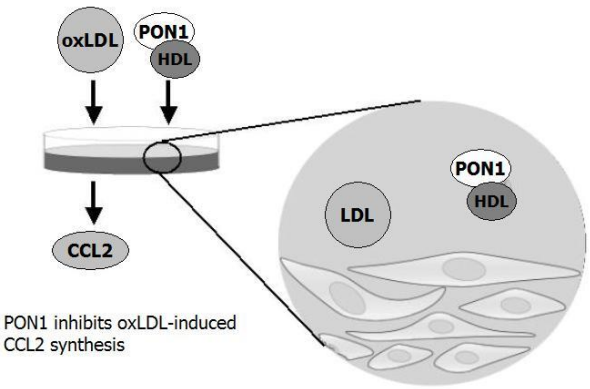

FIG. (1). PON1 is an anti-inflammatory enzyme. Incubation of endothelial cells with oxidized low-density lipoproteins (oxLDL) induces the synthesis of the pro-inflammatory chemokine (C-C motif) ligand 2 (CCL2). Co-incubation with PON1-containing high-density lipoproteins (HDL) or with recombinant PON1 leads to oxLDL degradation and inhibition of CCL2 synthesis

Following tissue injury, CCL2 is upregulated and expressed by inflammatory and stromal cells. Being ubiquitous and jointly localized with PON1 suggests a systemic and coordinated role for both molecules [7,31]. CCL2 induces endoplasmic reticulum (ER) stress leading to autophagy, while regulating the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) by catalyzing de-ubiquitination [32]. The inflammatory reaction is triggered by the activation of pattern-recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors (NLR), which recognize pathogens or pathogen-associated molecular patterns (PAMP). Products from damaged cells such as damage-associated molecular pattern (DAMP) initiate inflammation in a similar manner to pathogens [33]. The binding of PAMP/DAMP to a PRR leads to several molecular events resulting in inflammatory responses. NF-κB activation triggers the production of adhesion molecules and chemokines (including CCL2) that lead to infiltration of immune cells into the damaged tissue. There are other pathways which, when activated, result in similar outcomes. Examples are the mitogen-activated protein kinase (MAPK) pathway, the phosphoinositide 3-kinase (PI3K)-related signaling pathway, and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. These changes are linked to ER stress and the unfolded protein response (UPR). The UPR [34] is initiated via three ER-localized transmembrane proteins: inositol-requiring enzyme (IRE), protein kinase-like ER kinase (PERK), and activating transcription factor-6 (ATF6) (Figure 2).

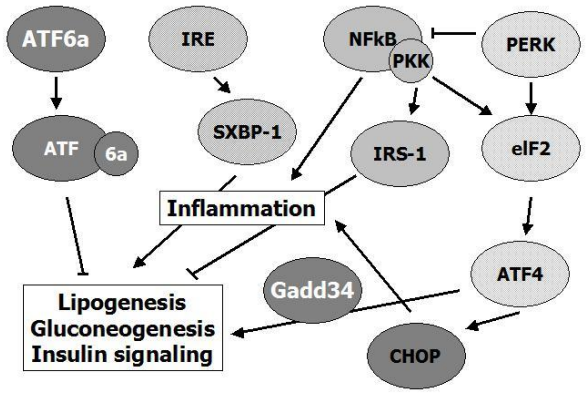

FIG. (2). The unfolded protein response (UPR) is a cellular stress response related to the endoplasmic reticulum, and has been found to be conserved in mammalian species.
The accumulation of unfolded, or misfolded, proteins in the lumen of the endoplasmic reticulum activates the response. It is designed, initially, to restore normal function of the cell by halting protein translation and activating the production of molecular chaperones involved in protein folding. If not achieved within a certain time-scale, or if the disruption is prolonged, the UPR drives toward apoptosis. Molecules involved in the three branches of this response are summarized in the figure.
Notes:
ATF: activating transcription factor; CHOP: CCAAT/enhancer binding protein (C/EBP), homologous protein; eIF2: E74-like factor 2; Gadd 34: growth arrest and DNA damage inducible protein 34; IRE: inositol-requiring enzyme; IRS-1: insulin receptor substrate 1; N-ATF: N-terminal ATF6; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; PERK: protein kinase-like ER kinase; PKK: protein kinase C-associated kinase; SXBP-1: spliced X-box binding protein 1.

Inflammatory cells generate chemokines and reactive oxygen species (ROS) which trigger ER stress. The UPR signal pathways are integrated with the inflammatory pathways through several mechanisms such as the regulation of intracellular calcium, the generation of ROS, or the production of acute phase proteins which upregulate the expression of the CCL2 receptor [35]. Nitrous oxide (NO)

can activate ER stress through disturbance of ER calcium homeostasis, or by enhancing ROS generation, or by inhibiting protein disulfide isomerase (PDI); the result being the subsequent accumulation of poly-ubiquitinated proteins [36]. All these effects are involved in obesity and diabetes. Several components of UPR signaling link ER stress to cell death and autophagy [37]. When the cell damage is affordable, autophagy helps the cells to survive through the lesion i.e. enabling full recovery and avoiding cell death by removing the toxic protein aggregates. However, when autophagy cannot maintain protein and organelle quality, the outcome is a form of non-apoptotic cell death. There is compelling evidence that ER stress is important in the instigation, development and progression of atherosclerosis, and that ER stress induced apoptosis in macrophages is a major contributor to the instability of atherosclerotic plaques [38].CCL2 links ER stress to cardiovascular diseases Further, CCL2 induces plaque destabilization by increasing the activity of the ubiquitin-proteosome system in inflammatory macrophages [39].

Processes in the heart such as ischemia/reperfusion, hypoxia, pressure overload, and inflammation result in activation of ER stress through mechanisms involving CCL2 [40,41]. The pathogenic role of this chemokine in atherosclerosis is well established. Many stimuli, particularly oxidative stress, cause the production of CCL2 in vascular cells and stimulate foam cell formation, inflammation, and progression of the atherosclerotic lesion (Figure 3) [42-45].

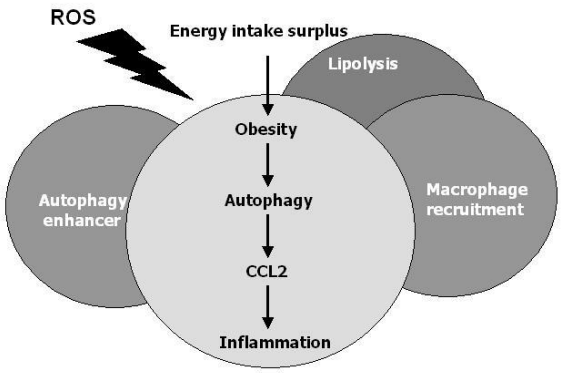

Fig. (3). Autophagy and inflammation are the consequences of oxidative stress and imbalanced nutrient intake, and are closely related to metabolic diseases, such as obesity, diabetes and cardiovascular diseases.

An autopsy study found strong associations of upregulation of ER stress markers in coronary arteries *versus* atherosclerotic plaque rupture [46]. Moreover, CCL2 induces plaque destabilization by increasing activity of the ubiquitin-proteosome system in macrophages [39]. CCL2 also plays a notable role in a variety of other inflammatory diseases. The nature of the genes induced by CCL2 is far from clear, but some experimental studies propose a novel class of zinc-finger proteins in monocytes triggered by CCL2 binding to CCR2. Termed MCPIP (MCP-1–induced proteins) they are expressed in macrophage-associated organs [47,48]. Monocyte-macrophage differentiation related to inflammation is involved in adipogenesis and angiogenesis and, as such, highlights a possible role for CCL2 in cardiovascular diseases, type 2 diabetes mellitus, tumor growth, and obesity, with the MCPIP system playing a crucial role in these processes. CCL2 is an angiogenic factor that differentiates bone marrow monocyte-lineage cells into endothelial-like cells [49]. An increased expression of CCL2 causes macrophage infiltration into adipose tissue while the acute increase in circulating concentration of CCL2 elicits systemic insulin resistance and, hence, type 2 diabetes. In humans, the increases in serum CCL2 levels correlate well with markers of metabolic syndrome [50,51].

Cell death and differentiation share common components, although the underlying mechanisms are unclear. In both processes, oxidative stress, ER stress, and autophagy have been described, but the relationships between them have yet to be elucidated. However, inflammation can lead to cell death and differentiation processes such as angiogenesis and adipogenesis. CCL2 is a plausible mediator in these processes [52] but the issue has not resolved although the involvement of macrophages appears to be critical. For instance, obesity is associated with cardiovascular disease, diabetes and cancer, and is frequently accompanied by metabolic disturbances in which the aforementioned processes are present. Obesity increases tissue infiltration by macrophages and proceeds via polarization to the pro-inflammatory M1 state of macrophages which is implicated in the development of insulin resistance [53]. Ascertaining the precise signal transduction pathways in macrophages that respond to these effects could present opportunities for new therapeutic procedures. The stress-responsive JNK signal transduction pathway (which is activated by obesity and is required for obesity-induced insulin resistance) has been identified recently as being important in the behavior of macrophages [54,55].

THE FATE OF PON1 AND CCL2 IS TO WORK COORDINATELY

As stated above, oxidized LDL induces endothelial cell CCL2 synthesis. Hence, an increase in oxidation leads to the activation of the inflammatory reaction that further increases the oxidation; somewhat like a vicious circle. This phenomenon is observed in many non-communicable diseases including obesity, diabetes, cardiovascular disease, and cancer. PON1 plays a key role in breaking this cycle, by degrading lipid peroxides in LDL and preventing increased CCL2 synthesis [30]. For this reason, alterations in the circulating concentrations or intracellular levels of PON1 and CCL2 are observed in many experimental models of disease, and in clinical studies. Depending on the circumstances of the process disease being studied, these changes can be unidirectional (PON1 and CCL2 proceeding to increase or decrease together) or bidirectional (CCL2 increase and PON1 decrease). For example, in normal C57BL/6J mouse tissues, PON1 and CCL2 gene and protein expressions have been found in the vast majority of tissues including heart, aorta, liver, and most epithelia; both molecules co-express in the same cells and tissue structures, which would suggest coordinated roles [7]. In the livers of

rats with experimental fibrosis, high levels of PON1 and CCL2 expression have been observed in hepatocytes surrounding the fibrous septa and inflammatory areas. However, while PON1 protein expression was enhanced, the enzyme activity was decreased, and it was suggested that this was due to enzyme inactivation [15,56]. Patients with chronic liver disease have high levels of CCL2 and low levels of PON1 in the circulation, which is in agreement with observations in experimental models [57,58]. In peripheral artery disease, a condition characterized by extensive atherosclerosis of lower extremities, PON1 and CCL2 co-localize at the sites of the atherosclerotic lesions. It was also evident that both proteins were increasingly expressed in progressively advanced lesions. However, serum PON1 activity was much lower than in normal subjects, and was similar to that observed in patients with liver disease [59,60]. Taken together, these data suggest that the PON1-CCL2 duo could constitute an important target for pharmacological intervention, in most non-communicable diseases.

MOLECULAR MODE-OF-ACTION OF METFORMIN IN THE ATHEROSCLEROSIS, OBESITY AND DIABETES PROCESSES

Metformin is a biguanide with two methyl groups attached to the nitrogen nucleus of biguanide (Figure 4). This compound is derived from galegine, extracted from the plant *Galega officinalis* [61]. The drug has been approved for use in the treatment of hyperglycemia, metabolic syndrome, and polycystic ovary [62-64]. Following ingestion, it is absorbed into the circulation and tissues within 1-3 hours, and 90% is eliminated by the kidneys.

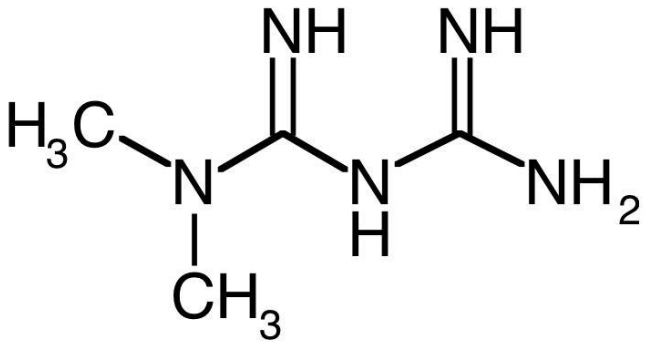

Fig. (4). Chemical structure of metformin

Metformin decreases glucose absorption by the intestine, and glucose production in the liver. However, insulin secretion in unaffected. It increases the uptake and utilization of glucose by muscle and adipose tissues. The lowering of blood glucose levels by metformin is only observed in patients with diabetes and insulin resistance, but has no effect on healthy individuals. Metformin also increases the affinity of the insulin receptor for insulin, reduces hyperinsulinemia and improves insulin resistance. Several days after its administration, insulin levels are reduced by 25-33% in both diabetic and non-diabetic patients. Metformin can also decrease fatty acid uptake and oxidation in muscle cells while lowering circulating levels of total cholesterol, LDL-cholesterol, and triglycerides. Additionally, metformin promotes weight loss. Generally, metformin is well tolerated, with only 5% of patients developing intolerance. The side effects are mild and reversible and include gastrointestinal perturbations (30%), metallic taste (3%), and decreased levels of vitamin B12 (in 6% of patients after 29 weeks of treatment). Metformin is considered to be a safe drug with a low risk of lactic acidosis, a reaction that affects about 3 of 100,000 people/year [65,66].

Metformin is a pleiotropic compound, but largely elicits its effects by activating 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK). AMPK is a major metabolic sensor involved in the regulation of cellular energy homeostasis (Figure 5). When cellular stress is present (e.g. glucose deprivation, hypoxia, oxidative stress or

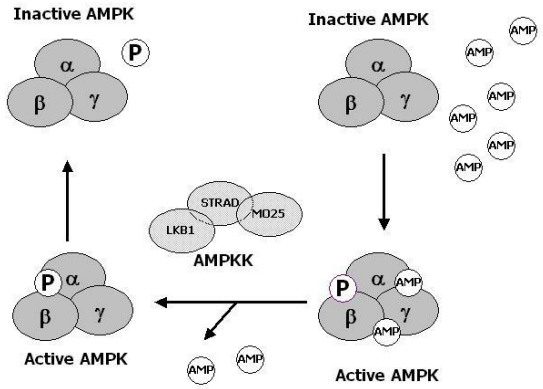

Fig. (5). AMPK or 5'-adenosine monophosphate-activated protein kinase, is a heterotrimeric protein formed by α, β, and γ subunits. Each subunit can be found in different isoforms: the γ subunit can exist as either γ1, γ2, or γ3 isoform; the β subunit can exist as either β1 or β2 isoform; and the α subunit can exist as either α1 or α2 isoform. Together, they make a functional enzyme that acts in cellular energy homeostasis. The increased concentrations of AMP produce a conformational change in the γ subunit of AMPK as two AMP bind the two Bateman domains located on that subunit. This exposes the active site (Thr-172) on the α subunit to the action of an upstream AMPK kinase (AMPKK). This modification increases the activity of AMPK. Conversely the loss of AMP and the lack of phosphorylation render the enzyme inactive. AMPKK is a complex of three proteins, STE-related adaptor (STRAD), mouse protein 25 (MO25), and liver kinase B1 (LKB1), which is a serine/threonine kinase.

ischemia), the ratio of AMP/ATP increases which, then, induces the activation of AMPK [62]. Once activated, AMPK inhibits anabolic processes that require energy and, instead, activates catabolic processes that produce energy. The activation of AMPK is mediated by other proteins. These include the enzymes serine-threonine kinase-11 (STK11), TGF-β-activated protein kinase 1 (TAK1), and calcium/calmodulin-dependent protein kinase (CaMKK) [67-71]. hyperinsulinemia-mediated loss and/or mutation of STK11 are predictors of sensitivity to metformin [72]. One

of the consequences of AMPK activation is the inhibition of lipogenesis in tissues. The effects of metformin on energy homeostasis include the repressed activation or expression of key enzymes of fatty acid biosynthesis (e.g. acetyl-CoA carboxylase, fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA reductase) and enhanced expression of regulators of mitochondrial biogenesis (e.g. peroxisome proliferator-activated receptor gamma coactivator 1-alpha) [73-78]. Together, these effects on energy homeostasis involve inhibition of endogenous fatty acid biosynthesis and a shift in cellular bioenergetics towards catabolism. In addition, the activated form of AMPK inhibits the mechanism of rapamycin (mTOR) activity via the phosphorylation and stabilization of the tuberous sclerosis complex 2 (TCS2). As such, metformin inhibits the mTOR-signaling pathway in an AMPK-dependent manner. It should be noted that mTOR inhibition might also occur in the absence of AMPK activation, for example, by inhibiting the insulin-like growth factor-1 (IGF1), the insulin receptor and the serine-threonine protein kinase, AKT [79]. Therefore, metformin can inhibit mTOR by decreasing the levels of insulin and/or IGF1 independently of AMPK.

METFORMIN TREATMENT INFLUENCES PON1 AND CCL2 LEVELS AND *VICE VERSA*

Few studies have investigated the interactions between metformin, PON1 and CCL2. Coll *et al.* [80] were the first to address this point. They studied the effect of treatment with 1g of metformin twice daily for 26 weeks in patients infected with the human immunodeficiency virus, and with lipodystrophy. They observed an increase of approximately 50% in serum PON1 activity, and a decrease of 25% in plasma CCL2 concentration. In addition, the postprandial levels of these molecules (PON1 and CCL2), following a high-fat meal, were not as strongly altered as in those patients receiving placebo. The methionine-choline-deficient rat is a model of non-alcoholic fatty liver disease which exhibits lower serum and liver PON1 activities than their corresponding controls. The administration of metformin of 200 mg/kg for 15 weeks in this model produced an increase in serum and liver PON1 and a decrease in oxidative stress markers [81]. A randomized trial in patients with type 2 diabetes mellitus showed that the administration of 1g of metformin daily over a period of 3 months was associated with a significant increase in serum PON1 activity. This was paralleled with a decrease in advanced oxidation protein products and advanced glycation end-products; two markers of oxidative stress [82,83]. Similar results were reported recently in a study of obese children with metabolic syndrome as well as in women with polycystic ovary syndrome [84-87]. Regarding CCL2, available information is even scarcer. Metformin administration was found to be associated with significant decreases in the plasma concentration of this chemokine in diabetic rats [88,89] and in women with polycystic ovary syndrome [90]. Overall, clinical and experimental studies suggest that metformin increases serum and liver PON1 activity, and decreases plasma CCL2 concentration. As such, these mediators may play a role in the observed beneficial effects of metformin.

The biochemical pathways linking metformin with PON1 and CCL2 are unclear. Since metformin decreases oxidative stress, there is the possibility that the observed increase in PON1 is merely an epiphenomenon. PON1 degrades lipid peroxides but the PON1 active site for this process requires a free sulfhydryl group at cysteine 284. The result is that lipid peroxides react covalently with this site leading to enzyme inactivation [91]. This implies that every PON1 molecule degrading an oxidized lipid becomes inactivated, with a resultant overall decrease in enzyme activity. The same scenario would explain an inhibited CCL2 synthesis. However, some data suggest an active influence of metformin on PON1 and CCL2 synthesis through AMPK activation; similar manner to that reported for β-carotene [92-94]. This terpenoid, when added to human endothelial cells, activates AMPK, up-regulates PON1, and down-regulates CCL2 gene expressions; these effects being reversed by STO-609, a CaMKK inhibitor. These findings indicate that β-carotene regulates the expression of PON1 and CCL2 via CaMKK and AMPK pathway activation [94]. That metformin acts via similar pathways is very likely, and warrants further investigation.

Very recent data suggest that PON1 is necessary for metformin to generate its beneficial effects, at least in the liver. Several cases of metformin-induced aggravation of hepatic damage have been reported in patients with liver disease, and improvement in liver function occurred following discontinuation of the drug [95-98]. Severe liver impairment is associated with low hepatic and circulating PON1 levels. Indeed, serum PON1 activity is strongly decreased in patients with chronic hepatitis or cirrhosis, and the magnitude of the decrease is related to the extent of liver damage [99,100]. Further, a study found that a decreased hepatic PON1 activity was related to increased lipid peroxidation and liver damage in rats with experimental fibrosis [101]. The question arises as to whether decreased hepatic PON1 activity is related to these toxic effects of metformin in patients with existing liver damage? This point was addressed by a study in PON1-deficient mice fed with either a standard chow diet or a high-fat and high-cholesterol diet. PON1-deficient mice presented with spontaneous hepatic steatosis resembling human non-alcoholic fatty liver disease [102]. In this experimental model, metformin administration was observed not to improve liver function but, conversely, increased the severity of steatosis, increased CC2 expression and the number of macrophages in the liver, and also increased the expression of the apoptosis marker caspase-9. Of note is that AMPK in *inactivated* by metformin in PON1-deficient mice [103]. These data suggest that PON1 plays a key role in metformin-induced AMPK activation, and the reported beneficial consequences of this drug.

CONCLUSION

Metformin is a well-established drug widely used since the 1960s in the treatment of diabetes and obesity. Recent studies indicate that some of the beneficial effects of metformin may be mediated by a stimulation of the synthesis of PON1 and inhibition of CCL2. However, the prescription

of metformin in patients with liver disease is controversial since, in some cases, this drug produces a worsening of liver function. Patients with chronic liver disease have decreased hepatic PON1 activity. A study in mice deficient in PON1 suggested that this enzyme is essential for the successful activation of AMPK in the liver, and for metformin to demonstrate its therapeutic function.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ABBREVIATIONS

AMP	=	Adenosine monophosphate
AMPK	=	AMP-activated protein kinase
AMPKK	=	AMPK kinase
ATF	=	Activating transcription factor
CaMKK	=	Ca/calmodulin-dependent protein kinase
CCL2	=	Chemokine (c-c motif) ligand 2
C/EBP	=	CCAAT/enhancer binding protein
CHOP	=	C/EBP homologous protein
DAMP	=	Damage-associated molecular pattern
elF2	=	E74-like factor 2
ER	=	Endoplasmic reticulum
GADD	=	Growth arrest and DNA damage inducible protein
HDL	=	High-density lipoprotein
IGF1	=	Insulin-like growth factor-1
IRE	=	Inositol-requiring enzyme
IRS-1	=	Insulin receptor substrate 1
JAK	=	Janus kinase
LDL	=	Low-density lipoprotein
LKB1	=	Liver kinase B1
MAPK	=	Mitogen-activated protein kinase
MCP-1	=	Monocyte chemoattractant protein-1
MCPIP	=	MCP-1-induced protein
MO25	=	Mouse protein 25
mTOR	=	Mechanistic target of rapamycin
NFκB	=	Nuclear factor κ-light-chain-enhancer of activated B cells
NLR	=	Nucleotide domain-like receptor
PAMP	=	Pathogen-associated molecular pattern
PDI	=	Protein disulfide isomerase
PERK	=	Protein kinase-like ER kinase
PI3K	=	Phosphoinositide 3-kinase
PKK	=	Protein kinase C-associated kinase
PON	=	Paraoxonase
PRR	=	Pattern-recognition receptor
ROS	=	Reactive oxygen species
STAT	=	Signal transducer/activator of transcription
STK11	=	Serine-threonine kinase-11
STRAD	=	STE-related adaptor
SXBP-1	=	Spliced X-box binding protein-1
TAK1	=	TGFβ-activated protein kinase-1
TCS2	=	Tuberous sclerosis complex 2
TLR	=	Toll-like receptor
UPR	=	Unfolded protein response

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UNIVERSITAT ROVIRA I VIRGILI
PERIPHERAL ARTERY DISEASE: THE SEARCH FOR A BIOLOGICAL MARKER
Anna Hernández Aguilera

Annex 2

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Review

Epigenetics and nutrition-related epidemics of metabolic diseases: Current perspectives and challenges

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ABSTRACT

We live in a world fascinated by the relationship between disease and nutritional disequilibrium. The subtle and slow effects of chronic nutrient toxicity are a major public health concern. Since food is potentially important for the development of "metabolic memory", there is a need for more information on the type of nutrients causing adverse or toxic effects. We now know that metabolic alterations produced by excessive intake of some nutrients, drugs and chemicals directly impact epigenetic regulation. We envision that understanding how metabolic pathways are coordinated by environmental and genetic factors will provide novel insights for the treatment of metabolic diseases. New methods will enable the assembly and analysis of large sets of complex molecular and clinical data for understanding how inflammation and mitochondria affect bioenergetics, epigenetics and health. Collectively, the observations we highlight indicate that energy utilization and disease are intimately connected by epigenetics. The challenge is to incorporate metabolo-epigenetic data in better interpretations of disease, to expedite therapeutic targeting of key pathways linking nutritional toxicity and metabolism. An additional concern is that changes in the parental phenotype are detectable in the methylome of subsequent offspring. The effect might create a menace to future generations and preconceptional considerations.

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1. Introduction

The rise in the prevalence of obesity and diabetes is commonly attributed to changes in dietary patterns, but this view does not explain their epidemic nature and the observed contribution of mitochondrial dysfunction, oxidative stress, inflammation, metabolism and epigenetics. There is a clear need for systematic efforts, methodology and mechanistic support to repurpose data to the concept of nutrient toxicity. The harmful excess of nutrients is not limited to short-term effects and public health concerns are currently focused on the effect of chronic excessive intake (Kristanc and Kreft, 2016). Fatness and the lack of fitness are only part of the problem; the association of obesity and diabetes ("diabesity") with non-communicable diseases such as cardiovascular disease and cancer is also relevant (Camps et al., 2016). Indeed, obesity is not only a "problem" or a "condition", but also a chronic disease. Contrary to what the "weight-loss industry" suggests, permanent weight loss is out with the reach of the majority of people with obesity. The word here is "permanent". Humans are the only species that become overweight and remain overweight. Losing weight is easy but keeping it off is not. Indeed, the 5-year relapse rate for weight re-gain is consistently greater than 90% and those who do experience (or will experience) the health consequences of obesity deserve better efforts than being simply told to "eat less".

There is a non-genetic transmission of obesity and insulin resistance (IR) (Huypens et al., 2016). It is appealing to consider the hypothesis that a nutrient-associated spillover of energy-related metabolites and inflammatory products from affected cells into circulation provides signaling molecules that regulate gene expression through epigenetic mechanisms. Crosstalk between epigenetic signals and cellular metabolism in chromatin would represent a sensor and a mechanism to convert metabolic changes into stable patterns of altered gene expression (Katada et al., 2012). But how is gene expression reprogrammed in response to metabolic stimuli?

In addition to their anabolic and catabolic functions, metabolites influence many cellular processes including cell migration and differentiation, and they can interact directly with transcription factors and modulate transmembrane ion channels (Cai et al., 2008; He et al., 2004; Martinez-Outschoorn et al., 2016; Tannahill et al., 2013). These actions are triggered by specific metabolomic patterns that initiate transcriptional regulation in cells (Takahashi and Yamanaka, 2006). Genes do not remain automatically activated or depressed if the metabolic event is not persistent, and the putative regulators need to be continuously present to maintain the state of expression (i.e., specificity and memory are both necessary). Addressing basic questions such as which epigenetic factors are involved in energy metabolism and whether epigenetic mechanisms are causally linked to changes in metabolomic phenotypes, may lead to novel therapeutic opportunities. Future research combining data from several "omics" platforms is therapeutically attractive because diabetes and obesity are potentially reversible through nutritional and/or surgical interventions. These questions require a comprehensive, systems-level understanding of disease mechanisms and molecular alterations. For such a comprehensive topic we found narrative review best suited to summarize primary studies and to draw holistic interpretations contributed by our own experience and existing models. The information was retrieved through PubMed without restrictions using combined search terms described in Fig. 1 and explicit criteria for inclusion.

2. Regulating food intake and health

There is a widespread claim that nutritionists are forever changing their advice. This is conceivably due to the majority of data relying on "associations" because of the obvious shortcomings of observational studies and free-living experimental trials (Gorder et al., 1986; Masana et al., 1991; Menotti, 1983). Studies linking food and health should be seen as preliminary, dealing with uncertainties, unknown confounding factors and without proving the whole chain of events. Moreover, presumably through mechanisms evolved to ensure adaptability, the clinical application of dietary changes tend to converge towards non-significance in the long-term and compliance is low (Bravata et al., 2003; Foster et al., 2010; Gardner et al., 2007; Sacks et al., 2009; Shai et al., 2008). These considerations are relevant because the abrupt increase in the prevalence of obesity and type 2 diabetes mellitus (T2DM) is now challenging former hypotheses. The main concerns are the frequent coexistence of both conditions and the undisputed associations with atherosclerosis and cancer (NCD Risk Factor Collaboration, 2016; Twig et al., 2016). These are all age-related diseases entangled with diet and linked to IR or obscure relationships (Joven et al., 2007).

Hippocrates said, "*persons who are naturally very fat are more apt to die suddenly than those who are slender*" (Aphorisms 2:44). This concept and the *Discourses on the sober life* (1558–1562) by Alvise Cornaro were highly influential for centuries to endorse caloric restriction as a means to achieve longevity and health (Darby, 1990; Howell, 1987). Clearly, these observations rebut the notion that obesity is a recent phenomenon. Indeed, it probably occurs in waves associated with affluence. A correlation between reduction of food intake and extension of lifespan has been demonstrated in a wide range of organisms (Fontana and Partridge, 2015), and it is accepted that excessive food intake, when linked with sedentary behavior, may result in obesity, T2DM and other non-communicable diseases (Hamilton et al., 2007). However, it is not clear whether obesity is truly preventable because there are neither options that work well for most people nor real success in prevention. The question of whether obesity will reverse the life-span gains made over decades is an emerging issue.

2.1. Nutrient sensing and the distribution of energy

Changes in food intake may alter metabolic strategies to reset

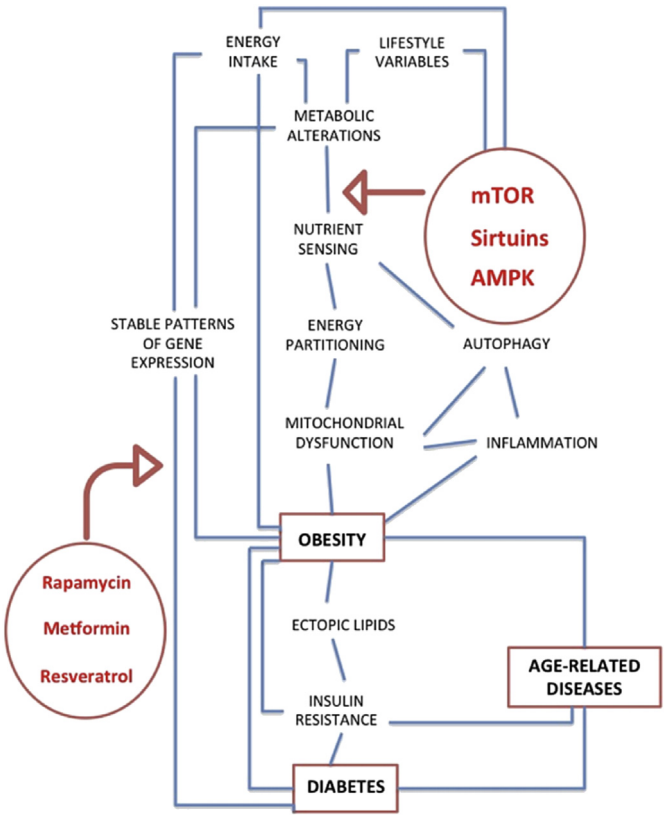

Fig. 1. Food intake participates in the pathogenesis of age-related diseases. Understanding the effects on key pathways linking nutrition, metabolism and disease may lead to preventive and therapeutic approaches. It will be important to determine the ability of insulin resistance and nutrient sensing to modulate gene expression in affected cells. mTOR, mechanistic target of rapamycin; AMPK, adenosine monophosphate activated protein kinase.

the distribution of energy into different tasks. These changes may or may not be relevant to disease, but, when excessive and continual, they may become toxic and trigger several deleterious events, including chronic inflammatory response, oxidative stress, mitochondrial dysfunction, adiposity, IR in skeletal muscle, and decreased insulin production by pancreatic β cells (Camps et al., 2016).

The perceived links and interactions (Fig. 1) can be interpreted as evidence that a growing number of chronic diseases are associated with IR in a vicious cycle. As tissues become unresponsive to insulin, more insulin is secreted by the pancreas and tissues grow ever more resistant. Determining the precise mechanisms of IR is complex because insulin is involved in the most fundamental processes of biology (Fitzgibbons and Czech, 2016).

Investigating the mechanisms of nutrient sensing is important to comprehend how food and metabolism are coupled to disease. In this context, several metabolic sensors have been well characterized. For example, the activated mechanistic target of rapamycin (mTOR) regulates events that modulate protein synthesis, insulin signaling, autophagic flux and mitochondrial function (Albert and Hall, 2015). Sirtuins are also controlled by nutrient availability and their activities regulate oxidative phosphorylation, fatty acid oxidation and mitochondrial oxidant production (Barger et al., 2015). Finally, adenosine monophosphate (AMP) activated protein kinase (AMPK) is a critical link between nutrients and health and regulates metabolic pathways that increase energy supplies and reduce energy demand (Hardie et al., 2016). These sensors have been successfully targeted pharmacologically. Notably, metformin has been used for decades to activate AMPK and, if epidemiological evidence is confirmed, has saved more lives from cancer than any other drug in history (Menendez and Joven, 2014). In the overall process of nutrient sensing, it is important to highlight the protective role of autophagy, the controlled degradation and recycling of cellular components. In particular, the specific autophagic targeting of dysfunctional mitochondria (mitophagy) eliminates oxidative stress and mitochondrial damage in obesity and T2DM. Mitophagy appears to be a crucial cellular process for the conversion of functionally mature mitochondria to an immature state and vice versa during reprogramming and differentiation, respectively (Vazquez-Martin et al., 2016). Autophagy also mediates exercise-induced increases in muscle glucose uptake, protects β cells against endoplasmic reticulum stress and promotes adipocyte differentiation. Conversely, decreased autophagic activity is implicated in the progression of obesity to T2DM (Barlow and Thomas, 2015; Sarparanta et al., 2016). Given that sirtuins, mTOR and AMPK all regulate autophagy and autophagy activators have demonstrable effects on age-related diseases, the search for activating compounds is an emerging field of investigation (Hubbard and Sinclair, 2014; Imai and Guarente, 2014; Kasznicki et al., 2014; Menendez and Joven, 2014; Menendez et al., 2014).

2.2. Insulin resistance: a multifactorial condition

It is generally accepted that IR is associated with overnutrition and the systemic response of poorly known metabolic feedback loops, but its role in causing disease might be controversial. For some investigators, proposed factors causing IR would work through one or more mechanisms sequentially triggered by excessive food intake: increased inflammation, changes in lipid metabolism, and changes in the gastrointestinal microbiota (Johnson and Olefsky, 2013). To pursue this causal chain may be scientifically sound and the amount of basic and preclinical knowledge supporting this hypothesis is compelling; however, this approach is clinically ineffective.

Pharmacologic therapy based on targets relevant to inflammation-induced IR has marked effects in rodents but has been disappointing in humans. This has been shown for tumor necrosis factor-α blocking agents (Bernstein et al., 2006; Solomon et al., 2011; Stanley et al., 2011), IL-1β inhibitors (van Asseldonk et al., 2011) and aspirin (Goldfine et al., 2010; Raghavan et al., 2014). More targeted anti-inflammatory approaches may improve efficacy in the future, but the role of tissue inflammation in causing IR remains speculative. For example, the usefulness of ligands for peroxisome proliferator-activated receptors (PPARs) has been curtailed because of potential toxicities, even before the precise mechanisms of action are known (Rull et al., 2014). Lipid accumulation in multiple tissues and the associated metabolic disturbances might be considered a biological marker, but a consequence rather than a contributing factor to IR (Calvo et al., 2015). Similarly, in mice, the gastrointestinal microbiota influences energy metabolism and systemic inflammation (Blumberg and Powrie, 2012; Burcelin et al., 2012; Henao-Mejia et al., 2012), and may produce bioactive metabolites, especially short-chain fatty acids, acetate and bile acids derivatives (Beltrán-Debón et al., 2015; Kau et al., 2011; Nicholson et al., 2012). Data from experimental models point to the potential of microbiota to modulate obesity and insulin sensitivity; however, the same data fail to consider that humans live in non-sterile conditions, are genetically heterogeneous, consume a range of different diets and have microbiota that is frequently perturbed by the administration of antibiotics.

Moreover, clinical measurements of insulin sensitivity are challenging and considerable variation exists in healthy individuals and in patients. Methodological flaws are not discarded because

laboratory procedures do not measure how individual tissues respond to insulin and most studies are performed with the patient fasting. These measurements are also limited by the observation that IR changes dramatically over the course of a day, from day to day, and in response to exercise and the quality and quantity of food intake, among other factors (Zaccardi et al., 2016). The use of indices combining glucose and insulin levels to predict insulin sensitivity may represent a simplistic assessment of actual glucose metabolism. Some investigators claim a significant (>10%) false-negative rate in assessing IR compared with glucose tolerance testing. These indices can certainly be used in clinical studies with a secondary interest in glucose metabolism, but considering and balancing possible inaccuracies (Lee et al., 2008; Martinez-Hervas et al., 2011; Pisprasert et al., 2013). Specifically, genetic influence, physical fitness and weight are recognized confounding factors that only partially explain why a significant proportion of severely obese patients are insulin sensitive. Thus, if obesity is not the cause of IR, the search for underlying factor(s) causing both obesity and IR needs to continue.

2.3. Mitochondria are not only providers of energy, but also signaling units

The detrimental effects of IR are associated with liver disturbances, and the deposition of lipids into non-adipose tissues interferes with insulin signaling. Several findings suggest that mitochondrial dysfunction is a cause rather than a consequence of IR. The distinction is important because diabetes and obesity are strong predictors of non-alcoholic fatty liver disease, which is characterized by damaged mitochondria and the progressive inhibition of fatty acid oxidation. Several mechanisms apparently converge to modulate the differential response of energetic and biosynthetic intermediates. The overall picture is unclear, but mitochondrial energetic efficiency, epigenetic signals and nutrient-sensing pathways are necessarily combined (Rull et al., 2009; Fontana and Partridge, 2015) to explain increased glucose oxidation, decreased glucose formation, mitochondrial dysfunction and the accumulation of metabolites that disturb glucose transport activity (Finkel et al., 2015).

In this context, the consideration of obesity and diabetes as likely mitochondrial diseases is clinically relevant. For instance, mitochondrial activity, inflammation and the infiltration of macrophages influence both the extent of atherosclerosis and the pathogenesis of cancer. All of these diseases involve mitochondria and are characterized by a decline in metabolic homeostasis and gene deregulation. This association provides grounds to justify that the detection of an unhealthy metabolic status requires novel, and possibly crucial, testable therapeutic approaches, especially those: 1) modulating the ability of cells to alter their metabolism to different energy requests, 2) therapeutically targeting glycolysis, and 3) directly modulating mitochondrial activity (Suliman and Piantadosi, 2016; Zaccardi et al., 2016a).

In the regulation of metabolism and energy production, mitochondria receive information from other parts of the cell and relay information via retrograde signaling molecules that are not of mitochondrial origin; in particular, reactive oxygen species (ROS), Ca^{2+}, and cytochrome C (Goodwin et al., 2009; Houtkooper et al., 2011; Sethe et al., 2006). Some investigators consider that mitochondrial ROS have evolved as a key communication method between the mitochondria and the cell to regulate homeostasis and normal cellular function (Sena and Chandel, 2012). Moreover, new findings suggest that mitochondria regulate metabolic homeostasis at the cellular and organismal level via peptides encoded within their genome. The mitochondrial transcriptome is a highly complex system and several mitochondria-derived peptides have been discovered. One such peptide, humanin, regulates critical processes such as aging, inflammation, and stress resistance (Guo et al., 2003). A second peptide, derived from mitochondrial 12S rRNA (MOTS-c), is involved in regulating metabolic homeostasis (Lee et al., 2015). Mitochondria modulate carbohydrate metabolism (Woo and Shadel, 2011) and MOTS-c is proposed as a key endocrine signal that systemically regulates in vivo glucose metabolism and muscle insulin action. MOTS-c has physiological similarities to the anti-diabetic metformin in terms of regulating glucose utilization, mitochondrial and fatty acid metabolism, and body weight (Ferguson et al., 2007) by targeting the folate cycle and one-carbon metabolism (Corominas-Faja et al., 2012; Ducker et al., 2016) and signaling via AMPK (Shaw, 2013). These data support an active role for mitochondria in the regulation of metabolism and weight homeostasis. Moreover, the significance of these peptides in the regulation of obesity, diabetes, exercise, and longevity represents a new frontier in mitochondrial signaling.

The appreciation of mitochondria as signaling organelles is also illustrated by very recent findings (Morton et al., 2016). The authors reasoned counterintuitively that because a substantial and stable proportion of individuals remain non-obese despite modern affluence, there might be genetic mechanisms for resistance to obesity and diabetes or genes that contribute to healthy low adiposity. To address this, the authors used a polygenic lean mouse line generated through selection for low adiposity over 60 generations (Morton et al., 2005) to identify mitochondrial thiosulfate sulfurtransferase as a beneficial regulator of adipocyte mitochondrial function that may have therapeutic significance for individuals with T2DM.

3. Metabolism and epigenetics: insights for an alternative working hypothesis

Systems biology encompasses many different approaches to systematically identify, analyze, control, and design metabolic systems. The convergence of data from these methodologies indicates that metabolites, which are directly related to the visible phenotype of biological systems (Novère, 2015), are organized in genetic- and signaling-regulated metabolic networks.

The relationship between epigenetics and metabolomics may provide immediate clinical applications; however, the extent to which epigenetic information is transmitted and whether the metabolic environment modulates this information are unanswered questions. Exploring how metabolic pathways are coordinated in diabesity might clarify the impact of inflammation, metabolic factors and nutrient excess on epigenetic pathways affecting genomic regulation (Finkel, 2015; Hernández-Aguilera et al., 2013; Katada et al., 2012). Key metabolites can accumulate in the plasma over time and if this is maintained specific metabolites have the capacity to regulate both epigenetic status and energy supply (Riera-Borrull et al., 2016; Rodríguez-Gallego et al., 2015; Menendez et al., 2016). Precisely how the effects of inflammation and mitochondrial dysfunction collectively work is unknown, but future investigations on chronic diseases should consider the consequence of excessive food intake for the balance of associated cellular pathways and biological mechanisms (Horng and Hotamisligil, 2011; Locasale, 2013, Fig. 2). The metabolite-driven changes in epigenetic regulation are mechanistically attractive and are supported by recent concepts that have revolutionized our understanding of chromatin-based epigenetic mechanisms and the relationship with gene regulation in the pathogenesis of human diseases. Nevertheless, interpreting the biological context and integrating data from metabolite measurements in clinical, epigenetic-guided studies is not an easy task (Dumas, 2012). The challenge is to provide biological explanations in humans before

and after therapeutic intervention.

3.1. Intermediates of metabolism influence chromatin structure: mechanisms of epigenetic inheritance

Epigenetic mechanisms control chromatin structure through posttranslational modifications, histone variants, RNA interference and DNA methylation (Fig. 3). Several interacting components of chromatin regulation, including enzyme kinases, acetyltransferases and methyltransferases, use cellular metabolites as sources of phosphate, acetyl or methyl groups, respectively. It is conceivable that these enzymes may interpret the metabolic state of a specific cell, but the level of a metabolite is unlikely to be the only determinant of enzymatic activity. Information on intracellular concentration and the dynamic changes in affinity or competition is sparse (Katoh et al., 2011), and diffusion-controlled reactions are unlikely in the viscous medium of nuclei. The concentration of proteins and DNA may reach 200 g/L and the sensitivity to metabolic alterations is not equally distributed in chromatin regions, favoring the heterogeneous occurrence of multiprotein complexes channeling reactions (Wei et al., 2011).

Changes in nutrition can impact gene expression patterns and memory of former metabolic disturbances may be involved in the progression of obesity and metabolic disease as shown in epidemiological studies examining the offspring of extreme nutritional deprivation during the periconceptual period or during fetal development. This has been extensively studied in cohorts suffering the Dutch Winter Hunger in 1944 (Kaati et al., 2002; Painter et al., 2005; Heijmans et al., 2008). DNA methylation signatures apparently link prenatal famine exposure to growth and metabolism and there are evidences suggesting that epigenetic modulation of pathways by prenatal malnutrition may promote an adverse metabolic phenotype in later life (Tobi et al., 2014). Similar data come from studies of offspring born during the severe Chinese famine in 1958–1961 (Li et al., 2010) but the negative findings obtained during the Siege of Leningrad suggest caution in comparing retrospective analysis with different exposure windows (Stanner et al., 1997). As discussed below (section 4.1) short-term high fat overfeeding may suggest transient epigenetic regulation in humans (Jacobsen et al., 2012).

The mechanisms directing the inheritance of these diseases are unknown but epigenetics is an attractive candidate in animal models. For example, studies in mice carrying the viable yellow allele of agouti (Avy) indicate that a specific mammalian gene can be subjected to germ-line epigenetic change (Cropley et al., 2006). The ablation of key epigenetic enzymes in mice also mimics the heritable effects of metabolic disturbances: mice with an inactive allele of the gene encoding the histone demethylase KDM3a

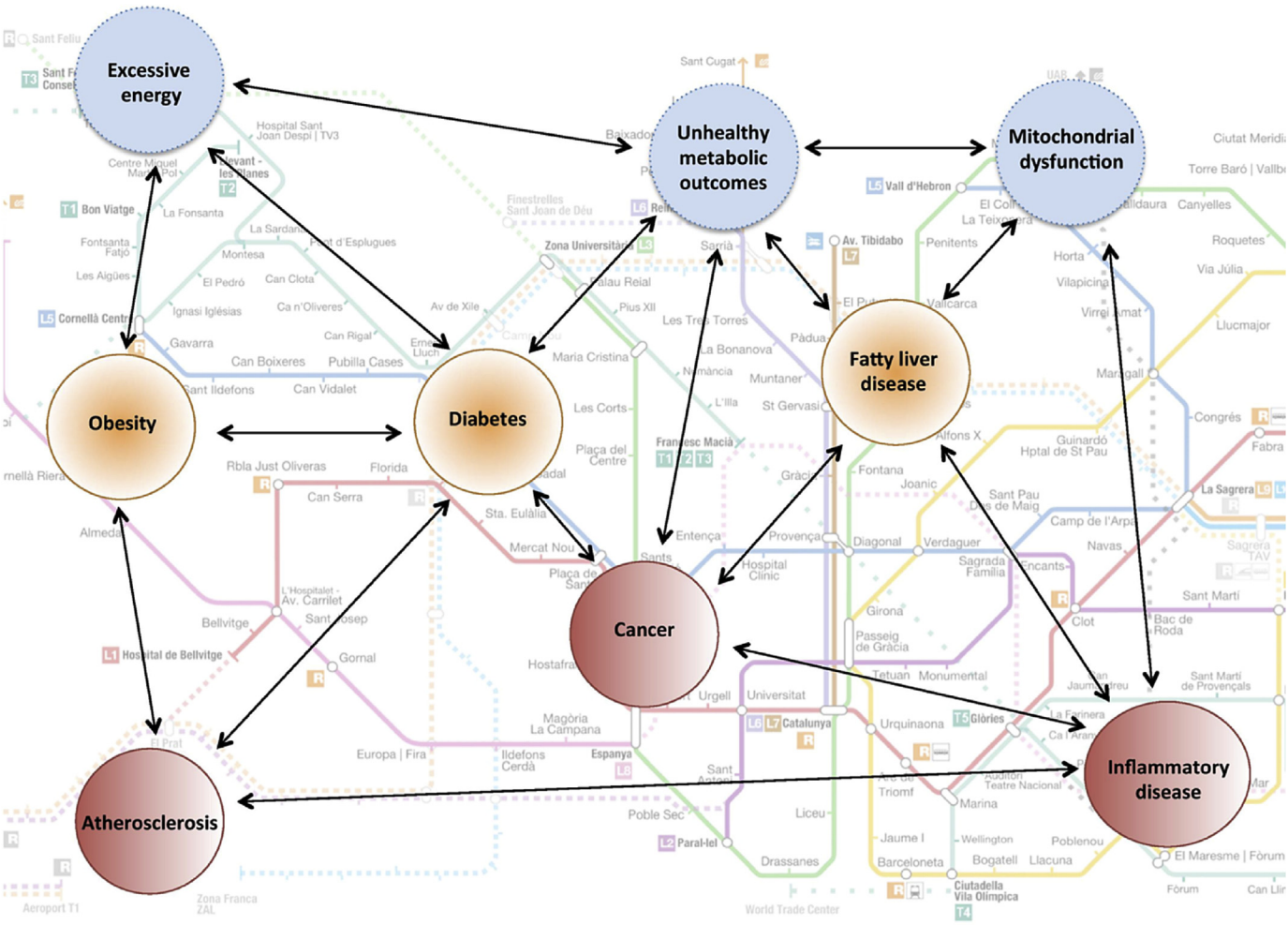

Fig. 2. The study of the complex network of cellular pathways and biological mechanisms altered by excessive food intake requires a detailed roadmap. The overall setting of dietary-favored diseases is inflammatory and entails the modulation of mitochondrial function, profound metabolic alterations and changes in epigenetic events. Chronic diseases tend to converge from disturbances in which food is a major contributing factor.

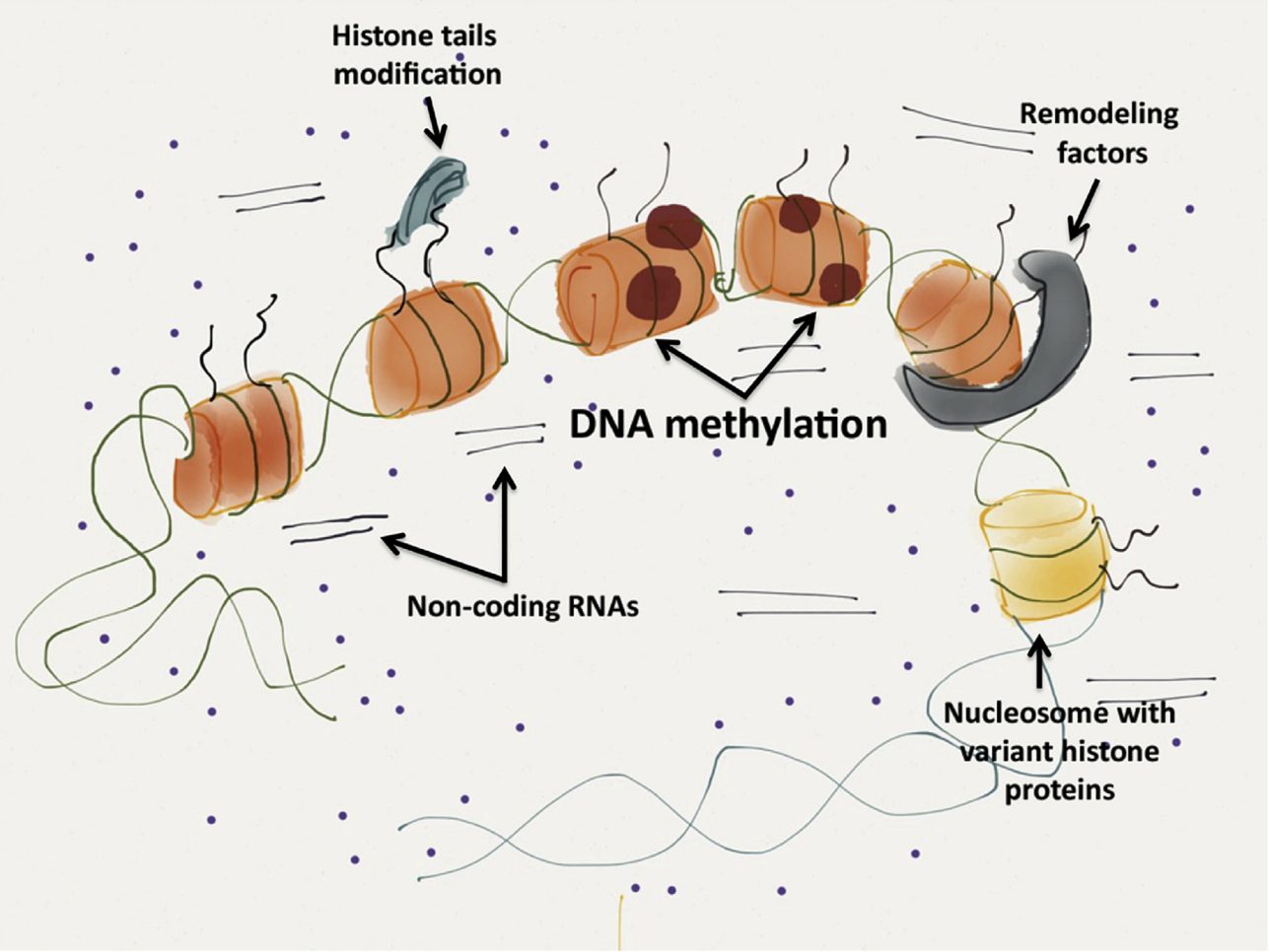

Fig. 3. Nutrition may influence epigenetic mechanisms. Epigenetics is the study of heritable changes in gene function not explained by changes in the primary DNA sequence. DNA modifications may include changes across the entire organism or may operate on a tissue-specific level.

become obese in adulthood and have increased levels of circulating lipids (Tateishi et al., 2009). A defect in the genes encoding metabolic enzymes also directly influences the enzymatic function of epigenetic regulators in cells with major metabolic alterations (Cuyàs et al., 2015; Menendez et al., 2016a). Some interesting questions arise as to how plastic is the genome to dietary changes, what magnitude of metabolic stimuli is required to switch between metabolic states, and whether these metabolic-triggered epigenetic changes are reversible.

During the germline cycle of development, the genetic material is replicated in each round of cell division. Information not replicated in the DNA sequence—epigenetic information—is lost in each generation. At least for epigenetic marks caused by DNA methylation, the information is not completely erased during germline development and may remain in the promoters of protein-coding genes resistant to demethylation (Tang et al., 2015). The evolution of mechanisms conferring long-term epigenetic memory, and that feedback between different epigenetic mechanisms contribute to long-term inheritance, are plausible concepts (Klosin and Lehner, 2016); that is, each epigenetic mechanism alone is unlikely to be used to transmit information reliably for more than one generation. Small RNAs are potential carriers of epigenetic information in animal germlines and their levels vary depending upon parental exposure to high-fat diets (Grandjean et al., 2015). Overexpression of histone demethylases during spermatogenesis alters histone modifications in sperm that impair offspring health transgenerationally (Siklenka et al., 2015). Indeed, the repression of repetitive DNA and transposons is likely the main function of DNA methylation and a barrier for the transmission of information. However, at least in mammals, this mechanism is insufficient and may also influence the expression of neighboring genes through generations (Blewitt et al., 2006). In flies, the specificity in the transcriptional response to low glucose diet is detected in the next generation before the heterochromatin resets. If demonstrated in humans, this mechanism could explain the inheritance of short-term epigenetic effects. In fact, there is evidence in mice and humans that a high glucose paternal diet can trigger obesity in offspring through deregulation of paternally inherited heterochromatin (Öst et al., 2014). Therefore, germline-transmitted mechanisms are conceivable and future research addressing if and how the diet-induced metabolic perturbations of obesity and diabetes can alter epigenetic information is warranted.

3.2. Energy metabolism and one-carbon metabolism: a targeted metabolomic approach

Several metabolites generated by mitochondrial respiration are implicated in stochastic chromatin remodeling. This is consistent with studies indicating that glucose and body weight homeostasis require an efficient management of energy. For instance, citrate can

modulate the global levels of histone acetylation, and other metabolites are obligatory co-substrates (α-ketoglutarate) or potent inhibitors (succinate) of relevant mitochondrial enzymes (Benayoun et al., 2015; Chin et al., 2014; Mentch et al., 2015). Excessive calorie intake leads to mitochondria fragmentation. Mitochondrial dynamics is abnormal in T2DM and the prevention of excessive mitochondrial division ameliorates insulin function. In the obese setting, changes in mitochondrial dynamics control appetite- and diet-regulated signaling pathways in neurons (Roy et al., 2015). Additionally, DNA methylation influences the expression of genes affecting energy homeostasis and is associated with an imbalance in mitochondrial dynamics and IR (Gut and Verdin, 2013).

In contrast to the genome, which remains unchanged in most cells, the combination of all chromatin modifications of a given cell type directs a unique gene expression pattern that is shaped by nutrition. Energy metabolism is important but distinguishing one-carbon metabolism is essential to understand the methylation of nucleic acids (Barth and Imhof, 2010). Accurate measurements of implicated metabolites require targeted metabolomics (Fig. 4) to establish the direct effect of one-carbon (and energy) metabolism on the output of a defined methylated state (Mentch et al., 2015).

Methylation of cytosine is the predominant epigenetic modification of DNA in vertebrates and DNA methylation inhibits the binding of transcription factors or recruits proteins with repressive properties (Tate and Bird, 1993; Bell et al., 2011). Methylation status is dependent upon changes in the enzyme activity of methyltransferases and demethylases, and alterations in genes that encode these enzymes are common in dietary-related diseases (Dawson and Kouzarides, 2012). S-adenosylmethionine (SAM) is the universal methyl donor in cells, yielding S-adenosylhomocysteine (SAH), and links metabolism and epigenetic status of cells (Gut and Verdin, 2013). Whether changes in the levels of SAM or SAH are sufficient to alter methyltransferase activity in vitro is controversial, but in mice threonine catabolism affects methylation status (Shyh-Chang et al., 2013) through indirect pathways involving energy production and acetyl-coA metabolism (i.e., pyruvate and glycine metabolism). Moreover, deprivation or restriction of essential amino acids causes profound transcriptional and metabolic responses (Anthony et al., 2013). In particular, dietary restriction of methionine produces responses that improve biomarkers of metabolic health, limit fat accumulation, and even prolong lifespan in rodents (Orentreich et al., 1993; Orgeron et al., 2014).

3.3. Metabolites are signaling molecules

Metabolites and transcriptional regulators are likely connected through as yet undefined mechanisms. Several G protein-coupled receptors (GPCRs) that impact immunity and inflammation are activated by intermediates of metabolism. For example, lactate, produced in the cytoplasm and secreted through the plasma membrane by solute carrier transporters, is recognized as a bioactive molecule with profound effects on immune and stromal cells. Although blood concentration of lactate is around 2 mM, it can reach up to 10 mM in inflammatory sites and up to 30 mM in tumor tissue. Among other effects, lactate is considered the driving force of tumor-associated macrophage development during epithelial-to-mesenchymal transition (Del Barco et al., 2011; Su et al., 2014). Lactate signaling has been also implicated in different features of chronic inflammatory diseases; for example, increased lactate concentration favors its internalization in activated T cells through $CD8^+$ and $CD4^+$ T cell-specific transporters, which causes inhibition of glycolysis and loss of responsiveness to chemokines and partly explains how T cells are entrapped in inflamed tissue (Haas et al., 2015). Acting as a ligand, signaling via lactate modulates insulin-induced reduction of lipolysis by binding to its cognate receptor, Gpr81, which is primarily expressed in adipocytes (Liu et al., 2009). Once considered a consequence of the lack of oxygen, it is now known that lactate is formed continuously in the presence of oxygen as an active part of mitochondrial metabolism (Hashimoto et al., 2006). Over the years, many laboratories have endeavored to identify ligands for orphan GPCRs (i.e., receptors unmatched to known ligands), but to date more than 100

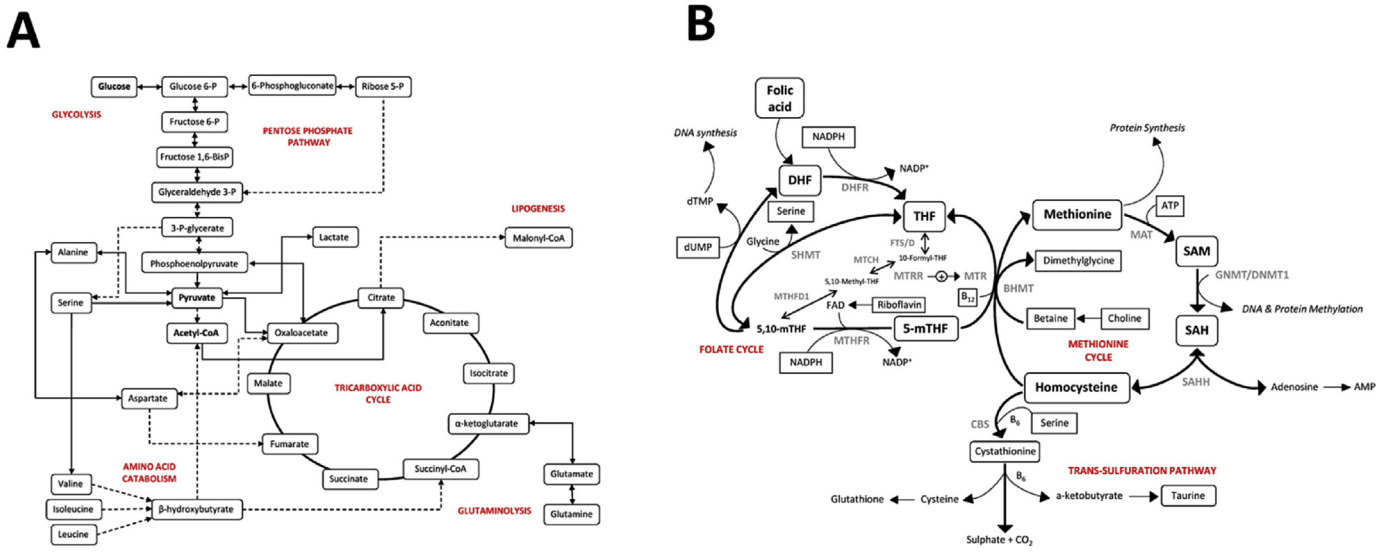

Fig. 4. Targeted metabolomics may increase the power of associations. Metabolites that regulate chromatin participate in pathways involved in intracellular energy balance (A) or enter one-carbon metabolism generating methyl donors (B). The tricarboxylic acid cycle links catabolic and anabolic pathways; glycolysis and β-oxidation generate acetyl-CoA, whereas removal of acetyl-CoA from mitochondria during glucose excess by the citrate shuttle fuels lipogenesis. Folate enters a cyclic reaction generating methyl donors for DNA methylation. BHMT, betaine—homocysteine S-methyltransferase; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; FAD, flavin adenine dinucleotide; GNMT, glycine *N*-methyltransferase; MAT, aminomethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, Methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, Tetrahydrofolate; UDP, uridine diphosphate.

of these receptors remain orphans. For example, Gpr91 is a receptor for succinate and Gpr80/99 is a receptor for alpha-ketoglutarate, revealing that dicarboxylic acids are active signaling molecules (He et al., 2004; Gonzalez et al., 2004). It is also known that ketone bodies (beta-hydroxybutyrate), produced mainly in the liver as a circulating glucose-sparing energy source, can also serve as signaling molecules in neurons (Shimazu et al., 2013). Other intermediates of glycolysis, the citric acid cycle and undoubtedly products of cellular fatty acid metabolism might also play significant roles in non-metabolic activities (Haas et al., 2016). For example, the polarization of macrophages into M2 cells is important because of their role in wound healing. Two critical pathways regulate this phenomenon: glutamine-related metabolism and the UDP-GlcNAc pathway. These are major connecting hubs between cellular metabolism and signaling. Certain dietary conditions might lead to an interplay between macrophage polarization, metabolism, and mTOR signaling, with the ability to manipulate macrophage function in clinically relevant settings (Wellen and Thompson, 2012; Jha et al., 2015). Similar concepts may be applied to the cancer metabolism program and the responses of healthy tissues during nutritional stress; the limitation of one energy source, glycolysis or mitochondrial metabolism, results in tissue vulnerability to the inhibition of the other energy source (i.e., treatment of metabolic diseases should include both factors).

4. Metabolism and DNA methylation: a search for therapeutic and diagnostic targets in obesity and diabetes

The discovery of endogenous metabolites signaling cell-fate decisions demonstrates the integration of multiple cellular functions. DNA methylation is the only epigenetic mark with strong mechanistic support for both heritability and response to dietary changes (Maddocks et al., 2016; Mentch et al., 2015; Mentch and Locasale, 2016; Rodríguez-Gallego et al., 2015), and represents a metabolo-epigenetic link that needs to be translated into clinical investigations. In particular, the measurement of metabolic states can be correlated with chromatin states and gene expression.

DNA methylation is currently used to construct models for predicting chronological age at a population level because the regulation of the chromatin landscape can alter lifespan (Benayoun et al., 2015). These models have practical implications for studying the role of methylation in age-related diseases, and to explain the association of complex metabolic and inflammatory states with early onset of diseases linked to aging, including atherosclerosis and cancer (Hannum et al., 2013; Horvath et al., 2015). Human immunodeficiency virus infection is characterized by early onset of age-related diseases (Deeks, 2011; Alonso-Villaverde et al., 2013) that are associated with changes in age-associated methylation sites (Gross et al., 2016). Also, obese patients not undergoing gastric bypass surgery (i.e., no changes in the metabolic state) have a worse long-term survival as they age than among those undergoing surgery, who present dramatic and beneficial metabolic changes (Davidson et al., 2016). These effects have been observed in the complexity of a whole organism, but it is now time to explore how specific metabolic changes may affect chromatin, transcription and consequences in health.

The chromatin landscape is dynamically configured throughout life, and changes in chromatin marks, defined as “epigenetic drift”, occur in response to nutritional, metabolic, environmental or pathological signals. Do changes in diet or in metabolism that are associated with obesity and diabetes lead to epigenetic drift? This has yet to be fully established in humans, but, if confirmed, known dietary manipulation or drugs that regulate methylation might be used to slow the aging process and influence the onset of age-related diseases. Alternatively, the assessment of DNA methylation might increase the accuracy of biomarkers for evaluating the risk of disease and may provide a mechanistic basis for chronic diseases. More importantly, transmitted molecules beyond DNA can modify human development (e.g., genomic imprinting). To which extent is phenotypic information transmitted? Might diet-induced changes in metabolic or phenotypic traits in one generation affect the next?

4.1. Methodological source of errors: critical reflections

To prove the hypothesis of diet-induced adverse epigenetic drifts is a demanding task. For example, epigenetic processes continuously interpret dietary-induced metabolic alterations and metabolomic studies and epigenome mapping should be concurrent. The choice of when to initiate the analysis is important because chronic diseases evolve through a sequence of metabolic stages over time, from a period when alterations are barely detectable to a stage with complications in multiple tissues. It is also important to consider which tissue to investigate (Rönn and Ling, 2015), but to be worthwhile, markers should be explored in blood where metabolic changes are readily detected and probably affect more rapidly circulating cells. This is plausible in blood cells in the context of DNA methylation involved in insulin secretion (Toperoff et al., 2012) and insulin sensitivity (Nilsson et al., 2014). Blood cells are also practical to explore differential DNA methylations during exposures to high-energy diets (Ling et al., 2007; Jacobsen et al., 2012).

The metabolic changes in plasma reflect the metabolic state of all body organs and each metabolite may be a functional intermediate trait or a correlated biomarker in relation to obesity and diabetes. The choice of analytical platforms and applications is also important and most are currently suitable to modern clinical laboratories. Applications are currently available for nuclear magnetic resonance and gas (GC) and liquid (LC) chromatography coupled to different mass spectrometry (MS) detectors, such as matrix-assisted laser desorption and ionization/time-of-flight (MALDI-TOF), quadrupole time-of-flight (QTOF) and triple quadrupole (QqQ) mass spectrometers. The analytes may be defined in advance to increase quality, but this comes at the cost of missing potentially interesting metabolites (Menendez et al., 2016; Beltrán-Debón et al., 2015). We favor an overall design similar to that suggested in Fig. 5. Current methods make it economically viable to analyze the metabolic profile of thousands of samples over extended periods of time. The main constraint of this scaled-up process is that samples cannot be run in a single analytical batch. To explore energy metabolism and mitochondrial status and function, GC-EI-QTOF-MS is the method of choice. Conversely, LC-MS/MS methods are preferred for the quantitative analysis of representative metabolites in one-carbon metabolism. Metabolically-related inflammatory stimuli should include secreted cytokines, growth factors and metalloproteinases, but there is no one individual marker that provides sufficient information (Puig-Costa et al., 2014).

The objective of the analysis is to compare changes in metabolism and DNA methylation with chances of error lower than 5% and criteria meeting genome-wide significance after Bonferroni correction for all tested loci and all metabolic traits. All methods should be combined in the quest of the ultimate goal, which is to provide epigenetic associations with a dynamic view of the metabolic phenotype (i.e., capturing the metabolome in its functional interactions). Longitudinal studies (i.e., stable genetic contribution) are currently favored with respect to other designs. A before-after design adds further power and reliability in patients with diabesity through successful dietary measures and bariatric surgery. Currently, most methods associated with global DNA methylation

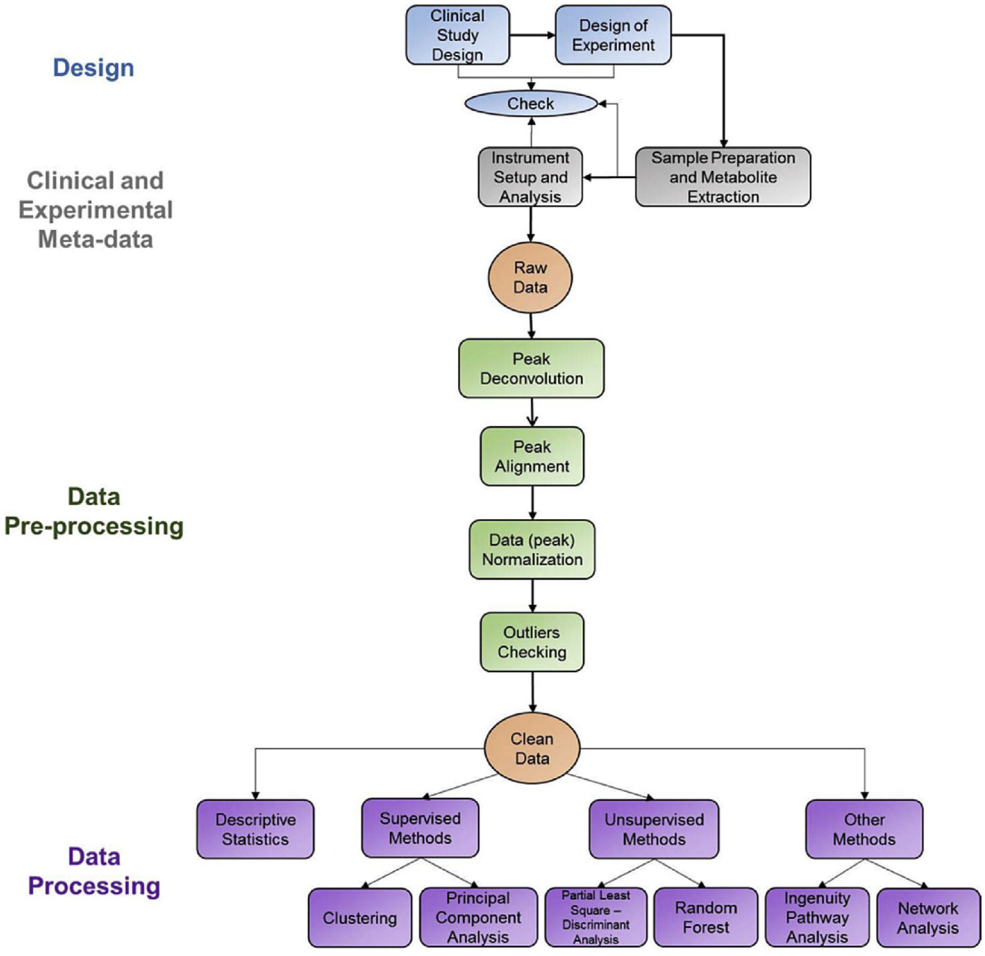

Fig. 5. The need for defined tasks in experimental analysis. The expected complexity in the interpretation of the relationships between metabolism and epigenetics requires activities arranged as a workflow. The figure depicts basic steps.

are performed with protocols from the reagents' manufacturers with minor variations, but some mainly detect variations in repetitive DNA or transposons (Fernández-Arroyo et al., 2016). There are also available methods to detect changes in individual methylated cytosine guanine dinucleotides (CpGs) and differentially methylated regions (DMRs) through array-based, bisulfite-converted, DNA methylation analysis (Ambatipudi et al., 2016; Chen et al., 2016; Glossop et al., 2016; Louie et al., 2016; van den Dungen et al., 2016).

This technique provides a subset of all potentially methylated sites in the genome. Because it is more selective and because of the limited tissue choices, the CpG—metabotype associations should be likely limited to processes of DNA methylation that are not cell-type specific. However, interpretation requires being unambiguous to distinguish between true functional associations and a mere correlation, and the need for complementary approaches is likely. Full sequencing may be used but the resulting loci will require further validation since polymorphisms in the detected region may provide potentially confounding associations. Typically, PCR products obtained through a Sequenom EpiTyper Assay are pre-treated and analyzed by MALDI-TOF MS. In particular, mechanisms linking the DNA methylation of certain genes and not others have yet to be fully established. Conversely, the replicated methylation sites could be within the proximity of known genes with a possible regulatory role in methylation. It is therefore necessary to compare global methylation profiling of normal tissue samples from publically available datasets, with DMRs. Finally, to establish correlations between DNA methylation and gene expression changes, confirmation is required at the RNA level. In summary, the combination of epigenetics and metabolomics involves decoding of the genome information, transcriptional status and later phenotypes. Data should be obtained in the complexity of a whole organism and bioinformatic analysis will be required (Cordero et al., 2015; Noureen et al., 2015; Preussner et al., 2015) with the objective of finding mechanistic links between the pathological outcomes and specific chromatin-based mechanisms.

4.2. Transgenerational epigenetic inheritance of obesity and diabetes: current evidence in humans

The reviewed findings raise a crucial question. How do specific nutritional or surgical interventions affect chromatin and transcription and lead to beneficial effects on metabolic health? The role of the epigenome in the development of obesity and diabetes, although plausible, is not yet established. Epigenetics is a comparatively new field of research and the first steps are now being taken to identify potential biomarkers to predict an individual's obesity/diabetes risk before the phenotypes develop. It is also clear that several epigenetic marks are modifiable, which implies that there is the potential for interventions to transform or rescue unfavorable epigenomic profiles (Kirchner et al., 2013; Cheng and Almeida, 2014; de Mello et al., 2014; van Dijk et al.,

2015). It should be clarified that in this review we only discuss the transmission of epigenetic alterations that occur in the absence of direct exposure to any specific environmental factor (i.e., the embryo is not exposed during gestation). The development of nutritional strategies and dedicated pharmaceuticals is plausible. For example, dietary manipulations may contribute to promoter-specific changes in DNA methylation and several clinical trials are investigating the efficacy of epigenetic modifiers already in the marketplace (Cooney et al., 2002; Foulks et al., 2012; Waterland et al., 2006; Weaver et al., 2005). The few studies that have assessed DNA methylation profiles in relation to weight loss interventions indicate substantial variation over time and future research will require establishing the relationships between DNA methylation and metabolomic profile considering potential inter-individual variation.

Interestingly, a recent issue of *Diabetes Care* is mostly devoted to support bariatric surgery as a new treatment option in the management of T2DM. The recommended guidelines endorse interventions initially designed to promote weight loss as an intentional treatment to improve glucose homeostasis, which is more effective than any known pharmaceutical or behavioral approach. Postoperative improvements in metabolic control occur rapidly and are out of proportion to weight loss, yet the physiological and molecular mechanisms underlying these beneficial glycemic effects remain unknown (Cefalu et al., 2016).

Children of obese fathers are at higher risk of developing obesity. Economic status and access to food are not clearly associated and some findings provide insight into how obesity may propagate metabolic dysfunction to the next generation. In particular, changes in metabolism lead to changes in chromatin with the potential of transgenerational inheritance. It has been recently found that whereas spermatozoal histone positioning is unaltered between lean and obese men, DNA methylation patterns are markedly different. Moreover, the sperm methylome is altered after bariatric Roux-en-Y gastric bypass (RYGB) surgery shortly after the procedure (Donkin et al., 2016). Thus, weight loss-induced changes in methylation are reversible. In this particular study, the consequences for the offspring were not examined, but it is known that children born after maternal bariatric gastrointestinal bypass surgery are less obese and exhibit improved cardiometabolic risk profiles carried into adulthood when compared with siblings born before maternal surgery (Guénard et al., 2013). Both studies indicate that human gametic epigenetic variation can be related to nutritional status and that changes in parental phenotype are detectable in the methylome of subsequent offspring. Interestingly, these methylation patterns are detected in circulating leukocytes.

Patients before and after weight-loss surgery have also been studied to demonstrate that methylation density in the leptin promoter may be a control level for cell type-specific leptin expression, and a main player in the regulation of energy homeostasis (Marchi et al., 2011). A study in obese women before and after RYGB surgery suggests that dynamic changes in DNA methylation may be an early event that orchestrates metabolic gene transcription involved in the regulation of insulin sensitivity in human obesity (Barres et al., 2013). Other studies reporting changes in methylation signatures before and after gastric bypass (i.e., with a substantial modification in metabolic state) provide additional evidence for the role of treatment-induced epigenetic organ remodeling in humans (Ahrens et al., 2013; Horvath et al., 2014; Benton et al., 2015; Dahlman et al., 2015; Nilsson et al., 2015).

Therefore, available data provide evidence that diet-induced metabolic changes might influence preconceptional behavior (Kirchner et al., 2013; Patti, 2013), but mechanistic insights are not sufficient to explain the overall picture. Other environmental stressors for the offspring should also be studied in combination, especially those associated with intra-uterine exposure.

4.3. Genetic predisposition in response to bariatric surgery: changes in the metabolome

It is apparent that there have to be internal underlying causes that influence obesity in addition to the environmental factors and excessive food intake. Genetic factors are known to play a role in weight gain and obesity and genome-wide scans have revealed several genes with altered transcriptional activity and/or epigenetic variations in obesity-related tissues (Levian et al., 2014). It is therefore likely that genetic factors may also be involved in how an individual loses weight following bariatric surgery. This issue, however, is unclear.

There are few studies about the effect of single nucleotide polymorphisms in body weight, body composition or weight gain during a follow-up period after bariatric surgery. Among others, variants in fat mass and obesity-associated (FTO) gene, leptin receptor gene, fatty acid amide hydrolase, Bsm1 vitamin D receptor, ghrelin receptor, and melanocortin 4 receptor are known to predispose for response to surgical intervention (de Luis et al., 2010a; de Luis et al., 2010b; Matzko et al., 2012; Hatoum et al., 2013; Mägi et al., 2013; Moore et al., 2014; Alexandrou et al., 2015; Rodrigues et al., 2015; Bandstein et al., 2016). However, these association studies are limited to Caucasians and Roux-en-Y gastric bypass-mediated weight loss. Bariatric surgery may also reverse obesity-related metabolic alterations and changes in serum metabolites as shown in women undergoing weight loss surgery (Gralka et al., 2015). As expected, these metabolites are mostly implicated in IR. For instance, circulating branched chain amino acids are reduced after bariatric surgery but this is apparently a procedure-dependent effect. (Mutch et al., 2009; Lips et al., 2014; Arora et al., 2015; Lopes et al., 2015, 2016; Gralka et al., 2015). Future studies should be conducted using metabolite profiling as a means to investigate adaptations associated with bariatric surgery and to identify molecular markers that could be use as surrogate markers of therapeutic response.

5. Concluding remarks

The study of epigenetic inheritance of complex traits characterized by metabolic disturbances, such as diabetes and obesity, is an exciting new frontier. The pivotal regulatory role of energy metabolism in transcriptional deregulation may suggest mechanisms on how toxic nutritional disequilibrium influences gene expression via cell metabolism, and may change the perception and pharmacological treatment of diabetes and obesity. We envision that the use of metabolomics to explore endogenous metabolites will reveal the existence of mechanisms accessible to intervention and will aid in the characterization of molecular mediators in the epigenetic information between generations.

The rising incidence of obesity and T2DM, major risk factors for severe comorbidities, is a major worldwide public health issue. These disorders threaten to reduce the length and quality of life of current and future generations and there is a strong need for safe and effective strategies for prevention and treatment. To improve such strategies, a better understanding of contributing factors is essential. We emphasize biological evidence indicating that living organisms continuously adapt to fluctuations in the availability of energy substrates. Consequently, the cellular transcriptional machinery and chromatin-associated proteins integrate inputs derived from food to mediate homeostatic epigenetic responses through gene regulation. Therefore, epigenetic mechanisms may exacerbate the epidemic of metabolic diseases by first contributing to the development of obesity and T2DM and second, by passing

modifications on to the subsequent generation. Fortunately, epigenetic modifications are not maintained over the lifetime and allow rapid adaptations. The challenge is to incorporate metabolo-epigenetic data in ways that will allow better biological interpretations, to provide clinical tools for diagnosis, prevention and treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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Annex 3

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Review Article

Mitochondrial Dysfunction: A Basic Mechanism in Inflammation-Related Non-Communicable Diseases and Therapeutic Opportunities

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Obesity is not necessarily a predisposing factor for disease. It is the handling of fat and/or excessive energy intake that encompasses the linkage of inflammation, oxidation, and metabolism to the deleterious effects associated with the continuous excess of food ingestion. The roles of cytokines and insulin resistance in excessive energy intake have been studied extensively. Tobacco use and obesity accompanied by an unhealthy diet and physical inactivity are the main factors that underlie noncommunicable diseases. The implication is that the management of energy or food intake, which is the main role of mitochondria, is involved in the most common diseases. In this study, we highlight the importance of mitochondrial dysfunction in the mutual relationships between causative conditions. Mitochondria are highly dynamic organelles that fuse and divide in response to environmental stimuli, developmental status, and energy requirements. These organelles act to supply the cell with ATP and to synthesise key molecules in the processes of inflammation, oxidation, and metabolism. Therefore, energy sensors and management effectors are determinants in the course and development of diseases. Regulating mitochondrial function may require a multifaceted approach that includes drugs and plant-derived phenolic compounds with antioxidant and anti-inflammatory activities that improve mitochondrial biogenesis and act to modulate the AMPK/mTOR pathway.

1. Background

The burden of noncommunicable diseases is increasing as such diseases are now responsible for more than three in five deaths worldwide. Atherosclerosis and cancer, in which tobacco use and excessive energy intake are determining factors, are the most frequently occurring of these diseases and are potentially preventable [1, 2]. Obesity and associated metabolic disturbances, which have been increasing worldwide in recent years, are the main factors that underlie noncommunicable diseases and are the consequences of unhealthy diets and physical inactivity [3]. Approximately 10–20% of patients with severe obesity, defined as a body mass index (BMI) > 40, present with no other metabolic complications. These patients are referred to by the oxymoronic designation of "metabolically healthy" obese [4–7]. Such a designation implies that most obese patients are not "metabolically healthy. " Hence, risk factors for the appearance of noncommunicable diseases have emerged. The reasons for these two phenotypes are unknown; the phenotypes might represent different transitions on a disease timeline, and different levels of either chronic inflammation or insulin resistance are likely contributors. Other contributors include gradual differences in glucose tolerance, inflammatory responses, adipose tissue distribution, patterns of adipokine secretion, and age.

Emerging obesogenic factors are likely to present with significant differences in the elderly, and consequently the

prevalence of obesity is expected to increase with increasing age. Therefore, it is likely not coincidental that most comorbidity associated with obesity and hence with noncommunicable diseases correlates with aging; the processes may share basic mechanisms, particularly mitochondrial age within an individual [7]. Of note, the prevalence of obesity is lower in people over 70 years of age, an effect attributed to the selective mortality of middle-aged people [8].

Current recommendations to decrease food intake and increase physical exercise do result in metabolic improvements, but such lifestyle changes are rarely sustained, despite strong motivation. However, several communities have undertaken initiatives to prevent noncommunicable diseases, and the lessons learned from the implementation of such initiatives should be examined further [9]. The active manipulation of energy sensors and effectors might be a possible alternative therapeutic procedure. Our aim is to provide a succinct review of the scarce and disseminated data that link mitochondrial dysfunction to the pathogenesis of energy-related complications and to discuss a possible multifaceted therapeutic approach.

2. Food Availability Links Mitochondrial Dysfunction and the Vicious Cycle of Oxidative Stress and Inflammation

Mitochondrial defects, systemic inflammation, and oxidative stress are at the root of most noncommunicable diseases such as cancer, atherosclerosis, Parkinson's disease, Alzheimer's disease, other neurodegenerative diseases, heart and lung disturbances, diabetes, obesity, and autoimmune diseases [10–16]. Obesity and obesity-related complications as well as impairment of mitochondrial function, which is required for normal metabolism and health (Figure 1), are universally associated with these conditions. The exact mechanisms that associate mitochondrial dysfunction, obesity, and aging with metabolic syndrome remain a topic of debate [17–22].

Body weight is controlled by molecular messengers that regulate energy status in a limited number of susceptible tissues, including the liver, adipose tissue, skeletal muscles, pancreas, and the hypothalamus [7, 23]. Mouse models of diet-induced obesity have revealed important morphological and molecular differences with respect to humans, particularly those related to the development of fatty liver (NAFLD: nonalcoholic fatty liver disease) or nonalcoholic steatohepatitis (NASH) [24–30] (Figure 2). High expectations for a human therapy after the generation of leptin-deficient animals (Ob/Ob) were countered by the determination that leptin is not a therapeutic option in humans [28].

Endoplasmic reticulum (ER) and mitochondrial stress, with the consequent oxidative stress, are immediate consequences of attempts to store excess food energy [23, 29]. Under normal weight conditions, adipose tissue-derived adipokines maintain the homeostasis of glucose and lipid metabolism; however, in obese conditions, the dysregulated production of adipokines favours the development of metabolic syndrome and related complications, particularly the accumulation of triglycerides in nonadipose organs that are not designed to store energy [19]. Other adipokines may cause inflammation and oxidative stress [31], but unknown factors are involved because interventions to ameliorate insulin resistance do not lead uniformly to clinical improvement [32]. It is of paramount importance to understand the mechanisms that disrupt ER homeostasis and lead to the activation of the unfolded protein response and mitochondrial defects in metabolic diseases in order to correctly manage noncommunicable diseases [33].

Incidentally, the role of genetics in low-energy expenditure and chronic food intake, although potentially significant, remains poorly understood [29, 30]. The genetic-selection hypothesis, which attempts to explain the high prevalence of obesity and diabetes in humans, remains controversial, since the recent abandonment of the "thrifty" gene hypothesis [34–38]. As a result, the roles of oxidative stress, inflammation, mitochondrial dysfunction, nutritional status, and metabolism might be reinforced in hypotheses regarding the pathogenesis of noncommunicable diseases (Figures 3 and 4).

Inflammation plays a vital role in host defence. Tissue damage, fibrosis, and losses of function occur under chronic inflammatory conditions. Growing evidence links a low-grade, chronic inflammatory state to obesity and its coexisting conditions as well as to noncommunicable diseases [10–16]. This low-grade inflammatory state is aggravated by the recruitment of inflammatory cells, mainly macrophages, to adipose tissue. Inflammatory cell recruitment is likely due to the combined effects of the complex regulatory network of cells and mediators that are designed to resolve inflammatory responses [7]. Anti-inflammatory drugs have shown to reverse insulin resistance and other related conditions that result from circulating cytokines that cause and maintain insulin resistance [19, 23, 39–42]. Therefore, it is likely that inflammation *per se* is a causal factor for noncommunicable diseases rather than an associated risk factor.

It is also important to highlight that adipose tissue is comprised of multiple types of cells that have intrinsic and important endocrine functions, particularly those mediated by leptin and adiponectin. Recruited and resident macrophages secrete the majority of inflammatory adipokines, specifically tumour necrosis factor α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), among others. The major roles of TNFα and other inflammatory cytokines in the progression of metabolic complications are likely related to oxidative stress [43, 44]. In adipose tissue macrophages, increased concentrations of saturated free fatty acids (FFAs) stimulate the synthesis of TNFα directly through the Toll-like receptor 4 (TLR4) or indirectly through cellular accumulation. Both macrophages and adipocytes possess TLR4 receptors that, upon lipid-dependent activation, induce NF-KB translocation to the nucleus and the subsequent synthesis of TNFα and IL-6 [7, 43, 44]. However, recruited macrophages have unique inflammatory properties that are not observed in resident tissue macrophages, and the recruitment of these cells is mainly modulated by MCP-1, the most important molecule of the CC chemokine family [7]. In this setting, the roles and polarisation of adipose tissue macrophages (ATMs) seem established [45]. M1 or "classically activated" ATMs

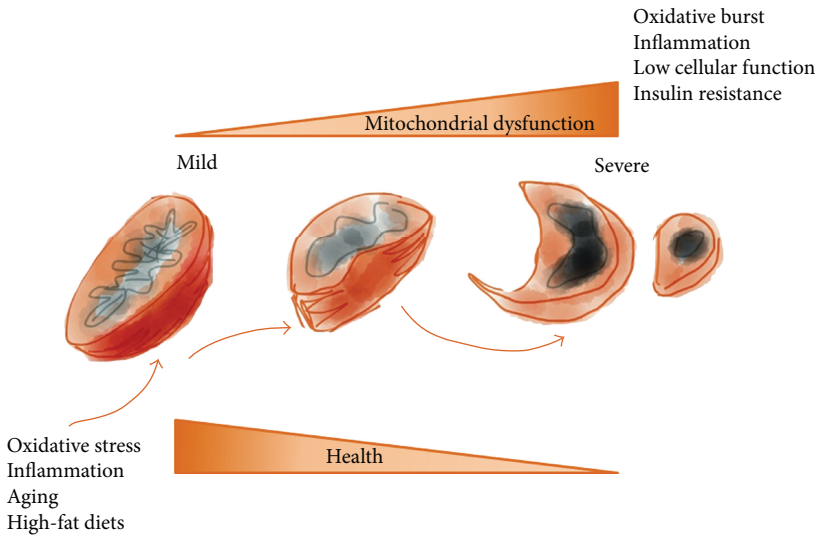
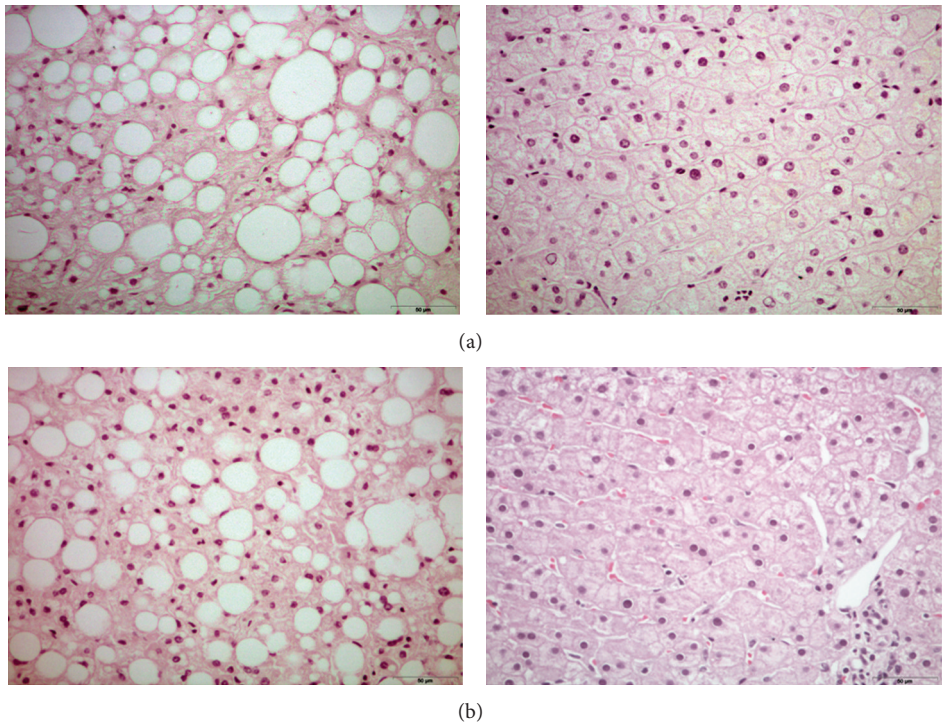


FIGURE 1: Mutations in mitochondrial DNA are accompanied by different disease-suggestive phenotypes (myopathies, neuropathies, diabetes, and signs of reduced lifespan and premature aging). Severe mitochondrial dysfunction triggers a high level of oxidative and inflammatory damage, impairs tissue function, and promotes age-related diseases.



(a)

(b)

FIGURE 2: Clinically, it is evident that, in severe obesity, (a) the presence of liver steatosis may vary from more than 80% to less than 5% of patients. Conversely, in most obese patients with some degree of liver steatosis (b), this condition disappeared in a relatively brief period of time after significant weight loss due to bariatric surgery.

are increased, and M2 or "alternatively activated" ATMs are decreased in the adipose tissues of both obese mice and obese humans, as discussed below [46, 47].

It is frequently assumed that, in contrast to hormones, chemokines influence cellular activities in an autocrine or paracrine fashion. However, chemokines may be relevant effectors in chronic systemic inflammation as the confinement of these molecules to well-defined environments is unlikely. Specifically, alterations in plasma MCP-1 concentrations in metabolic disease states, the presence of circulating chemokine reservoirs, recent evidence of novel mechanisms of action, and certain unexplained responses associated with

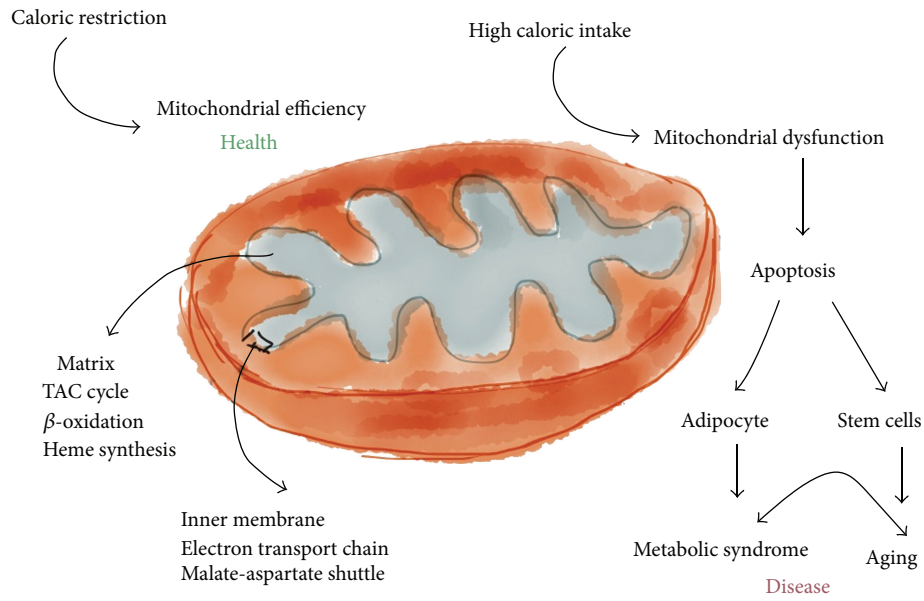


Figure 3: The mitochondrial matrix hosts the mitochondrial metabolic pathways (TAC cycle, β-oxidation, and haem synthesis), and the inner membrane contains the electron transport chain complexes and ATP synthase. Exchange carriers such as the malate-aspartate shuttle are also essential. Under caloric restriction, the mitochondrion achieves the highest efficiency, and high caloric intake produces dysfunction and a consequent increase in apoptosis, which promotes metabolic syndrome and age-related diseases.

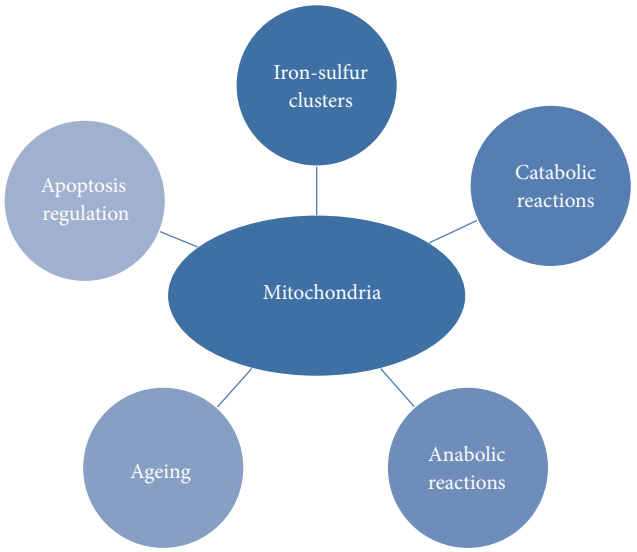


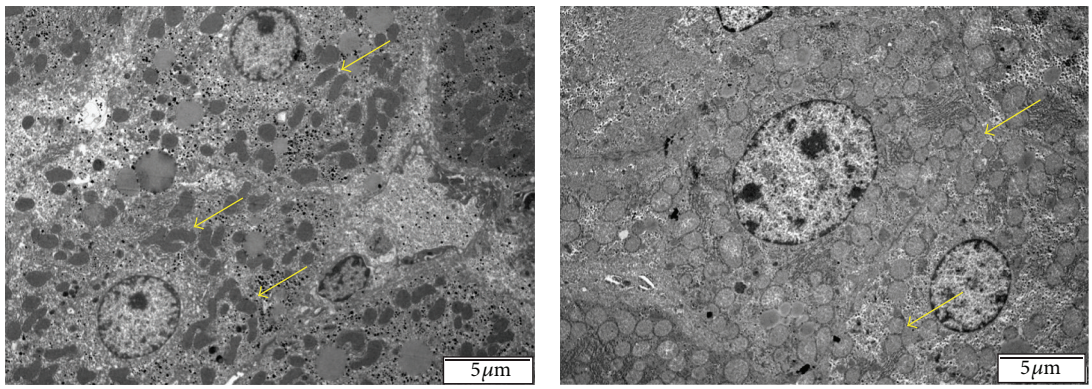
Figure 4: Schematic and abridged representation of the multiple roles of mitochondria in cellular processes that are associated with the pathogenesis of the more prevalent diseases.

metabolic disturbances suggest that MCP-1 might have a systemic role in metabolic regulation [48–50]. How and when obesity might initiate an inflammatory response remains controversial, but the underlying mechanism likely depends on the activation of the c-Jun N-terminal kinase (JNK) in insulin-sensitive tissues, as JNK is likely the principal mechanism through which inflammatory signals interfere with insulin activity [7].

ER stress responses and mitochondrial defects are also linked to the mTOR pathway, discussed below, which is essential for the regulation of numerous processes, including the cell cycle, energy metabolism, the immune response, and autophagy. Therefore, the specific cellular changes associated with metabolic alterations, particularly mitochondrial dysfunction, require further attention.

3. Mitochondria: Bioenergy Couples Metabolism, Oxidation, and Inflammation

Mitochondria are essential organelles that, among other functions, supply the cell with ATP through oxidative phosphorylation, synthesise key molecules, and buffer calcium gradients; however, they are also a source of free radicals (Figures 1, 3, and 4). It is not surprising that mitochondrial health is tightly regulated and associated with the homeostasis and aging of the organism. Within these processes, the antagonistic and balanced activities of the fusion and fission machineries constantly provide adequate responses to events caused by inflammation (Figure 5) [23, 50–54]. A shift towards fusion favours the generation of interconnected mitochondria, which contribute to the dissipation and rapid provision of energy. A shift towards fission results in numerous mitochondrial fragments. Apparently, the mixing of the matrix and the inner membrane allows the respiratory machinery components to cooperate most efficiently. Furthermore, fusion maximises ATP synthesis. In quiescent cells, mitochondria are frequently present as numerous morphologically and functionally distinct small spheres or short rods [51, 55, 56]. Upon the exposure of cells to stress, fusion optimises mitochondrial function and



(a) (b)

Figure 5: Mitochondrial fusion (a) and fission (b) processes in the liver (arrows). Mitochondrial morphology is basically controlled by metabolism and inflammation, and each change in morphology is mediated by large guanosine triphosphatases of the dynamin family, consistent with a model in which the capacity for oxidative phosphorylation is maximised under stressful conditions.

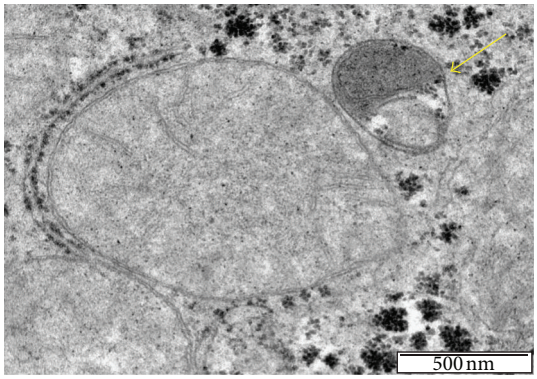


Figure 6: The complete elimination of mitochondria by autophagy (arrow) is a process linked to mitochondrial fission and fusion. Mitochondria also employ quality-control proteases to eliminate damaged molecules through the transcriptional induction of chaperones or the ubiquitin proteasome quality-control pathway.

plays a beneficial role in the maintenance of long-term bioenergetics capacities. In contrast, the mitochondrial fission machinery contributes to the elimination of irreversibly damaged mitochondria through autophagy [55–58]. This process, also called mitophagy, is extremely important under both physiological and pathological conditions (Figure 6). A detailed discussion of the importance of mitophagy is beyond the scope of this review; however, as an example of its importance, recall that amino acids are not stored in the body but are instead mobilised by proteolysis under conditions such as starvation, reduced physical activity, and disease [59]. Furthermore, intense exercise may modulate hepatic metabolism through similar mechanisms [60]. More recently, the mitochondrial E3 ubiquitin protein ligase 1 (Mul 1) was identified as a key protein that promotes mitophagy and skeletal muscle loss [61]. Mitochondrial fission *per se* triggers organelle dysfunction and muscle loss. The opposite is observed when mitochondrial fission is inhibited. The same authors [61] also demonstrated that the overexpression of Forkhead box O3 (FoxO3) induces mitochondrial disruption via mitophagy.

Therefore, it is not surprising that mitochondrial diseases often have an associated metabolic component, and consequently mitochondrial defects are expected in inflammation, aging, and other energy-dependent disturbances [58, 62]. In such disturbances, cellular oxidative damage caused by the generation of reactive oxygen species (ROS) that exceed the natural antioxidant activity is likely an initiating factor in inflammation and aging [63, 64]. Several potential therapeutic approaches are currently available to slow down age-related functional declines [65], including antioxidant treatments [66]; however, the effectiveness of existing antioxidants is likely suboptimal because these antioxidants are not selective for mitochondria [67]. However, recent experiments with a mitochondria-targeted antioxidant have been successful in animal models [67]. Similar assumptions can be made for endothelial cells, in which oxidation and the accompanying inflammation are recognised factors for atherosclerosis. Oxidative stress, which is mainly derived from mitochondrial dysfunction, decreases NO synthesis, contributes to hypertension, upregulates the secretion of adhesion molecules and inflammatory cytokines, and is responsible for the oxidation of low-density lipoproteins [68, 69].

Defective mitochondrial function in muscle tissues leads to reduced fatty acid oxidation and the inhibition of glucose transport, indicating that insulin-stimulated glucose transport is reduced. This is a hallmark of insulin resistance and type 2 diabetes. The chronic production of excess ROS and inflammation result in mitochondrial dysfunction potentially inducing lipid accumulation in these tissues and the endless vicious cycle of insulin resistance [70–74]. Mitochondrial ROS have also been related to the increased activity of uncoupling proteins (UCP), which uncouple ATP synthesis from electron transport. UCP activity leads to heat generation without ATP production, and long-term reductions in ATP

levels affect cellular insulin signalling. The roles of the UCPs and the metabolically relevant differences between brown and white adipose tissues were reviewed recently [75–77].

The mitochondria of obese individuals are different from those of lean individuals. Alterations in mitochondrial morphology, impaired mitochondrial bioenergetics, increased mitochondrial lipid peroxides, decreased ATP content, and mitochondrial dysfunction further increase the risks of developing metabolic complications [78, 79]. In comparison to those of lean individuals, mitochondria in obese individuals have lower energy-generating capacities, less clearly defined inner membranes, and reduced fatty acid oxidation. These differences might promote the development and progression of obesity and might also have therapeutic implications [80, 81]. Impaired mitochondrial function could account for the insulin resistance that is closely associated with increased lipid content in the muscles of patients with type 2 diabetes. Altered mitochondrial function is the major factor that leads to increased muscular lipid accumulation and decreased insulin sensitivity [80, 81]. More recently, a model was created in which the amount of mitochondrial activity in adipocytes and hepatocytes can be altered based on the properties of the mitochondrial protein mitoNEET, which is located at the outer membrane [70]. Despite the prevalence of obesity in this model, mitoNEET overexpression during periods of high caloric intake resulted in systemwide improvements in insulin sensitivity, thereby providing a model of a "metabolically healthy" obese state with minimal tissue lipotoxicity that is similar to the clinically observed condition [82]. Alterations in mitoNEET expression might modulate ROS concentrations and mitochondrial iron transport into the matrix [70, 82, 83]. The mitochondrial fusion protein mitofusin-2 (Mfn-2), another useful protein in studies of mitochondrial dysfunction, regulates cellular metabolism and controls mitochondrial metabolism. In cultured cells, mitochondrial metabolism was activated in Mfn-2 gain-of-function experiments, whereas Mfn-2 loss-of-function reduced glucose oxidation, mitochondrial membrane potential, oxygen consumption, and mitochondrial proton leakage [84]. It is defective in the muscles of obese and type 2 diabetes patients in which mitochondrial size is reduced [71].

Therefore, a detailed characterisation of the proteins involved in mitochondrial fusion and fission and studies of the mechanisms that regulate these two processes are relevant to human pathology and might have a great therapeutic potential to improve metabolism and to decrease the generation of oxidative stress and excessive inflammatory response [85].

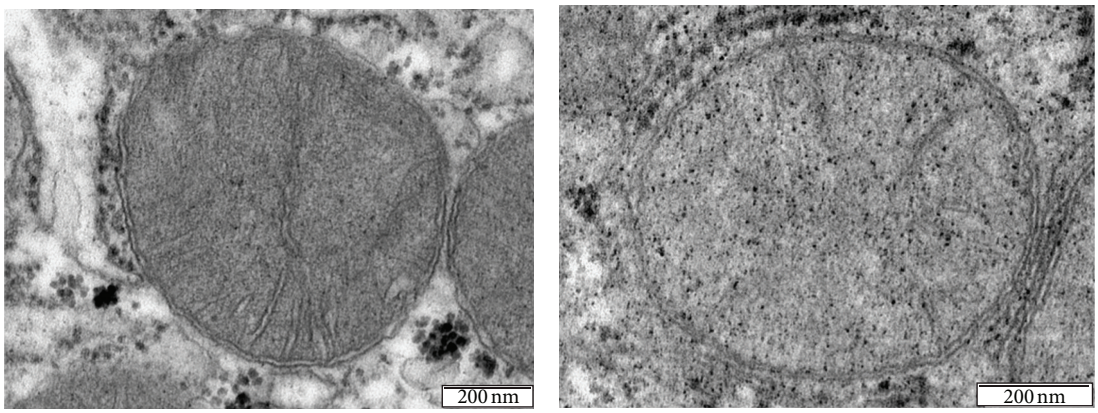
4. Is There a Link between Mitochondria and Nutrient Availability? The Possible Roles of Inflammation and Apoptosis

Apoptosis is another basic process to consider in metabolic diseases. Excess food intake leads to mitochondrial dysfunction and higher apoptotic susceptibility. Mitochondria specialise in energy production and cell killing. Only 13 proteins are encoded by the mitochondrial DNA, a circular molecule of 16 Kb. The remaining necessary proteins are encoded in the nuclear DNA [86]. Mitochondria are composed of outer and inner specialised membranes that define two separate components, the matrix and the intermembrane space [87]. Mitochondria regulate apoptosis in response to cellular stress signals and determine whether cells live or die [88]. Thus, it is conceivable that the availability or ingestion of nutrients could be a main candidate in the regulation of cell death and that mitochondria could have been selected as a nutrient sensor and effector. This could explain the influence of apoptosis-related proteins on mitochondrial respiration [89].

A common laboratory finding is that the morphology of the mitochondria changes when mice are supplied with a high-fat diet (Figure 7) and that optimal mitochondrial performance is achieved under conditions of calorie restriction. Excess food intake impairs respiratory capacities, likely through mTOR, and increases the susceptibility of the cell to apoptosis and additional stress [90, 91]. Of note, apoptotic protein levels are increased in the adipocytes of obese humans, and the depletion of proapoptotic proteins protects against liver steatosis and insulin resistance in mice fed a high-fat, high-cholesterol diet [92]. These conditions are relevant to the development of metabolic syndrome, as nutritional imbalances in Western diets lead to mitochondrial dysfunction and higher susceptibilities to inflammation, apoptosis, and aging [22].

5. AMP-Activated Protein Kinase (AMPK) Not Only Influences Metabolism in Adipocytes but Also Suppresses the Proinflammatory Environment

AMPK has anti-inflammatory actions that are independent of its effects on glucose and lipid metabolism [93]. The action of AMPK is not necessarily identical in all tissues. In adipose tissues, the role of AMPK is largely unknown because laboratory techniques to explore the action of this kinase in terminally differentiated adipocytes have not been fully established. Several agents have been used to activate AMPK experimentally, including AICAR (5′-aminoimidazole-4-carboxamide ribonucleoside), metformin, rosiglitazone, resveratrol and other polyphenols, statins, and several adipocytokines. In adipocytes, AMPK appears to increase the insulin-stimulated uptake of glucose, likely by increasing the expression of GLUT4, yet inhibits glucose metabolism [94]. Studies of the effects of AMPK on lipolysis in adipocytes have been controversial; some authors have reported an antilipolytic effect, while others have suggested that AMPK stimulates lipolysis [95, 96]. However, the activation of AMPK by metformin in human adipose tissues increases the phosphorylation of acetyl-CoA carboxylase (ACC) and decreases the expression of lipogenic genes, leading to reductions in malonyl-CoA, which is the precursor for fatty acid synthesis; malonyl-CoA also regulates fatty acid oxidation through the inhibition of carnitine palmitoyl-transferase 1, the rate-limiting enzyme for fatty acid entry into the mitochondria [97, 98]. Adipose tissue secretes adipocytokines, which influence metabolic and inflammatory pathways through the recruitment of



(a) (b)

Figure 7: The nutrient availability of food in "natural" conditions for mice is likely low and near the condition known as calorie restriction. In the laboratory, however, mice are usually fed *ad libitum*, and certain biases cannot be discarded. However, mitochondria from mice fed a chow diet (a) display rapid morphological changes when mice are fed with high-fat diets (b).

macrophages and the consequent transition from the M2 state to M1 [7, 41]. These actions contribute to the development of disease (Figure 8). Conversely, adiponectin has been reported to induce adipose macrophages to switch to the anti-inflammatory M2 state [99]. AMPK is anti-inflammatory, as it inhibits the synthesis of proinflammatory cytokines and promotes the expression of IL-10 in macrophages; adiponectin and leptin levels may also be regulated by AMPK [100] (Figure 8). Finally, brown adipocytes contain high numbers of mitochondria that express UCP1, which permit thermogenesis. Exposure to cold temperatures stimulates AMPK and may play a role in the differentiation of fatty oxidising brown adipose tissue, thus leading to greater energy expenditure [101]. Therefore, we hypothesise that the chronic manipulation of the AMPK/mechanistic target of rapamycin (mTOR) pathway might represent a therapeutic approach for preventing noncommunicable diseases (Figure 8). Metformin, along with salicylate, polyphenols, and rapamycin, has a long history of safe and effective use, but other modulators are currently under development and will likely permit the design of tissue-specific activators of this pathway.

6. Metformin and/or Rapamycin and Plant-Derived Polyphenols: An Apparent Treatment of Choice for Metabolic Syndrome and Obesity-Related Complications?

The first therapeutic approaches to metabolic disturbances are reduced caloric ingestion and increased physical activity. The effects are based mainly on weight reduction, but usefulness in other common complications remains incompletely explored [102]. Bariatric surgery is also effective, even in "metabolically healthy" patients [103, 104]. The effectiveness of surgery for the treatment of metabolic disturbances is surprisingly higher than expected, and mechanisms associated with surgical effects are not completely understood.

Insulin resistance and mitochondrial dysfunction appear to be the most significant alternative therapeutic targets. Metabolic abnormalities are associated with inflammation. Normally, glycolysis yields pyruvate, which is further oxidised in the mitochondria. When oxygen becomes limiting, mitochondrial oxidative metabolism is restricted. The induction of an inflammatory response is an energy-intensive process, and the involved cells rapidly switch from resting to highly active states. This is observed in diseases such as cancer, atherosclerosis, or autoimmune diseases, and mechanistic insights suggest the common involvement of the transcription factor hypoxia-inducible factor 1α, AMPK, and the mTOR pathway. In addition, the activation of sirtuins, which act as NAD+ sensors that connect nutrition and metabolism to chromatin structure, is anti-inflammatory [105] (Figure 8).

The use of metformin, an AMPK activator used extensively to treat type 2 diabetes, has been indicated for other metabolic conditions based on the rationale that insulin-sensitising agents might be effective [106], and the mode of action of metformin has guided our own experiments on cancer, aging, and viral infection [65, 107, 108]. We have shown that the beneficial effects of this biguanide class drug, which was initially obtained from *Galega officinalis*, are universal in patients with metabolic complications and negligible in patients without such complications. The primary effect is thought to be the suppression of hepatic glucose production and hepatic lipogenesis [109]. Metformin activates AMPK in hepatocytes, resulting in the phosphorylation and inactivation of ACA, a rate-limiting enzyme in lipogenesis [110], and theoretically might be useful and safe in the treatment of NAFLD [111]. Surprisingly, the beneficial clinical effects seem to be limited, despite the effects of metformin on insulin resistance, most likely because long-term treatment is an absolute requirement for the prevention of progressive disease. Our own current experiments in animal models suggest new insights into this phenomenon. Metformin activates AMPK, but AMPK deficiency does not abolish

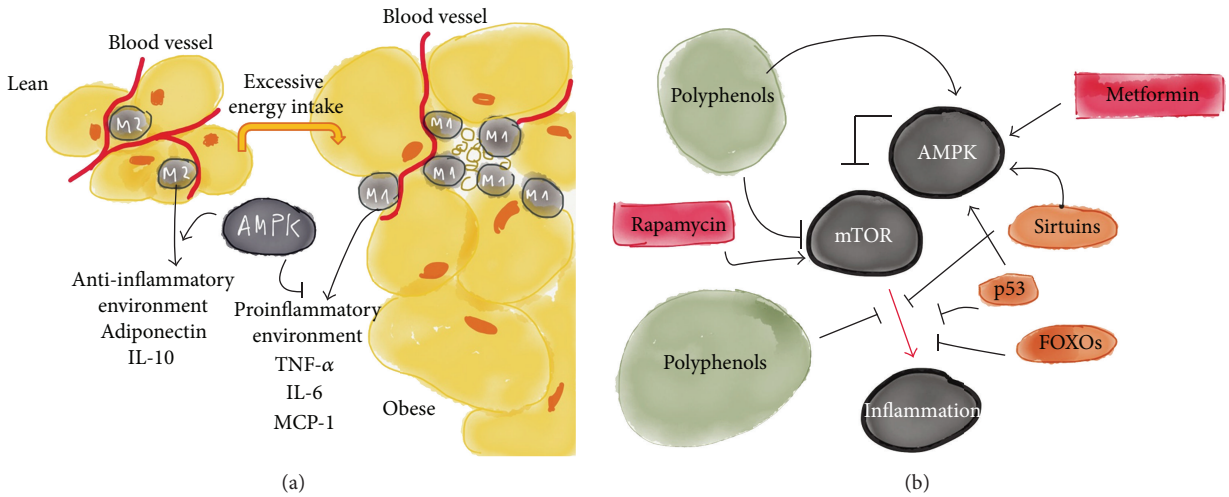


Figure 8: Activation of AMPK in macrophages promotes the switch from a proinflammatory to an anti-inflammatory phenotype by inducing a shift from glycolysis towards mitochondrial oxidative metabolism. In obesity, there may be a shift towards proinflammatory states, whereas in dietary restriction the balance may shift towards anti-inflammatory phenotypes through the activation of AMPK (a). The activation of AMPK implies the inhibition of mTOR, and several compounds are known to regulate this pathway (b). The inhibition of mTOR extends lifespan in model organisms and confers protection against a growing list of age-related pathologies. Several characterised inhibitors are already clinically approved, and others are under development.

the effects of metformin on hepatic glucose production, indicating that the role of AMPK is dispensable, as indicated previously [112]. This suggests that the overall effect of metformin is mediated through actions on mitochondrial function through decreases in the hepatic energy state and intracellular ATP content. Other studies suggest that metformin inhibits Complex I of the mitochondrial respiratory chain, but the exact mechanisms and pathways involved are unclear [113]. Sirtuin 3 (SIRT 3), a member of the family of nicotinamide adenine dinucleotide (NAD+) dependent deacetylase proteins, is a crucial regulator of mitochondrial function that controls the global acetylation of the organelle (all sirtuins regulate energy production and the cell cycle; Figure 8). SIRT3 induces the activity of Complex I and promotes oxidative phosphorylation. In SIRT3 knockout mice, mitochondrial proteins are hyperacetylated, and cellular ATP levels are reduced, effects that are aggravated by fasting [114]. As a complement, peroxisome proliferator-activated receptor gamma coactivator 1-alpha induces the expression of SIRT3 in the liver [115]. Therefore, mitochondrial function appears to be the key target of metformin; reductions in ATP production may mediate the hepatic and antihyperglycemic actions of the drug and downregulate SIRT3 expression [116]. However, metformin distinctively regulates the expression of different sirtuin family members [117, 118]. In summary, metformin acts against both insulin resistance and mitochondrial dysfunction and is currently an attractive candidate agent of choice in the management of metabolic disorders. We have recently reviewed this complex scenario and found the following: (1) the unique ability of metformin to activate AMPK while leading to the increased utilisation of energy occurs because metformin inhibits AMP deaminase; and (2) in metabolic tissues, metformin can inhibit cell growth by functionally mimicking the effects of a multitargeted antifolate [119].

Based on these and other findings, we have also demonstrated that plant-derived phenolic compounds interact with numerous targets and multiple deregulated signalling pathways that may be useful in the management of metabolic conditions [120–123]. The proposed mechanisms are direct antioxidant activity, attenuation of endoplasmic reticulum stress, blockade of proinflammatory cytokines, and blockade of transcription factors related to metabolic diseases [120]. Most polyphenols modulate oxidative stress and inflammatory responses through relevant actions in the process of macrophage recruitment. Interactions between the chemokine/cytokine network and bioenergetics, likely through the mTOR pathway, may also represent potential mechanisms for the prevention of metabolic disturbances [121]. Moreover, polyphenols attenuate the metabolic effects of high-fat, high-cholesterol diets when administered continuously at high doses, and we have described beneficial actions associated with the expression of selected microRNAs [122].

Inflammation lies at the heart of many diseases because the entire body is under metabolic stress, which induces symptoms and causes morbidity. Targeting altered metabolic pathways in inflammation may enhance our understanding of disease pathogenesis and point the way to new therapies. As mentioned, metformin, polyphenols, AICAR, salicylates, and corticoids all activate the AMPK/mTOR pathway. New compounds such as A-769662 are under scrutiny. Finally, rapamycin, which is also known as sirolimus and was first isolated from *Streptomyces hygroscopicus*, and several derivative compounds, including everolimus, temsirolimus, ridaforolimus, umirolimus, and zotarolimus, have been approved for a variety of uses, including posttransplantation

therapy, the prevention of restenosis following angioplasty, and as a treatment for certain forms of cancer. Drugs that inhibit the mTOR pathway could one day be used widely to slow aging and reduce age-related pathologies in humans [124]. The development of chemical inhibitors of mTOR, as well as drugs that target other components of the mTOR pathway, promises to aid research greatly while also providing drugs with potential therapeutic value.

7. Perspectives and Implications

Obesity, metabolic alterations, and age-related diseases are complex conditions that require a multifaceted approach that includes action on both the chemokine network and energy metabolism [123, 125]. The underlying mechanisms are far from being understood [126] although the association between obesity and insulin resistance seems to be well substantiated. However, obesity is not distributed normally throughout the population, and type 2 diabetes mellitus is not associated closely with increased body weight; also, the relationship with noncommunicable diseases is not straightforward. A working hypothesis is that adipose tissue has a limited maximum capacity to increase in mass. Once the adipose tissue has reached the expansion limit, fat is deposited in the liver and muscle tissues and causes insulin resistance. This process is also associated with the activation of macrophages, oxidative stress, and inflammation which produce cytokines that have negative effects on insulin sensitivity, induce the secretion of adipokines that cause insulin resistance, and suppress those that promote insulin sensitivity. However, a host of other mechanisms must be involved because metabolic responses are different among patients with maximum adipose tissue expansion. A more popular and recent hypothesis suggests a differential effect of lipophagy, which implies a tissue-selective autophagy with cellular consequences from the mobilisation of intracellular lipids. Defective lipophagy is linked to fatty liver tissues and obesity and might be the basis for age-related metabolic syndrome [127]. Increased adipose tissue autophagy may be responsible for more efficient storage. Autophagy also affects metabolism, oxidation, and proinflammatory cytokine production. Very recent evidence suggests that autophagy is increased in the adipose tissues of obese patients [128]. Inexpensive and well-tolerated molecules such as chloroquine, metformin, and polyphenols already exist and could be used to fine-tune the metabolic alterations derived from an excess of energy and, more specifically, to modulate autophagy in the liver. Whether these therapies will dampen the genetic expression of factors that affect the development of noncommunicable diseases remains to be ascertained.

Acknowledgments

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