PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE

Ajla Alibalic

DOCTORAL THESIS

PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE

Ajla Alibalic

UNIVERSITAT ROVIRA i VIRGILI

Department of Medicine and Surgery

Faculty of Medicine and Health Sciences

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Protheomic and adipo/cytokine biomarker analysis of unstable carotid atheroma plaque

Doctoral Thesis

Directed by Prof. Cristóbal Manuel Richart Jurado

and

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Tarragona 2022

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I STATE that the present study, entitled "Protheomic and adipo/cytokine biomarker analysis of unstable carotid atheroma plaque", presented by Ajla Alibalic 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.

Tarragona, 1 of June of 2022

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Mojoj familiji

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“Lo que conocemos es una gota, lo que no conocemos es un océano”

Isaac Newton

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A

ABCA1 - ATP binding cassette transporter

ABCA-1 - ATP-binding cassette transporter-1

ACC/AHA - American College of Cardiology/American Heart Association

ACT- Angiographic computed tomography

AGEs - advanced glycation end products

AHA - American Heart Association

AHA/ASA - American Heart Association/American Stroke Association

Apo- apolipoprotein

ATP III - Adult Treatment Panel III

B

BH4- tetrahydrobiopterin

BMI - body mass index

BMT - Best medical therapy

C

CAC - coronary artery calcium

CAPs - carotid atherosclerotic plaques

CAS - carotid artery stenting

CASt - carotid arteries stenosis

CC - Common Carotid method

CD40- cluster of differentiation 40

CDUS - carotid duplex ultrasound

CEA - carotid endarterectomy

CEMRA- contrast-enhanced magnetic resonance angiography

CRP - C-reactive protein

CTA - computed tomography angiography

CVD - Cardiovascular Disease

D

DAPT - Dual antiplatelet therapy

Dos-DE - two-dimensional electrophoresis

DSA - d igital subtraction angiography

E

ECST - European Carotid Surgery Trial

ELISA - enzyme-linked immunosorbent assays

EPCs - endothelial progenitor cells

ESC - European Society of Cardiology

ESC/ESH - European Society of Cardiology and European Society of Hypertension

ESI - electrospray ionization

ET-1- endothelin-1

ETB - endothelin B

F

FDR - false discovery rate

FPG - Fasting plasma glucose

H

HbA1C - glycated hemoglobin

HDL - high-density lipoprotein

HEPES - hydroxyethyl piperazineethanesulfonic acid

HEPES - hydroxyethyl piperazineethanesulfonic acid

HOMA2-IR - homeostasis model assessment of IR

Hs-CRP - high-sensitivity C - reactive protein

Hsp70 1A - heat shock protein-70 1A

I

IAA - Iodoacetamide

ICAM - intercellular adhesion molecule

ICAM-1 - Intercellular adhesion molecule-1

IEF - isoelectric point called isoelectric focusing

IL - interleukin

IL-10- interleukin 10

IL-4 - Interleukin 4

IL-6 - interleukin-6

IR - Insulin resistance

L

LC - liquid chromatography

LC–MS - liquid chromatography separation

LDL - low-density lipoprotein

LDL (-)-electronegative LDL

LP(a) - lipoprotein (a)

Lp-PLA_2 - Lipoprotein-associated phospholipase A_2

LRS - Lifetime Risk Score

M

MA - Mammary arteries

MAs - Mammary arteries

MCP - monocyte chemotactic protein;

MCP-1 - Monocyte Chemoattractant Protein-1

M-CSF - monocyte colony-stimulating factor

MESA - Multi-ethnic Study of Atherosclerosis

miRNAs - microRNAs

MMP - matrix metalloproteinase

MP - microparticles

MRA - magnetic resonance angiography

MS - mass spectrometry

MtDNA - Mitochondrial DNA

N

NADPH - nicotinamide adenine dinucleotide phosphate

NASCET - North American Symptomatic Carotid Endarterectomy Trial

NCEP - National Cholesterol Education Program

NIH - National Institutes of Health

NO - nitric oxide

NOS - Nitric oxide synthase

O

OPG - osteoprotegerin;

ox-LDL - oxidized low-density lipoprotein

P

PAF-AH - platelet-activating factor acetylhydrolases

PAPP-A - Pregnancy-associated plasma protein-A

PBS - phosphate-buffered saline

PBS - phosphate-buffered saline ()

PCA - Principal component analysis

PCSK9i - subtilisin/kexin 9 protein convertase inhibitors

PMNs - polymorphonuclear neutrophils

PON1- paraoxonase-1

PTX - pentraxin

PTX3 - Pentraxin-3

R

RDW - red blood cell distribution width

ROS - reactive oxygen species

RP - reversed-phase

RPMI - Roswell Park Memorial Institute medium

S

SAA - serum amyloid-A protein

SAPT - single antiplatelet therapy

SCORE - Systematic COronary Risk Evaluation

sdLDL - small and dense LDL cholesterol particles

SDS - sodium dodecilsulphate

suPAR - plasma-soluble urokinase plasminogen activator receptor

T

T2DM - type 2 diabetes mellitus

TCD - transcranial Doppler

TCEP - tris (2-carboxyethyl) phosphine

TF - Tissue factor

TIAs - transient ischemic attacks

TIMP - tissue inhibitors of metalloproteinase

TLR-4 - Toll-like receptor 4

TNF - tumor necrosis factor

TNF-α - tumor necrosis factor alpha

TOF -three-dimensional time-of-flight

TRL - triglyceride-rich lipoprotein

V

VCAM - vascular cell adhesion molecule

VCAM- 1-vascular cell adhesion molecule 1

VEGF - vascular endothelial growth factor

VSMCs - Vascular Smooth Muscle Cells

W

WBC - white blood cell

WHO - World Health Organization

Z

ZAG - zinc-alpha-2-glycoprotein

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INTRODUCTION

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1.1. Definition of Atherosclerosis

Atherosclerosis (in Greek, athere means "gruel," and skleros means "hard") is a chronic pathological process that affects arteries of medium and large calibre. Literally, the translated term "atherosclerosis" means the hardening of blood vessels. Historically, the first acknowledgements of this phenomenon were in ancient Egypt. In 1575, Fallopius wrote about "ossified arteries" describing the evolution of arteries into bones. In 1740, Johann Friedrich Crell described those "ossified arteries" as not bony but rather derived from pus. In fact, Von Haller was the first to identify blood vessel lesions as an "atheroma". The first use of the term "arteriosclerosis" can be attributed to Jean Frederic Martin, who analysed the composition of calcified arteria[1].

The actual classification begins from a short editorial by de Rabson published in January 1954 in the *American Journal of Clinical Pathology*. His article suggests that the name "arteriosclerosis" could be used as a generic name. He defines atherosclerosis as "arteriosclerosis with atheromatosis". This definition, provided by Rabson, is the one that is officially used by pathologists in their everyday descriptions[1].

Arteriosclerosis, atheroma plaque formation, is a highly prevalent pathological process that starts in childhood and progresses over the years.

Atherosclerosis is the underlying cause of important clinical manifestations of vascular diseases. This process is associated with cardiovascular events such as myocardial infarction, stroke or peripheral arterial occlusive disease.

1.2. Blood Vessel Structure

To better understand the atherosclerotic process, we must first briefly review anatomohistology.

The arterial wall normally consists of three well-defined concentric layers[2]:

Tunica intima (inner layer): formed from a single continuous layer of endothelial cells and supported by a subendothelial layer of connective tissue and supportive cells. The tunica intima is surrounded by a thin membrane comprising elastic fibres running parallel to the vessel (lamina basal).

Tunica media: Surrounding the tunica intima is the tunica media, comprising circularly arranged smooth muscle cells and elastic and connective tissues. The thickness of this layer depends on the type of blood vessel. This layer is much thicker when comprised of arteries rather than veins. The fibre composition also differs; veins contain fewer elastic fibres and function to control the calibre of the arteries. The tunica media and tunica intima are separated by an internal elastic sheet (lamina elastica interna).

The tunica externa (outermost layer) comprises connective fibres entirely and is surrounded by an external elastic lamina that functions to anchor vessels with surrounding tissues.

The tunica externa and tunica media are separated by an external elastic sheet (lamina elastica externa) (Figure 1).

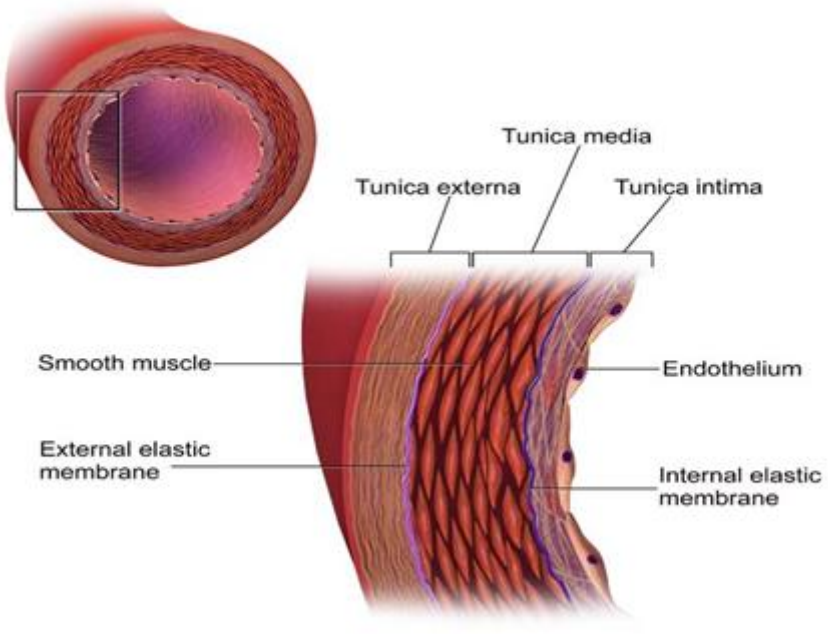

Figure 1. Artery Wall Structure: Cross section of an artery, the layer structure and its cellular composition.

1.3. Pathogenesis of Atherosclerosis

The basic research of atherosclerosis in the 20th century has allowed us to change the concept of this disease, which is traditionally considered a mere deposit of lipids and currently known as a disease of a clear inflammatory nature[3]. Several modifiable and unmodifiable environmental and genetic factors are associated with atherosclerosis[4], and they are described below.

1.3.1. Unmodifiable Risk Factors

Age

This risk factor has a greater predictive influence on cardiovascular disease, as demonstrated in the famous Framingham Heart Study. In this study, risk factors with a score of up to 14 points predicted the risk of cardiovascular disease at 10 years. Of these 14 points, 7 of them can be attributed only to age[5].

Sex and Gender

The most commonly used definition of sex and gender is the one used by the Canadian Institutes for Health Research Panel on Sex and Gender. Sex refers to a set of biological attributes in humans and animals and is usually categorized as female or male. It is primarily associated with physical and physiological features, including chromosomes, gene expression, hormone levels and function, and reproductive/sexual anatomy. Gender, usually conceptualized as a binary (girl/woman/femininity and boy/man/masculinity), refers to the socially constructed roles, behaviours, opportunities, expectations, expressions and identities of girls, women, boys, men, and gender-diverse people. It influences how people perceive themselves and each other, how they act and interact, and the distribution of power and resources in society[6].

Sex differences could be understood as biological differences, while gender differences are the social differences. Several observational studies have detected an increased risk of atherosclerosis in the male population. In women, higher levels of both oestrogen and high-density lipoprotein (HDL) are considered protective against atherosclerosis development. Incident cardiovascular disease is less common in premenopausal women than in their age-matched male counterparts[7].

Biological differences between men and women determine the size of the arteries. Women have smaller carotid arteries[8,9] with less plaque but more apparent stenosis, which could explain the sex differences in the diagnosis of acute coronary syndrome[10].

Race

The incidence of atherosclerosis in African-Americans is almost double that in Caucasian individuals of the who live in the same region [11].

Recent findings from the Multi-ethnic Study of Atherosclerosis (MESA) are consistent with previous reports that non-Hispanic white adults have a greater presence and quantity of coronary artery calcium (CAC) than non-Hispanic black (black), Hispanic, and Chinese-American (Chinese) adults[12–14].

Heritage

In recent years, several trials have promoted advances in understanding the influence of genetic factors on the development of atherosclerosis. Studies of genetic susceptibility to atherosclerosis in mice suggest that these factors may be more important than traditional risk factors[15].

Single-gene (Mendelian) disorders with large effects are the most dramatic examples of genetic contributions to atherosclerosis[16,17]. Disease expression is not mainly a consequence of only one gene influence. Most forms of the disease are the product of many genes with small effects[18]. Moreover, a family history of stroke increases the risk of suffering from atherosclerosis[19]. In the same sense, greater concordance has been observed in cerebral infarction in monozygotic twins [20].

1.3.2. Modifiable Risk Factors

Modifiable Factors

Modifiable factors are those that may undergo some changes after the application of therapeutic measures. The most common modifiable factors are related to metabolic syndrome, obesity, hypertension, diabetes, high cholesterol levels, smoking, low physical activity and a high-fat diet. The presence of obesity, hypercholesterolemia, hypertension and insulin resistance is associated with an increased risk of cardiovascular disease.

Obesity

Defined as an excess of fat, obesity is a chronic, global epidemic disease that is an indicator of poor health in rich countries. Obesity is associated with a significant increase in morbidity and mortality. The first step to measuring the degree of overweight is the determination of body mass index (BMI)[21]. To measure BMI, we used the following formula:

$$\text{BMI} = \text{body weight (in kg)} \div \text{height (in metres) squared}$$

The recommended classifications for BMI adopted by the National Institutes of Health (NIH) and World Health Organization (WHO)[22] for Caucasian, Hispanic, and black individuals are as follows:

- Severely underweight - BMI less than 16.5kg/m2
- Underweight - BMI under 18.5 kg/m^2
- Normal weight - BMI greater than or equal to 18.5 to 24.9 kg/m2
- Overweight – BMI greater than or equal to 25 to 29.9 kg/m2
- Obesity – BMI greater than or equal to 30 kg/m2
    - Obesity class I – BMI 30 to 34.9 kg/m2
    - Obesity class II – BMI 35 to 39.9 kg/m2
    - Obesity class III – BMI greater than or equal to 40 kg/m2 (also referred to as severe, extreme, or massive obesity)
- Asian and South Asian populations
    - Overweight - BMI between 23 and 24.9 kg/m2
    - Obesity - BMI greater than 25 kg/m2

To assess abdominal obesity, measurements of waist circumference are recommended. A waist circumference of ≥102 cm for men and ≥88 cm for women is considered elevated and indicative of increased cardiometabolic risk[23]. Waist circumference measurement is unnecessary in patients with BMI ≥35 kg/m^2, as almost all individuals with this BMI also have an abnormal waist circumference and are already at a high risk from their adiposity.

Arterial Hypertension

Since the early 1950s and the Framingham study, hypertension has been considered a high-risk factor for the development of atherosclerosis, particularly in the coronary and cerebral regions[24].

The European Society of Cardiology and European Society of Hypertension (ESC/ESH), as well as the National Institute for Health and Care Excellence guidelines, defines hypertension, using office-based blood pressure, as a systolic pressure ≥140 mmHg or diastolic pressure ≥90 mmHg[25,26].

In 2017, the American College of Cardiology/American Heart Association (ACC/AHA) defined normal blood pressure as when the systolic blood pressure is <120 mmHg and the diastolic blood pressure is <80 mmHg and elevated blood pressure as when the systolic blood pressure is 120 to 129 mmHg and the diastolic blood pressure is >80 mmHg. Hypertension is divided into two stages: stage 1 (systolic 130 to 139 mmHg or diastolic 80 to 89 mmHg) and stage 2 (systolic at least 140 mmHg or diastolic at least 90 mmHg).

The measurement technique is of paramount importance when identifying patients as having hypertension[27].

Dyslipidaemia

Abnormal lipoprotein metabolism plays a critical role in the development of atherosclerosis[28].

High levels of low-density lipoprotein (LDL) cholesterol, lipoprotein a (Lpa), or triglycerides or low levels of high-density lipoprotein (HDL) cholesterol might promote atheroma formation. LDL accumulates in macrophages enriched in cholesterol esters, forming foam cells. These LDL must be previously oxidized such that the receptors (CD36 or also called receptor B scavenger) of macrophages can capture them[29,30].

Accumulation of cholesterol in foamy cells causes dysfunction that leads to apoptosis and necrosis, releasing proteases, inflammatory cytokines and prothrombotic molecules[31].

Oxidized lipoproteins, including LDL, HDL, remnant lipoproteins and phospholipids, disrupt the endothelial cell surface and promote inflammation by releasing cytokines from macrophages, reducing cholesterol efflux and leading to the development of atherosclerosis. Oxidized LDL may also play a role in plaque instability (Figure 2). Antibodies to oxidized LDL have been found in human atherosclerotic plaques and in the plasma of patients with atherosclerosis [32,33].

HDL has antiatherogenic properties that include reverse cholesterol transport, maintenance of endothelial function and protection against thrombosis. There is an inverse relationship between plasma HDL-cholesterol levels and cardiovascular risk. However, cardiovascular disease event reduction from increasing HDL-cholesterol has

not been established, particularly in patients with well-controlled LDL-cholesterol levels [34,35].

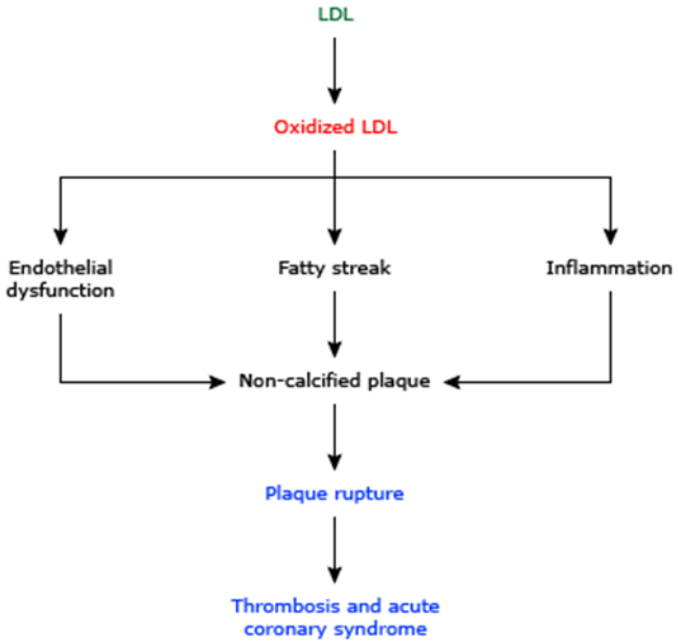

Figure 2. Schematic representation of LDL and oxidized LDL effects of in the pathogenesis of atherosclerosis [36].

Diabetes Mellitus

Diabetes is usually associated with other risk factors, such as hypertension and dyslipidaemia. However, diabetes mellitus appears to be the one that contributes most to increased cardiovascular risk [37].

Atherosclerosis and type 2 diabetes mellitus (T2DM) share similar pathological mechanisms, including elevation of cytokines (monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), that contribute to underlying inflammation in both diseases. Insulin increases the expression of CD36 and decreases ATP-binding cassette transporter-1 (ABCA-1) expression, which may promote cholesterol accumulation in human monocyte-derived macrophages [38]. Low concentrations of adiponectin increased the phosphorylation of Akt (Ser436) to the same degree as insulin and had the same modulating effect on CD36 and ABCA-1 as insulin [38,39].

Sedentary Lifestyle

While a sedentary lifestyle is significantly associated with cardiovascular disease, aerobic exercise reduces cardiovascular risk. Regular exercise causes a decrease in body fat, higher HDL cholesterol, lower LDL and triglyceride levels, higher insulin sensitivity, lower glucose levels and lower blood pressure [40].

High-fat diets and cholesterol are the factors that predispose the body most to the development of atherosclerosis in animal experiments [40].

Smoking

Cigarette smoking is another major risk factor associated with an increased level of multiple inflammatory markers, including C-reactive protein, IL-6, and tumour necrosis factor alpha (TNF-α), in both male and female smokers[41]. Cigarette smoking increases the oxidative modification of LDL[42] and decreases the plasma activity of paraoxonase, an enzyme that protects against LDL oxidation[43]. As a cardiovascular risk factor, cigarette smoking is not only an important but also reversible. The risk of cardiovascular events is proportional to tobacco consumption in both men and women and is higher in inhalers than in noninhalers[44].

Additionally, cigarette smoking has a prothrombotic effect that has an impact on different stages of atherosclerosis, from endothelial dysfunction to the appearance of clinical events[45]. Tobacco damages vasodilatation dependent on the endothelium, probably due to a mechanism of decreased bioavailability of nitric oxide. Moreover, it increases platelet activation and adhesion due to a decrease in nitric oxide and, at the same time, decreases the sensitivity of nitric oxide to platelets[46].

Metabolic Syndrome

Metabolic syndrome is defined as the cooccurrence of metabolic risk factors for both T2DM and cardiovascular disease (CVD): abdominal obesity, hyperglycaemia, dyslipidaemia, and hypertension. The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) definition is one the most widely used[47,48].

ATP III criteria define metabolic syndrome as the presence of any **three** of the following five traits:

- Abdominal obesity, defined as a waist circumference ≥102 cm (40 in) in men and ≥88 cm (35 in) in women.
- Serum triglycerides ≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides.
- Serum high-density lipoprotein (HDL) cholesterol <40 mg/dL (1 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL cholesterol.
- Blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure.
- Fasting plasma glucose (FPG) ≥100 mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose.

1.3.3. Other Risk Factors

Endothelial Dysfunction

The endothelium forms a thromboresistant layer between the blood and all other tissues. Endothelial dysfunction is associated with many of the traditional risk factors for atherosclerosis, including hypercholesterolemia, diabetes, hypertension, and cigarette smoking. The initial step in atherosclerosis is endothelial vasodilator dysfunction, induced by oxidized LDL [49], principally caused by loss of endothelium-derived nitric oxide [50]. It can be improved with correction of hyperlipidaemia by diet or by therapy with a statin (HMG-coenzyme A reductase inhibitor), which increases the bioavailability of nitric oxide [51,52].

Inflammation

Inflammation plays a key role in atherosclerosis[53] Oxidized LDLs modify macrophages, which release inflammatory substances such as cytokines and growth factors (Figure 3)[54].

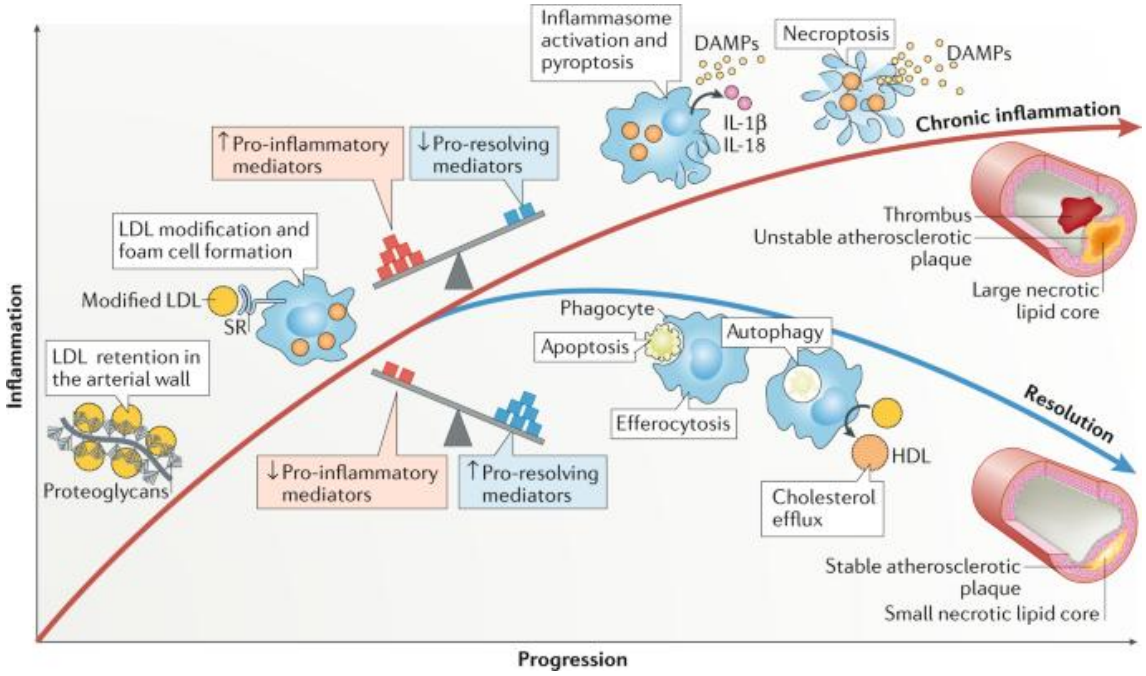

Figure 3. The balance of pro-inflammatory and antiinflammatory processes controls the resolution of the lipid-driven inflammation in atherosclerotic lesions. Retention of LDL particles by arterial wall proteoglycans and subsequent modification of the retained LDL induce inflammation in the arterial wall. Macrophages ingest the modified LDL particles via scavenger receptor (SR)-mediated endocytosis and become foam cells. If the balance between pro-inflammatory and pro-resolving mediators is tilted towards inflammation, the resolving mechanisms fail. Under these conditions, pyroptosis (mediated by inflammasome activation) or necroptosis can ensue. These pro-inflammatory forms of cell death further promote inflammation and generation of a large necrotic lipid core. These unstable atherosclerotic plaques might ultimately lead to plaque rupture and a local occluding arterial thrombus. Conversely, if the balance between the mediators is tilted towards pro-resolving mediators, apoptosis and autophagy-associated cell death and cholesterol efflux from the lesions are favoured, and efferocytosis of the dead cells can lead to resolution of inflammation. These processes promote the formation of a stable plaque with a small necrotic lipid core. DAMPs, damage-associated molecular patterns. IL, interleukins [55].

Several inflammatory molecules and cells are involved in the pathogenesis of atherosclerosis.

- C-reactive protein (CRP) is the most extensively studied biomarker of inflammation. CRP is a nonspecific marker that is increased as part of the acute phase response to inflammatory stimuli. This marker is highly involved in the atherosclerotic process.

Several epidemiologic studies have shown a significant association between elevated serum or plasma concentrations of CRP and the prevalence of underlying atherosclerosis, the incidence of first cardiovascular events among individuals at risk for atherosclerosis and the risk of recurrent cardiovascular events among patients with established disease[56–61]. CRP may have a direct effect on the development of atherosclerosis based on the finding of CRP in atherosclerotic lesions. CRP binds to LDL, allowing LDL to be taken up by macrophages without the need for modification[62–64]. CRP induces the expression of adhesion molecules and the production of IL-6 and MCP-1 in the human endothelium, which attracts macrophages and lymphocytes, increasing the inflammatory response in atherosclerotic plaques[65–67].

A normal value of CRP is not clearly defined. According to the European Society of Cardiology guidelines for cardiovascular disease prevention in clinical practice, high-sensitivity C-reactive protein (hs-CRP) levels may be measured as part of refined risk assessment only in patients with an unusual or moderate risk profile (class IIb/B recommendation) but not in asymptomatic low-risk or high-risk individuals (class III/B recommendation)[68]. The American College of Cardiology/American Heart Association guidelines state that hs-CRP measurement may be considered if, after quantitative risk assessment, a risk-based treatment decision is uncertain (class IIb/B recommendation)[69].

- Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a macrophage-secreted enzyme that may perpetuate plaque inflammation[70].

- Cytokines - Not all cytokines are involved in atherogenesis. In experimental studies, interleukin 4 (IL-4) and interleukin 10 (IL-10) are antiatherogenic interleukins[71]. Interleukin 1 (IL-1) and TNF-α cytokines have atherogenic effects. Cytokines enhance the expression of cell surface molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), cluster of differentiation 40 (CD40), and selectins on endothelial cells, smooth muscle cells, and macrophages[72].

- Pregnancy-associated plasma protein-A (PAPP-A) - PAPP-A is a high-molecular-weight zinc-binding metalloproteinase. It has been identified in vulnerable coronary plaques but not in stable ones. PAPP-A degrades the proteins that maintain the integrity of the protective fibrous cap of atherosclerotic plaques[73].

- Toll-like receptor 4 (TLR-4) is involved in the inflammatory response against gram-negative pathogens and possibly against other ligands. A polymorphism of this receptor gene, specifically Asp299Gly, seems to be related to lower levels of inflammatory markers, such as CRP, adhesion molecules and IL-6. Asp299Gly is associated with a diminished inflammatory response to Gram-negative pathogens[74].

- Leukocytes - Leukocytes (circulating monocytes and, to a lesser extent, T lymphocytes) accumulate early in atherosclerotic lesions, providing evidence for the role of local inflammation[75]. Inflammatory cells, including macrophages and T-lymphocytes, have often been found at the immediate site of intimal rupture or erosion of a thrombosed coronary artery in patients who die from acute myocardial infarction.

Infections

Atherosclerosis has been associated with chronic infections such as Chlamydia pneumoniae[76], Cytomegalovirus[77], and Helicobacter pylori[78]. Some viral infections, such as enterovirus (Coxsackie virus), hepatitis B virus, hepatitis A virus, and herpes simplex virus type 1 and type 2, are also involved in the pathogenesis of atherosclerosis[79].

Genetic Association

Two large genetical studies recently investigated the genetic influences and provided a better understanding of the molecular mechanism of atherosclerosis.

The first study investigated the roles of genes in atherosclerosis *in vitro, in vivo* and in association studies[80,81]. The second studied atherogenesis-regulating quantitative trait loci. This method has the potential to identify new atherosclerosis genes. The availability of whole-genome sequences in humans and mice has made it possible to perform genome-wide association studies, another unbiased approach, to identify disease genes relatively quickly compared with traditional genetic methods[82].

All of these studies have helped to identify newer therapeutic targets in atherosclerosis. For example, the discovery of genetic variants of PCSK9 in regulating LDL-C levels has led to the rapid development of PCSK9 inhibitors as a potential therapy for lowering LDL-C and reducing cardiovascular events[83–87].

Tissue Factor

Tissue factor (TF) is the primary initiator of coagulation and enhances platelet activity, and plaque disruption promotes thrombosis. Tissue factor also plays a role in the progression of atherosclerosis via coagulation-dependent and coagulation-independent mechanisms. In one study, TF overexpression increased neointimal area and plaque size by increasing mural thrombus and smooth muscle cell migration and accelerating endothelial regrowth over the plaque after rupture[88].

Endothelin-1

Endothelin-1 is a potent vasoconstrictor and mitogen for vascular smooth muscle cells, stimulating their migration and growth and contributing to the pathogenesis of atherosclerosis at all stages, even when the plaque is clinically imperceptible[89,90].

Angiotensin II

Increased plasma concentrations of angiotensin II, particularly when combined with hyperlipidaemia, contribute to atherosclerosis[91]. Angiotensin II may modulate vascular smooth muscle cell proliferation and the production of extracellular matrix[92,93].

Adhesion Molecules

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are cell surface endothelial glycoproteins. ICAM-1 is expressed on normal endothelial cells. VCAM-1 is present in the microvessels of human atherosclerotic lesions with inflammation. VCAM-1 stimulates leukocyte adherence to the endothelium[94].

Mitochondrial DNA damage

This condition has been investigated in experimental animals, mostly mouse models[95,96].

Mitochondrial DNA (MtDNA) lesions are found in developed aortic plaques. More mtDNA lesions and larger plaque sizes were detected in the aortas of mice expressing mitochondrial mutations[97,98]. In the VIVA trial (Virtual Histology in Vulnerable Atherosclerosis), an association between mtDNA of leukocytes and plaque vulnerability was detected. Mitochondrial DNA damage is uniquely associated with thin cap fibroatheroma, which is associated with a high risk of cardiovascular events[98,99].

Flow Characteristics

Altered blood flow and low shear stress can play a role in the development of atherosclerosis. Disturbed flow can alter endothelial cell function. These changes may be mediated by inhibition of the release of nitric oxide from endothelial cells[33,100].

1.4. Atherogenesis

The basic research of atherosclerosis in recent years has allowed us to change the concept of this disease, traditionally considered merely lipid deposition, which we currently known as a disease of a clearly inflammatory nature.

Atherogenesis is the process of forming atherosclerosis. It is characterized by a proliferative inflammatory response to LDL that has been deposited in the subendothelial sheet of the vascular wall, mainly in the intimal layer, in response to different damaging agents.

The first atherosclerotic lesion is located in the tunica intima and is popularly called the fat striatum. It is the first lesion visible macroscopically and consists of the accumulation of lipids. It is produced by endothelial dysfunction initiated by the influence of several factors[101,102](Figure 4).

Endothelial dysfunction is a primary step in the development of atherosclerosis. In the early stage of atherosclerosis, the accumulation of advanced glycation end products (AGEs) due to oxidative stress is perceived. The accumulation of AGEs causes endothelial damage by activating cytokines and increasing reactive oxygen species (ROS) and oxidized low-density lipoprotein (ox-LDL) retention via the macrophage-SR pathway. Dysfunction of the vascular tone balance is noted by decreased nitric oxide (NO) and increased endothelin-1 (ET-1)[103,104]. NO is produced from the conversion of L-arginine from endothelial cells to L-citrulline. The activity of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NO synthase (NOS) is mediated by calcium, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin (BH4) as cofactors[105–108] ET-1 is a 21-amino acid peptide that regulates vasoconstriction, inflammation, and the proliferation of endothelial cells by interactions with NO[109]. ET-1 expression can be both inhibited and stimulated by eNOS.

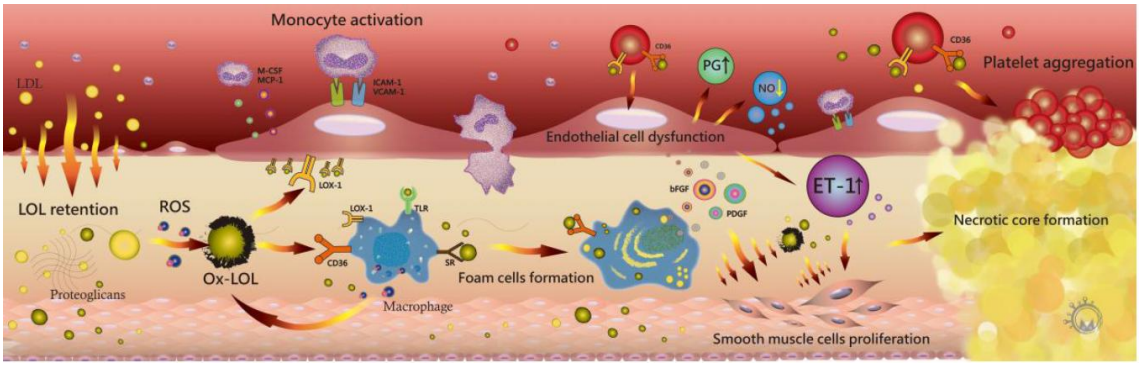

Figure 4. Small low-density lipoproteins (LDL) penetrate the endothelial barrier, bind to proteoglycans through apolipoprotein B100, and are retained in the subendothelial space. LDL is oxidized (ox-LDL) and induces several pro-inflammatory conditions via lectin-like oxidized LDL receptor-1 (LOX-1). The upregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1) by ox-LDL increase monocyte and inflammatory cell adhesion on the endothelium. Ox-LDL particles stimulate endothelial cells and smooth muscle cells (SMCs) to secrete monocyte chemotactic protein-1 (MCP-1) and monocyte colony stimulating factor (M-CSF), both factors induce monocyte recruitment. Ox-LDL promotes an increased in reactive oxygen species (ROS) and inhibits nitric oxide production. Monocytes differentiate into macrophages and express scavenger receptors (SRs), cluster of differentiation 36 (CD36), LOX-1, and Toll-like receptors (TLRs). Ox-LDL– CD36 interaction induces monocyte differentiation, macrophage activation, and macrophage Retention.Macrophage SRs increase ox-LDL uptake and foam-cell formation. The retention of ox- LDL leads to foam cell apoptosis and inflammatory progression. Ox-LDLs also increase the expression of growth factors, including platelet-derived growth factor (PDGF) for migration and basic fibroblast growth factor (bFGF) for proliferation, on SMCs. SMC proliferation contributes to the thickening of atherosclerotic plaques and formation of a necrotic core. The ox-LDL–CD36 interaction in resting platelets causes platelet aggregation and activation, with activated platelets expressing LOX-1 to mediate adhesion to endothelial cells[110].

Inflammatory response activation starts with ox-LDL. Ox-LDL stimulates the release and enhanced tissue levels of ET-1 in endothelial cells, vascular smooth muscle cells (VSMCs), and inflammatory cells. Other proinflammatory factors, such as IL-1, thrombin and TNF-α, induce adhesion of molecules such as selectins, E-selectin, P-selectin, immunoglobulins, ICAM-1 and VCAM-1[111,112]. They cause the accumulation of T-lymphocytes and monocyte chemoattractant protein-1 (MCP-1) in the lesion[113]. When monocytes pass the endothelial barrier, they are converted to macrophages. Macrophages release cytokines and growth factors and absorb LDL. They convert to lipid-filled cells (foam cells). Foam cells are “the heart” of the central lipid nucleus. Foam cells produce ET-1, which can act on macrophages by binding to endothelin B

(ETB) receptors[114,115]. Foamy cells and the lipids produced from destroyed cells accumulate in the central nucleus of atherosclerotic plaques. VSMCs are located around the lipid nucleus and form a fibrous capsule due to their active synthesis of extracellular matrix rich in collagen.

Adhesion molecules induce adhesion of leucocytes and monocyte colony-stimulating factor (M-CSF) to the endothelium. M-CSF is responsible for the differentiation of monocytes to macrophages. This differentiation entails the expression of scavenger receptors. These receptors help macrophages to collect large amounts of modified LDL, transforming them into foam cells (Figure 5).

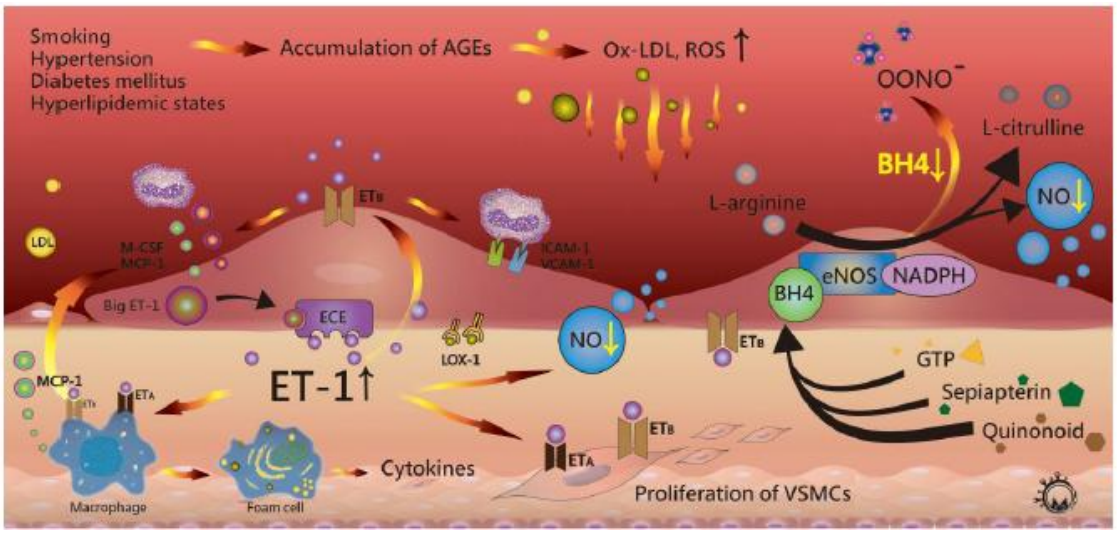

Figure 5. Hyperlipidemic status and other conditions can induce the accumulation of advanced glycation end products (AGEs), leading to increase reactive oxygen species (ROS) and retention of oxidized low-density lipoprotein (ox-LDL). Oxidative stress causes endothelial dysfunction and impairs the release of nitric oxide (NO) and endothelin-1 (ET-1). In atherosclerotic lesions, elevated tissue levels of ET-1 bind to ETB receptors on endothelial cells and cause expression of endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular-cell-adhesion molecule-1 (VCAM-1). ET-1 promotes monocyte migration and activation by monocyte chemoattractant protein-1 (MCP-1), which is released from activated macrophages and endothelial cells. ET-1 also activates vascular smooth muscle cells (VSMCs) via ETA receptors to promote SMC proliferation. Oxidative stress also causes lower tissue levels of BH4 and induces the uncoupling of endothelial nitric oxide synthase (eNO and superoxide) [110]

1.5. Plaque Rupture

Formed atherosclerotic plaque can evolve towards a stable or unstable situation. This mainly depends on its composition and the thickness of the fibrous layer.

Stable plaques can remain silent without causing damage. Unstable plaques can break and cause clinical manifestations.

The most frequent clinical manifestations of atherosclerosis are produced by occlusion of the flow due to the formation of a thrombus or clot. The ability of the lipid nucleus to form thrombi depends highly on tissue factor, and it is an important initiator of the coagulation cascade.

Initially, Virmani et al.[116] established three main causes of thrombus formation: plaque rupture, plaque erosion, and calcification of the nodules. Further investigations included a fourth cause, plaque haemorrhages. Peter Libby et al.[117] noted that the fibrous layer undergoes changes that include thinning before rupture occurs. This rupture is an injury consisting of a necrotic heart surrounded by a thin fibrous layer, generally <65 μm, which, when ruptured, releases highly thrombogenic substances into the bloodstream.

The second complication that atheroma plaque can cause is plaque erosion. In this case, the thrombus has a rich base in VSMCs and proteoglycans. The third cause, and the most exceptional cause for thrombus formation, is the erosion of calcified nodules. Plaques with calcification are characterized by a discontinuous fibrous layer with an irregular luminous surface with the absence of CDs and an underlying thrombus. Currently, a fourth cause is intraplate haemorrhages[118]. The reason for why the degradation of the layer occurs appears to be related to degradation in the extracellular matrix[119] and a subsequent mechanical stress on the weakened plate[120] (Figure 6).

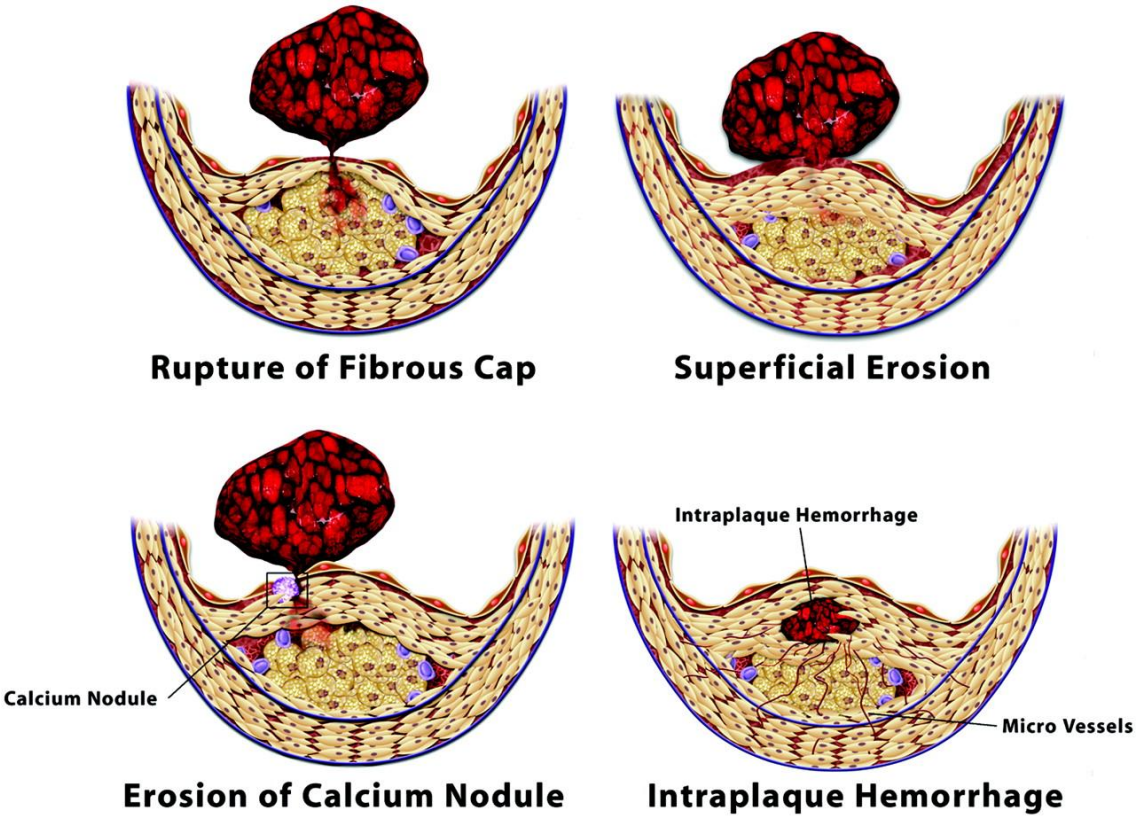

Figure 6. Anatomy of the main causes of thrombus formation in order of incidence (A> B> C> D). A: Rupture of Fibrous Cap. As plaque ruptures, it releases highly thrombogenic material into the bloodstream. B: Superficial Erosion. Erosion in the endothelial cells brings the thrombogenic material into contact with the blood. C: Calcification of the nodules. These calcified plaques are characterized by discontinuity, which allow the release of thrombogenic material. D: Intraplaque hemorrhage. Plaque internal ruptures result increases a plaque size. The consequence is reduced blood vessel lumen. Adapted from[121]

Atheroma plaques are classified into stable and unstable plaques. A stable plaque is structured by a lipid nucleus, a thick fibrous capsule rich in smooth muscle cells, extracellular matrix and inflammatory infiltrate.

The unstable plaque contains a high lipid concentration in the nucleus, with a fine fibrous capsule and a large inflammatory component consisting of macrophages, lymphomonocytes and T cells. In addition, vulnerable plaques express a greater number of markers, such as calprotectin, inflammatory cytokines, and intimal cell apoptosis, that lead to an increase in the necrotic nucleus in the plaque[122]. The American Heart Association (AHA) proposed an atherosclerotic plaque staging system in 1994 based on its structure and histological composition (Table 1).

AHA grade	Criteria	Comments and corresponding gross classification
0	Normal artery with or without intimal thickening; no hold	Normal tissue
1	Isolated MFCs containing lipid; variable adaptive intimal thickening grossly with lipid staining	Initial atherosclerosis lesion, sometimes visible grossly with lipid staining
2	Numerous MFCs, often in layers, with fine particles of extracellular lipid; no distinct pools of extracellular lipid: variable adaptive intimal thickening	Fatty streak, visible grossly with III staining
3	Numerous MFCs with > pools of extracellular lipid: no well-defined core of extracellular lipid	Fatty plaque, raised fatty streak, intermediate lesion, or transitional lesion
4	Numerous MFCs plus well-defined core of extracellular lipid, but with luminal surface covered by relatively normal intima	Atheroma, fibrous plaque, or raised lesion
5	Numerous MFCs, well-defined core or multiple cores of extracellular lipid, plus reactive fibrotic cap, vascularization, or calcium	Fibroatherema, fibrous plaque, or raised lesion
6	All of the above plus surface defect, hematoma, hemorrhage, or thrombosis	Complicated lesion

Table 1. Criteria for American Heart Association lesion classification system and correspondence with classification of gross arterial specimens. MFC: macfrophage foam cell; AHA: American Heart Association.

The first histological change is the intimal thickening and proliferation of smooth muscle cells and extracellular matrix, which is called grade 1.

In grade 2 lesions, both extracellular and intracellular lipid accumulation is observed. Macrophages accumulate intracellular lipids (known as "foam cells"), and they mainly consist of LDL-cholesterol. Both T lymphocytes and LDL-cholesterol form the "fatty stretch".

Grade 3 lesions comprise foam cells and products of necrosis of macrophages. They contain small extracellular lipid deposits (cholesterol esters).

Grade 4 lesions comprise type II lesions and core extracellular lipids.

In grade 5 lesions, one capsule can be relatively acellular (formed by dense collagen) or may have abundant smooth cells. As the plaque develops, this lesion acquires its own microvascular network (vasa vasorum), which extends from the adventitia and reaches

the thickened intima. These thin-walled vessels are prone to rupture, causing bleeding inside the plate. Grade 6 lesions have a structure characterized by calcified fibrosis areas, formed by the accumulation of connective tissue with an increase in lipid-filled smooth muscle cells, frequently with extracellular lipids, with areas of visible ulceration. These types of lesions are frequently associated with symptoms or embolization (Figure 7)[123–125].

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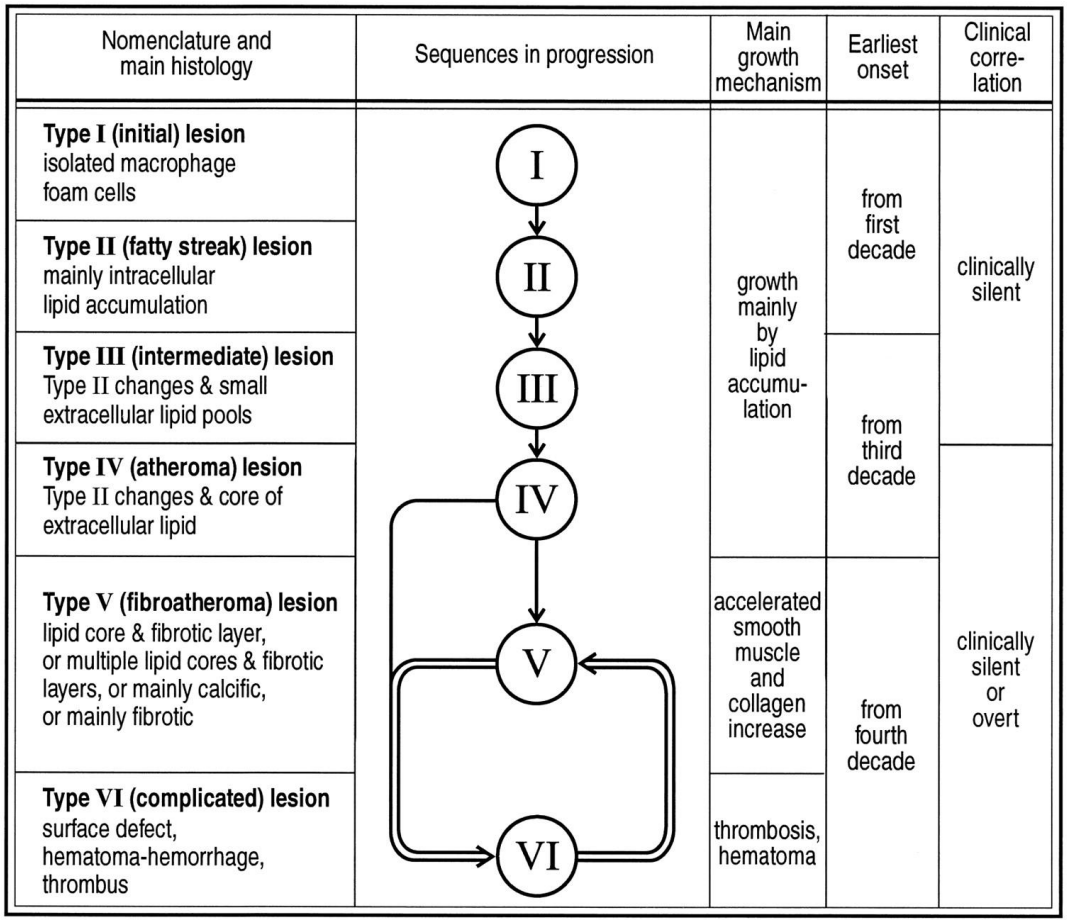

Figure 7. Staging system based on a histological composition: Flow diagram in center column indicates pathways in evolution and progression of human atherosclerotic lesions. Roman numerals indicate histologically characteristic types of lesions enumerated in Table 2 and defined at left of flow diagram. The direction of arrows indicates sequence in which characteristic morphologies may change. From type I to type IV, changes in lesion morphology occur primarily because of increasing accumulation of lipid. The loop between types V and VI illustrates how lesions increase in thickness when thrombotic deposits form on their surfaces.

Thrombotic deposits may form repeatedly over varied time spans in the same location and may be the principal mechanism for gradual occlusion of medium-sized arteries.

Terms for Atherosclerotic Lesions in Histological Classification		Other Terms for the Same Lesions Often Based on Appearance With the Unaided Eye	
Type I	Initial lesion		Early lesions
Type IIa	Progression-prone type II lesion	Fatty dot or streak	
Type IIb	Progression-resistant type II		
Type III	Intermediate lesion (preatheroma)		
Type IV	Atheroma	Atheromatous plaque	
Type Va	Fibroatheroma (type V lesion)	fibrolipid plaque	
		fibrous plaque	
Type Vb	Calcific lesion (type VII lesion)	Calcified plaque	Advanced lesions
Type Vc	Fibrotic lesion (type VIII lesion)	Fibrous plaque	raised lesions
Type VI	Lesion with surface defect, and/or hematoma-hemorrhage, and/or thrombotic deposit	Complicated lesion, complicated plaque	

Table 2. Terms Used to Designate Different Types of Human Atherosclerotic Lesions in Pathology

1.6. Estimation of cardiovascular risk

Atherosclerosis is the underlying cause of most cardiovascular complications. As cardiovascular complications represent the main cause of death in the world, early diagnosis and assessment of cardiovascular risk are essential and are the basis for the prevention and treatment of cardiovascular events[126].

The primary evaluation of the risk of cardiovascular complications is based on the use of several equations based on epidemiological studies. Currently, we have up to seven risk equations. The most commonly used equation in clinical practice used to be the Framingham equation (Figure 8A)[127], but the REGICOR[128] equation and the SCORE (Systematic COronary Risk Evaluation) and SCORE2-OP (Figure 8B)[129,130] equations are now more commonly used. REGICOR has been validated in Catalonia[131]. In the general population between 35 and 75 years of age, REGICOR correctly estimates the occurrence of coronary episodes, with no significant differences between the number of events estimated by the equation and those observed at the 5-year study period[132].

The Framingham study[133,134] estimates the risk of cardiovascular disease in a mean period of ten years using equations that combine risk factor values: age, sex, cholesterol levels, diabetes, hypertension, smoking, healthy lifestyle behaviours and the use of medical treatments to control any of these risk factors. These equations give great importance to sex and age and give less value to other risk factors, especially in the case of men under 45 and women under 65. Furthermore, they may overestimate or underestimate the risk based on different ethnic groups or populations. This makes their individual value limited.

In Europe, the European Society of Cardiology (ESC) guide recommends the use of SCORE, which gives value to the statistical data of each country, thus generating an ideal equation for each European country [130,134].

The Lifetime Risk Score (LRS) evaluates risk throughout life. The main variations from the previous risk calculators' estimates are that LRS counts the duration of exposure of individuals to risk. LRS gives more importance to small changes during childhood than to large changes in older people[135] LIFE-CVD demonstrates that increasing age is a negative prognosticator of cardiovascular disease (CVD) risk. LIFE-CVD reveals that

with increasing age, although a higher 10-year absolute CVD risk reduction is observed after implementation of lifestyle or pharmacological interventions, a lower benefit is achieved with the gain in CVD-free life expectancy[123].

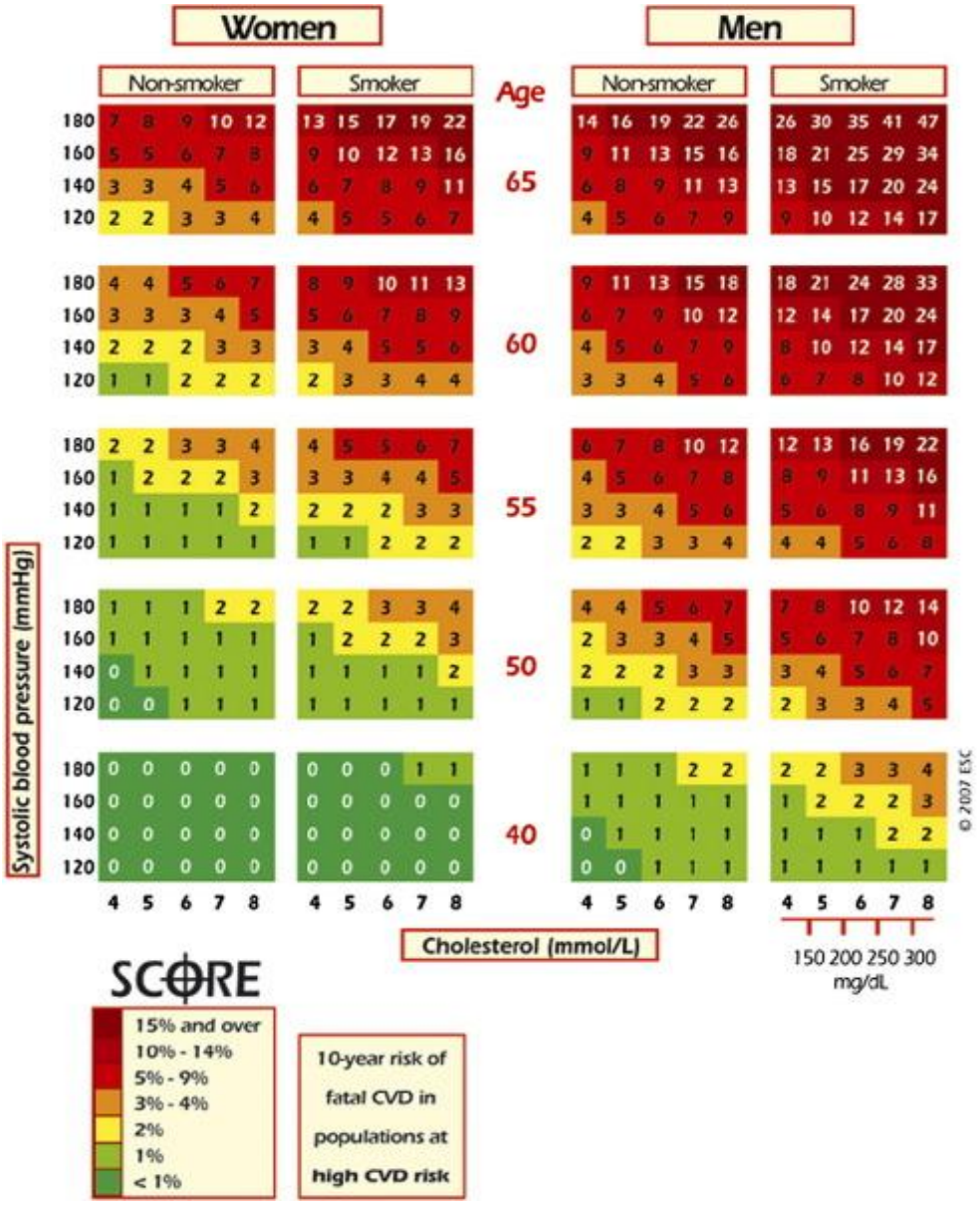

Figure 8A. Estimator from the Framingham study.

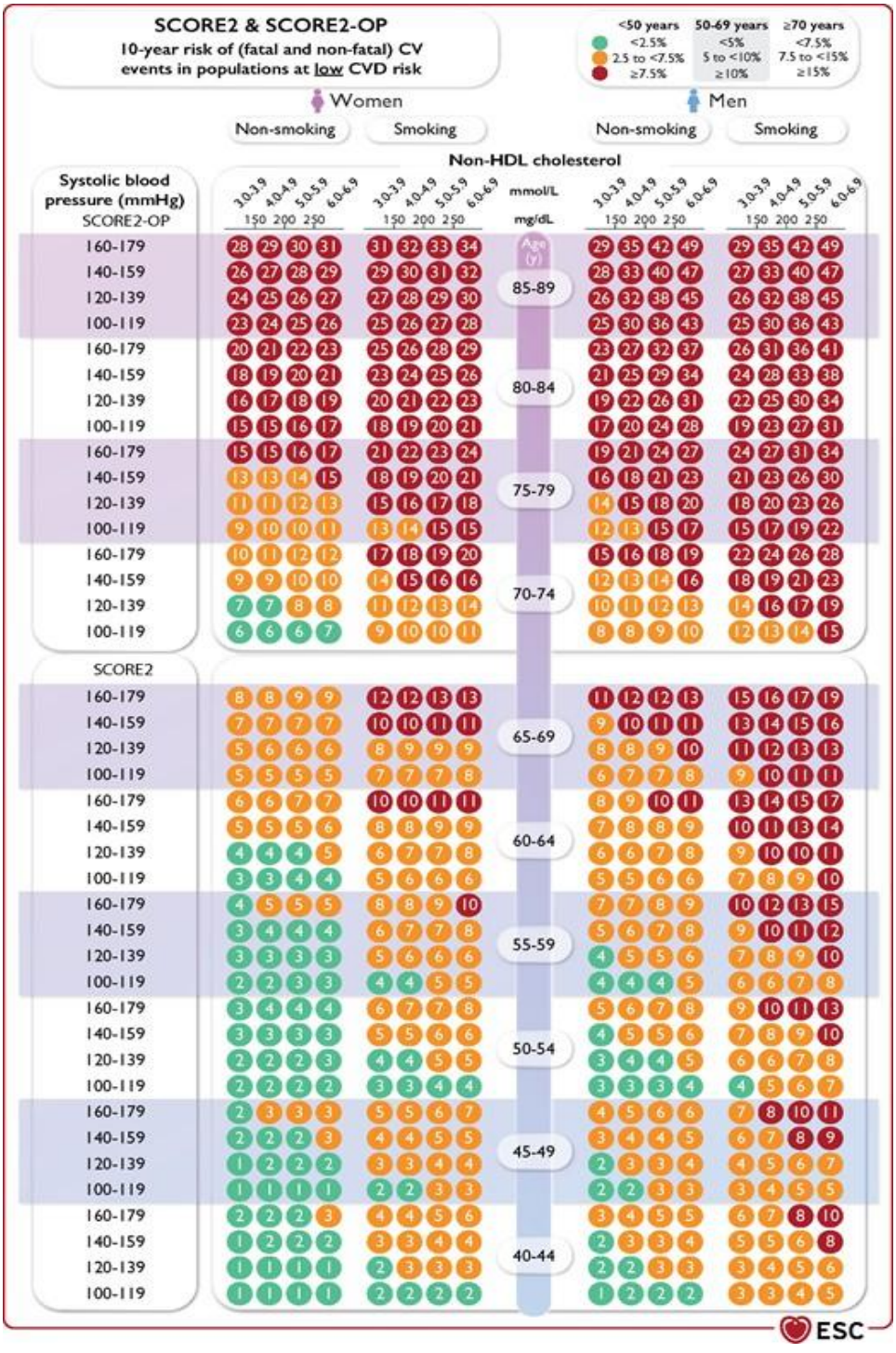

Figure 8B. SCORE and SCORE -OP estimator.

Figure 8. Cardiovascular risk estimators. A: Estimator from the Framingham study. It is indicated to assess an individual's risk of suffering a CVE in the next 10 years. It is indicated for adults from 20 years old who have not had a previous CVD or Diabetes. B: SCORE estimator. Calculate the risk of suffering a CVE at 10 years based on age, sex, smoking, blood pressure and total cholesterol, with age and sex being the factors with the greatest weight in the estimation.

1.7. Carotid Atherosclerosis: Pathophysiology of Symptoms

Atherosclerosis is a pathologic process that causes pathological changes not only in the coronary and peripheral arteries but also in cerebral arteries. Atherosclerosis is the most common cause of *in situ* local disease within the large extracranial and intracranial arteries that supply the brain.

Carotid atherosclerosis is usually located 2 cm above the carotid bifurcation and predominantly involves the posterior wall of the vessel. The blood vessel reduces its diameter as a function of plaque growth. Not all patients with atherosclerotic carotid plaque are symptomatic. Symptomatic patients present several clinical manifestations, from carotid bruit to ischaemic symptoms. The mechanism of stroke may be embolism of the thrombotic material or low flow due to stenosis with inadequate collateral compensation[136].

Carotid bruit is an important sign of carotid stenosis and is heard over the site of the stenosis[137]. However, carotid bruit in asymptomatic patients is a poor predictor for the presence of underlying carotid stenosis and for the subsequent development of stroke. In symptomatic patients, carotid bruit is useful for diagnostic screening.

Ischaemic symptoms may be transient, representing transient ischaemic attacks (TIAs), or permanent, resulting in cerebral infarction. TIAs may be due to either low flow, manifesting as brief, repetitive, stereotyped spells, or embolization. Embolic TIAs are usually singular and more prolonged. Symptomatology depends on the vascular territories involved. When the internal carotid artery occludes completely, it can also cause low flow or embolic ischaemic events depending upon the adequacy of collateral flow through the orbit and across the circle of Willis. The prognosis of patients with stroke due to carotid occlusion depends on collateral flow[138].

1.8. Evaluation of carotid artery stenosis

As mentioned, carotid stenosis can be symptomatic and asymptomatic. The classification has been used depending on whether there are signs or symptoms of ischaemia in the carotid territory. We use four diagnostic techniques to directly image the internal carotid artery: cerebral angiography, carotid duplex ultrasound, magnetic resonance angiography and computed tomographic angiography. These diagnostic modalities have their own advantages and disadvantages.

Currently, three predominant methods are used for evaluating the degree of angiographic stenosis: the North American Symptomatic Carotid Endarterectomy Trial (NASCET), the European Carotid Surgery Trial (ECST) and the Common Carotid method (CC). NASCET measures the residual lumen diameter at the most stenotic portion of the vessel and compares this with the lumen diameter in the normal internal carotid artery distal to the stenosis[139]. ECST measures the lumen diameter at the most stenotic portion of the vessel and compares this with the estimated probable original diameter at the site of maximum stenosis[140]. CC measures the residual lumen diameter at the most stenotic portion of the vessel and compares this with the lumen diameter in the proximal common carotid artery (Figure 9)[141].

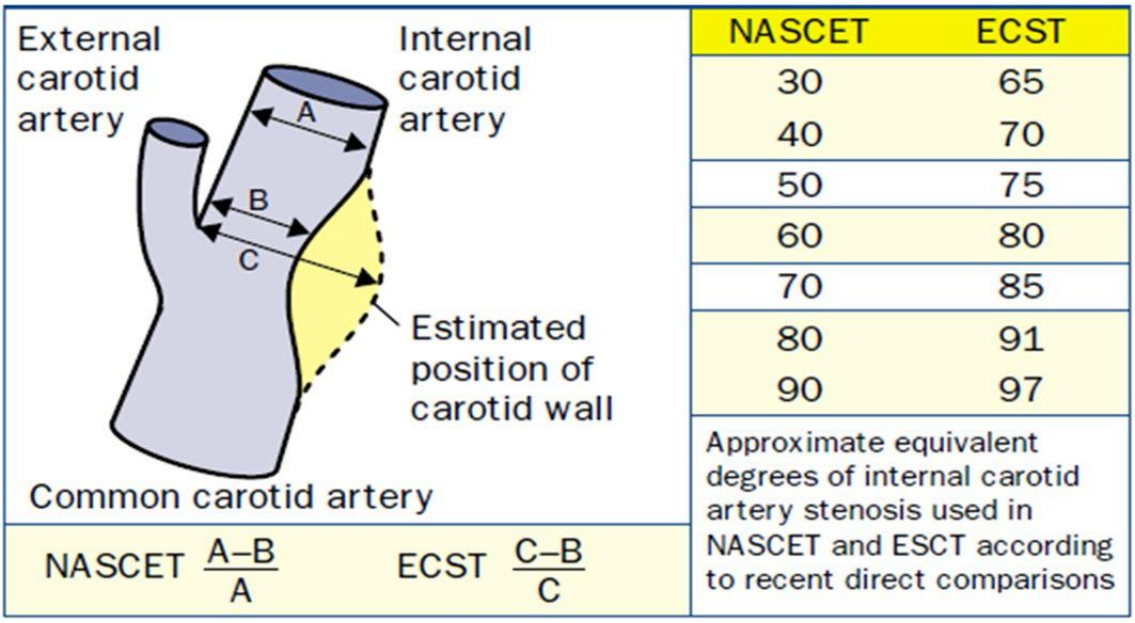

Figure 9. NASCET and ECST measurements of internal carotid artery stenosis[142]

There are some differences in the results of measured stenosis by these three methods. The ECST and CC methods quantify a higher degree of stenosis than the NASCET method. ECST methodology requires an assumption of the true lumen, which increases the risk of interobserver variability. Despite these differences, the results of all three methods have a nearly linear relationship with each other and provide data of similar prognostic value[141].

Four imaging modalities are used to visualize the internal carotid artery: cerebral angiography, carotid duplex ultrasound (CDUS) with transcranial Doppler (TCD), magnetic resonance angiography (MRA) and computed tomography angiography (CTA).

Cerebral angiography

Cerebral angiography is the *gold standard* for imaging carotid arteries and permits an evaluation of the entire carotid artery system. This method provides information about tandem atherosclerotic disease, plaque morphology and composition, and collateral circulation, which may affect its management[143]. The disadvantages of cerebral angiography are the high cost, invasive nature and risk of morbidity and mortality. The development of intraarterial digital subtraction angiography (DSA) reduces the dose of contrast, uses smaller catheters, and shortens the length of the procedure[144]. DSA helps to identify patients who can benefit from carotid endarterectomy[145] (Figure 10).

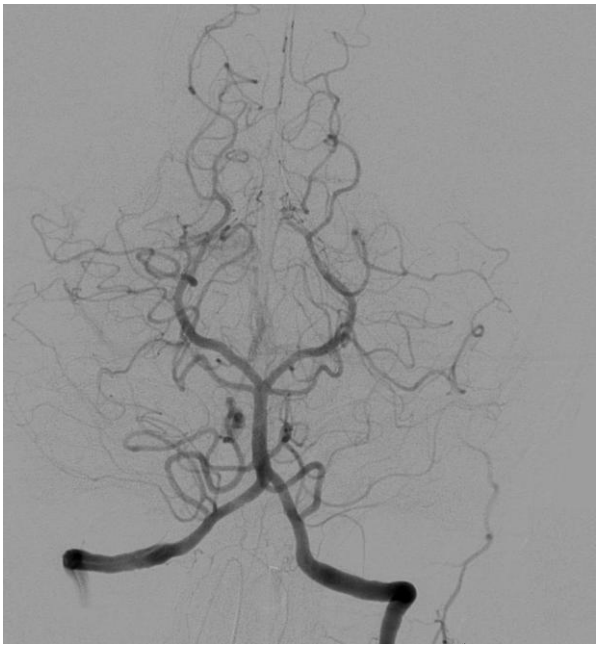

Figure 10. Cerebral angiography, injection in the left vertebral artery, with retrograde flow in the contralateral vertebral artery, the basilar artery and the posterior communicating artery. The posterior cerebral circulation can be seen, including the posterior part of the Circle of Willis[146]

Carotid Doppler ultrasound

Carotid Doppler ultrasound is a noninvasive, safe and relatively cheap imaging technique for evaluating the carotid arteries, with a sensitivity of 81-98% and specificity of 82-89% in detecting significant internal carotid artery stenosis[147,148].

As it is very important to diagnose carotid artery stenosis (CAS) at an early stage, carotid ultrasound can help to detect it[149]. Carotid ultrasound has limited utility in obtaining information about plaque composition and intraplaque haemorrhage. This method uses B-mode ultrasound for morphological characterization of the plaque. Ultrasound Doppler detects the blood flow speed, which plays a role in determining the degree of stenosis[147]. The velocity during the systolic peak is the most widely used measure for estimating the severity of stenosis. The speed at the end of diastole, the spectrum configuration and carotid index (speed ratio in the internal carotid artery and speed in the common carotid artery) could provide additional information about the plaque (Figure 11).

The most frequently used echo Doppler classification is the Gray–Weale classification, which classifies atherosclerotic plaque into four types:

Type I: Predominantly echolucent.

Type II: Mainly echolucent but with echogenic areas.

Type III: Mainly echogenic but with echolucent areas.

Type IV: Uniformly echogenic

Type V is reserved for plaques that are extremely calcified or those that cannot be well visualized.

Using this classification, we could associate symptomatic patients with type I and II plaques and asymptomatic patients with type III and IV plaques. However, many conditions, especially depending on the plaque structure, do not permit clear classification. During echo Doppler practice, it is very important to obtain a correct plaque description. These classifications can only be used as an additional classification because of their several limitations[150].

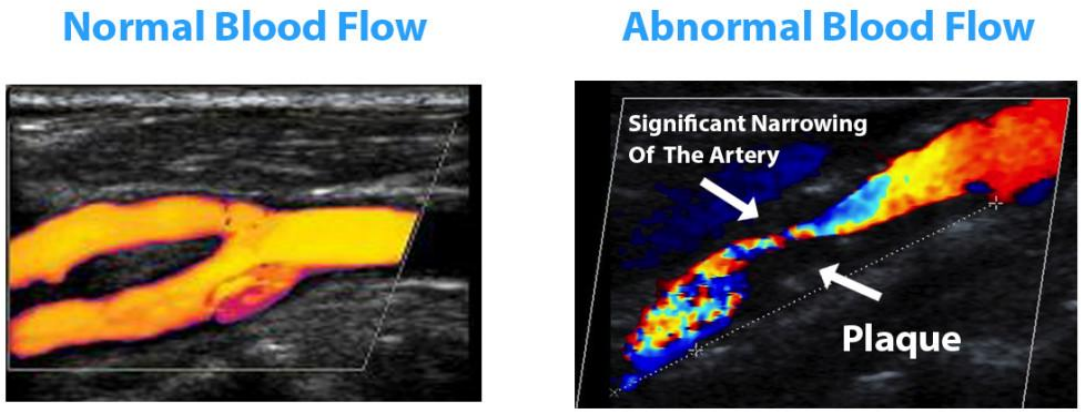

Figure 11. Carotid Doppler ultrasound: Normal Blood flow (left); Abnormal Blood Flow[151]

Magnetic Resonance Angiography

Magnetic resonance angiography (MRA) techniques utilize either two- or three-dimensional time-of-flight (TOF) MRA or gadolinium-enhanced MRA (also known as contrast-enhanced MRA or CEMRA). The CEMRA technique is more sophisticated than the TOF technique. MRA has good sensitivity for detecting high-grade carotid stenosis.

MRA imaging (Figure 12) has many advantages. MRA is less operator-dependent and provides very objective imaging. However, MRA cannot be performed if the patient is

critically ill and unable to lie supine. Furthermore, it cannot be performed if the patient has claustrophobia, a pacemaker or ferromagnetic implant[152,153].

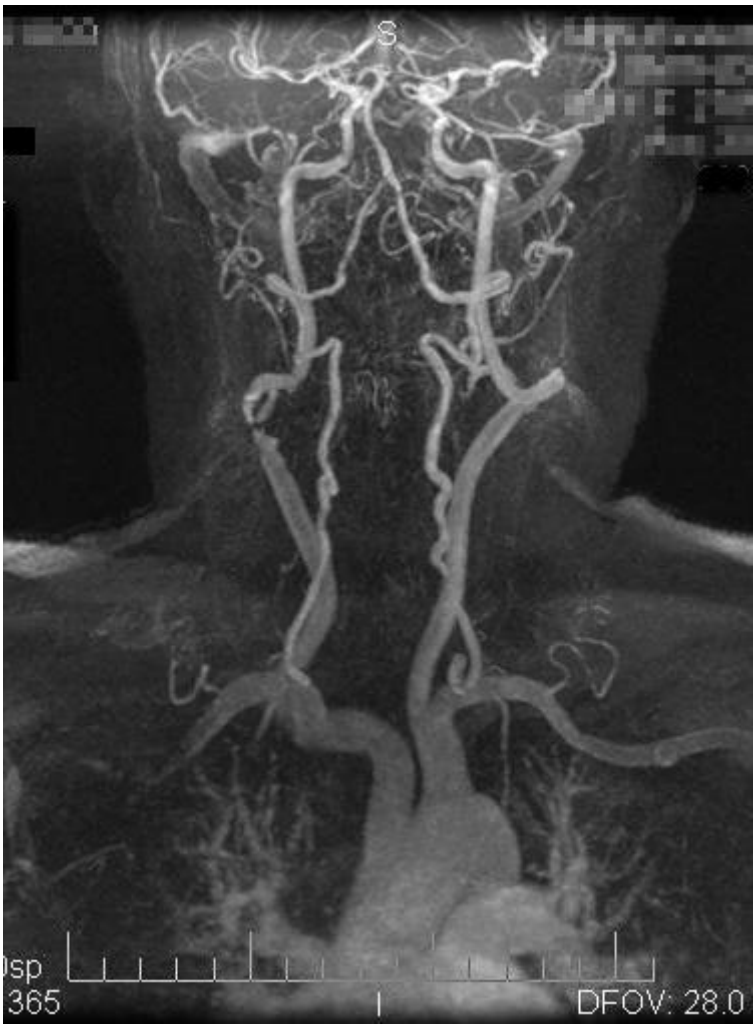

Figure 12. Magnetic resonance angiography neck and head circulation[154]

Angiographic Computed Tomography

Angiographic computed tomography (ACT) is especially useful when carotid duplex ultrasound cannot be performed, e.g., in cases with severe kinking, calcification, short neck, or high bifurcation or when whole-field imaging is needed (bone and muscle structure included).

Computed tomography angiography provides an anatomic depiction of the carotid artery lumen. It requires a contrast bolus comparable with that administered during a conventional angiogram. It cannot be performed in cases of contrast allergies and renal failure (Figure 13).

A meta-analysis published in 2006 concluded that ACT compared with intra-arterial cerebral angiography for the diagnosis of 70 to 99% carotid stenosis had a sensitivity of 0.77 (95% CI 0.68-0.84) and a specificity of 0.95 (95% CI 0.91-0.97)[155].

An earlier systematic review and meta-analysis that compared ACT with arteriography or digital subtraction angiography concluded that ACT is an accurate method for the detection of severe carotid artery disease, particularly for the detection of carotid occlusion, where CTA had a sensitivity and specificity of 97 and 99%, respectively[156].

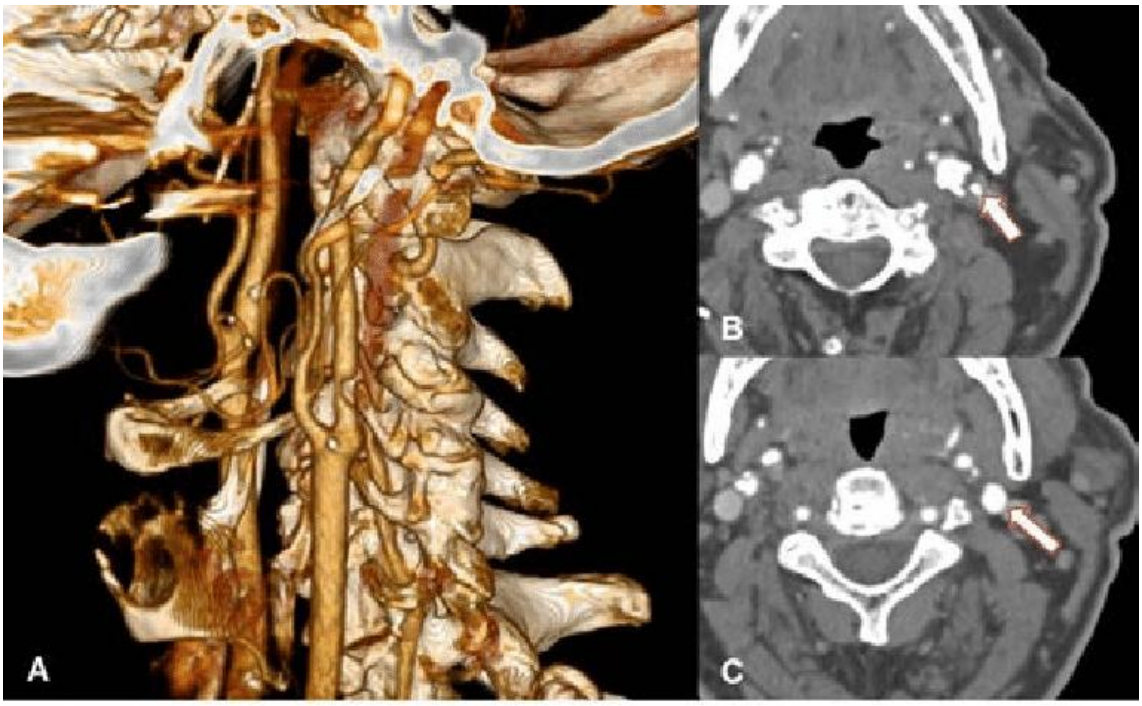

Figure 13. NASCET method for measuring carotid artery stenosis. Volume rendered CTA axial (A) and MPR images (B and C). The volume rendered post-processed CTA image (A) show the anatomic sites of measurement in the carotid artery for calculating percent stenosis for the NASCET method. It was calculated as the ratio between the residual luminal surface at the stenosis (B) and the surface of the distal normal lumen where there is no stenosis (C)[157]

1.9. Management and Treatment of Carotid Atherosclerosis

1.9.1. Treatment of Symptomatic Carotid Atherosclerosis

Symptomatic disease is defined by acute onset of focal neurological symptoms in the ipsilateral distribution territory of the affected carotid artery. These symptoms could be presented by transient ischaemic attacks characterized by focal neurological dysfunction, transient monocular blindness (amaurosis fugax) or ischaemic heart attacks. The definition is based on a symptom evaluated during the last six months [139,140,158].

The treatment of symptomatic carotid atherosclerosis includes medical or surgical options. Surgical options include revascularization by carotid endarterectomy (CEA) or carotid artery stenting (CAS) in significant carotid artery stenosis. Medical treatment could be practised if the stenosis is not significant (<50%).
CEA is recommended in patients with symptomatic carotid atherosclerosis with 70-99% stenosis and at least five years of life expectancy. Patients should meet the following conditions: surgically accessible lesion; absence of cardiac, pulmonary or other pathology that meet surgical or anaesthesia contraindications; and no history of previous ipsilateral endarterectomy[159].Carotid artery stenting (CAS): Transfemoral carotid artery angioplasty and stenting is the standard for endovascular carotid intervention[160].

CAS reduces the risk of embolization, thrombosis, carotid artery recoil, and long-term restenosis. CAS procedures are performed with local anaesthesia and minimal or no sedation, but they can be performed with total anaesthesia.
Guidelines from the American Heart Association/American Stroke Association (AHA/ASA) define the following:
Patients with symptomatic carotid stenosis that is less than 50 percent could benefit only from medical management rather than CEA or CAS[161]

For men with recently symptomatic carotid stenosis of 50 to 69 percent who have a life expectancy of at least five years, CEA rather than medical management alone is suggested[162]

For women with recently symptomatic carotid stenosis of 50 to 69 percent, medical management rather than CEA is suggested[162]

For patients with recently symptomatic carotid stenosis of 70 to 99 percent who have a life expectancy of at least five years, CEA rather than CAS is suggested.
For patients with total or near total occlusion of the symptomatic ipsilateral internal carotid artery, medical management rather than CEA or CAS is suggested.
For patients selected for treatment with CEA, CEA should be performed within two weeks (but not within the first two days) of the last symptomatic event rather than a later time. Observational evidence suggests that CEA in the first 48 hours after stroke onset is associated with increased risk compared with CEA performed 3 to 14 days after symptom onset.
For select patients with recently symptomatic carotid stenosis of 70 to 99 percent, CAS rather than CEA is suggested if any of the following conditions are present: a carotid lesion that is not suitable for surgical access; radiation-induced stenosis; or clinically significant cardiac, pulmonary, or other disease that greatly increases the risk of anaesthesia and surgery.

Medical management: All patients with atherosclerotic carotid artery stenosis in any location and regardless of symptoms should receive antithrombotic and antilipemic therapy and risk factor modifications.

1.9.2. Treatment of Asymptomatic Carotid Atherosclerosis

Regarding the treatment of asymptomatic carotid atherosclerosis, it is important to identify personal risk factors, change lifestyles and maintain medical treatment. The selection of asymptomatic patients who can benefit from revascularization therapy is another important issue highlighted by the AHA[163]

Prophylactic CEA could be considered in selected asymptomatic patients with a minimum of 60% stenosis measured by angiography or 70% stenosis measured by echo Doppler with less than 3% morbidity and mortality.

Prophylactic stents could be performed in asymptomatic patients with a minimum stenosis of 60% due to angiography, 70% by Doppler ultrasound or 80% by CT angiography or MRI angiography if the ultrasonic stenosis is 50-69%.

General CV prevention and multidisciplinary management in symptomatic and asymptomatic patients is of the utmost importance. Best medical therapy (BMT) includes CV risk factor management, such as best pharmacological therapy, as well as nonpharmacological measures such as smoking cessation, healthy diet, weight loss and regular physical exercise. The pharmacological component of BMT includes antihypertensive, lipid-lowering and antithrombotic drugs[68,134,164,165], and in diabetic patients the optimal glucose level control should be obtained as recommended[166]

The use of platelet antiaggregation is shown in Figure 14. While the benefit of single antiplatelet therapy (SAPT) for preventing stroke in asymptomatic patients with carotid artery stenosis >50% has not been demonstrated through a randomized clinical trial, lifelong low-dose aspirin should be part of BMT to reduce the risk of stroke and other cardiovascular events[167], as these patients are also at twice the risk of myocardial infarction[168]. In symptomatic extracranial carotid stenosis, antiplatelet monotherapy is recommended[169]Clopidogrel (75 mg/day) is an alternative in patients with aspirin intolerance[170]

Dual antiplatelet therapy (DAPT): The randomized Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management and Avoidance (CHARISMA) trial showed no benefit between DAPT and SAPT[171]. The Clopidogrel and Aspirin for the Reduction of Emboli in Symptomatic carotid Stenosis (CARESS) study, conducted in 108 patients, demonstrated that DAPT vs. aspirin reduced silent cerebral microemboli by 37% after 7 days[172]. No life-threatening intracranial or major bleeding was observed, but the sample size was small. For these reasons, DAPT may be considered within 24 h of a minor ischaemic stroke or transient ischaemic attack (TIA) and may be continued for 1 month in patients treated conservatively[173].

DAPT is recommended in patients undergoing carotid artery stenting (CAS). Two small RCTs comparing aspirin alone with DAPT for CAS were terminated prematurely due to high rates of stent thrombosis and neurological events in the aspirin-alone group[172,174]. These data were obtained at 30 days. Most events were procedure related. The optimal

duration of DAPT following CAS is unknown. Recent studies showing late brain lesions on diffusion-weighted MRI after CAS question whether DAPT beyond the first month may be needed. However, potential risks include haemorrhagic transformation in patients with recent stroke and intracranial bleeding in patients at risk of reperfusion injury following revascularization. DAPT may be prolonged beyond 1 month after CAS in the presence of recent (<12 months) MI and low bleeding risk[175].

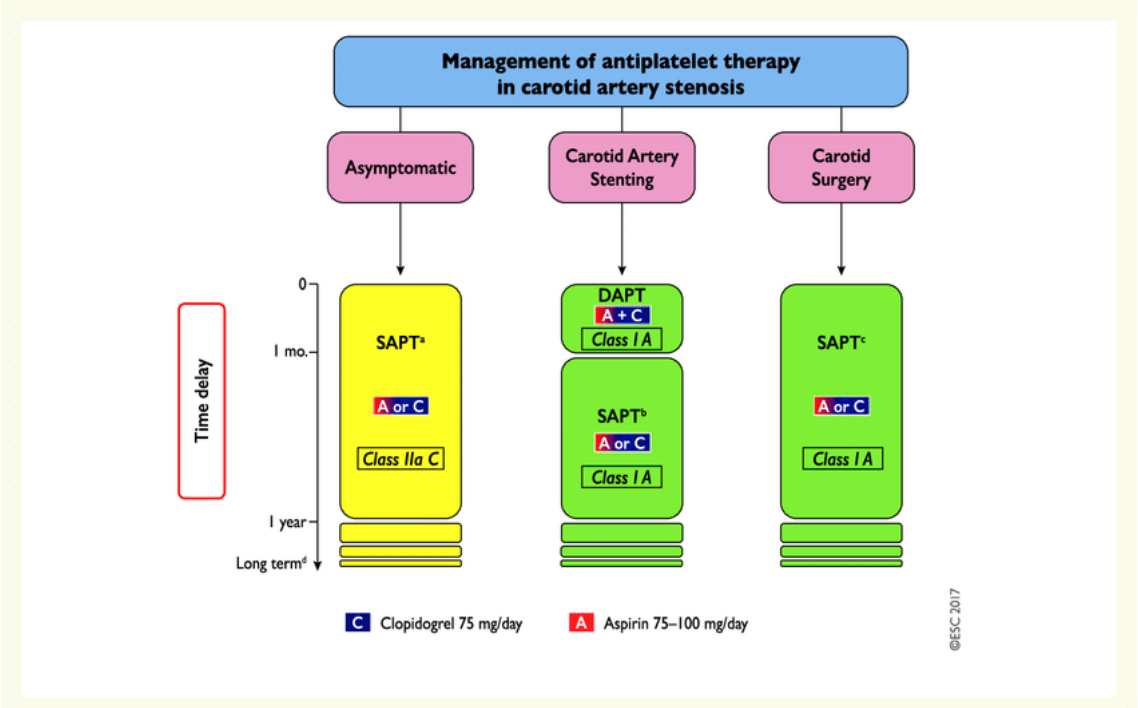

Figure 14. Management of antithrombotic treatment in patients with carotid artery stenosis. DAPT = dual antiplatelet therapy, a daily combination of aspirin (75–100 mg) and clopidogrel (75 mg); CAS = carotid artery stenting; SAPT = single antiplatelet therapy; TIA = transient ischaemic attack. a- At the exception of patient at very high bleeding risk. b- DAPT may be used if another indication supersedes that of carotid artery stenting such as acute coronary syndrome or percutaneous coronary intervention of less than 1 year. c- In case of recent minor stroke or TIA. A loading dose of aspirin (300 mg) and/or clopidogrel (300/600 mg) is recommended at the acute phase of stroke/TIA or during CAS. d- Stands for as long as it is well tolerated[175]

The use of antilipemic drugs: Lipid-lowering drugs (resins, statins, ezetimibe, and subtilisin/kexin 9 protein convertase inhibitors (PCSK9i)) in patients with established cardiovascular disease reduce serious cardiovascular events and mortality[176]. The indication for lipid-lowering treatment is based on the concentration of LDL-C and

global cardiovascular risk[177]. Lipid-lowering treatment aims to obtain at least the cLDL objectives[178,179]. High-intensity hypocholesterolaemic therapies that include statins are recommended. The efficacy of fibrates in lowering cholesterol is generally moderate.

All treatment for dyslipidaemia should be personalized to achieve the objectives and calculated after individual assessment of CVR and LDL levels. In patients with atherogenic dyslipidaemia, the main objective is to achieve LDL-C or non-HDL cholesterol in therapeutic objectives according to the level of risk, using treatment with statins or high-intensity therapeutic combinations[178](Figure 15).

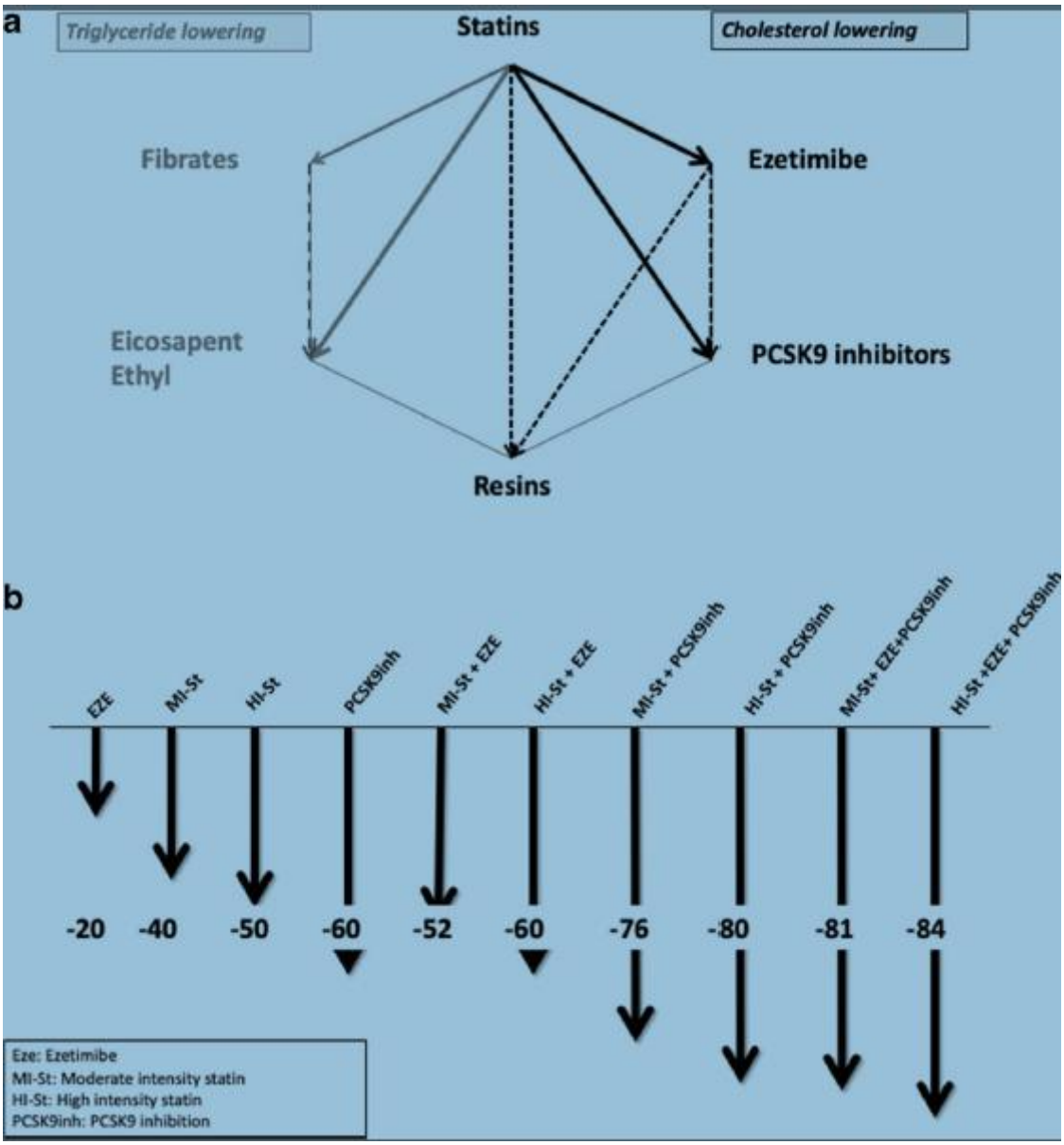

Figure 15. Recommended lipid lowering therapy combinations and its efficacy. a Appropriated lipid-lowering combination therapies according scientific evidence. Thicker continuous lines indicate that at least one RCT supports the association. Thinner continuous lines indicate that

combination is supported by subgroup analyses. Discontinuous lines indicate that combination potentiates lipid lowering therapy. Triglyceride lowering square indicates that drugs below could be combined with statins in patients with hypertriglyceridemia. Cholesterol-lowering square indicates that drugs below could be combined with statins to reduce LDL-cholesterol. B Theoretical percentage reduction on LDL cholesterol concentrations [178]

1.10. Searching for Biomarkers of Carotid Atheroma Plaque

Despite important advances in our understanding of atherosclerotic disease and its derived complications, atherosclerosis remains the leading cause of death in developed countries. With the increasing incidence of atherothrombosis and the risk of carotid atheroma plaque rupture, the search for novel therapeutic approaches and early biomarkers is a priority. Moreover, new biomarkers should have the potential to improve risk stratification, diagnosis or treatment.

Circulating biomarkers have the great advantage of allowing quantification through noninvasive procedures, but studies performed with plasma are complicated due to the protein complexity of the sample, its high dynamic range of concentrations, and the difficulty of detecting low-abundance proteins[180].

There are a low number of atherosclerotic plaque studies because of the difficulty in obtaining samples. The small calibre of the artery makes sample collection laborious, especially because of the quantity of material suitable for this type of analysis. The carotid artery is the artery most frequently used in studies on atherosclerosis. The reasons are the significant accessibility and great quantity of material obtained from endarterectomy.

Studies of the secretome represent an intermediate methodology. The study sample has less complexity than plasma and allows the direct identification of potential proteins, probably biomarkers of the disease released into the blood[181].

The term secretome was first introduced in 2000 in a genomic study that described the proteins secreted by Bacillus subtilis[182]. Secretome is the set of proteins released by a cell or tissue under certain conditions and in a concrete time interval. One of its main advantages is that it can be considered a plasma subproteome. Secretome allows direct

analysis of the proteins involved in atheroma plaque pathology. Most secretome studies have been performed in tumour investigations[183].

In the secretome analysis strategy, we must be aware of two types of contaminants that may appear: a) intracellular proteins released by cell damage and b) serum (from blood in tissue or foetal bovine serum in cultures) (Figure 16).

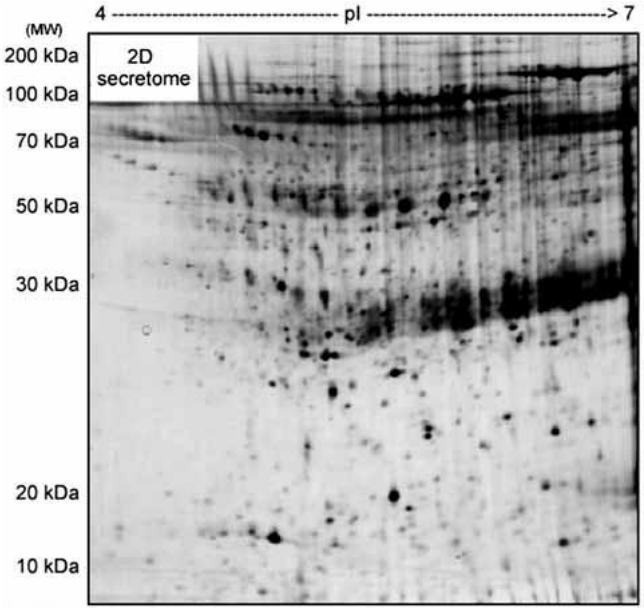

Figure 16. 2D-analysis of the secretome from a complicated atherosclerotic plaque. Carotid artery biopsies from atherosclerotic patients are incubated in culture media. Next, media with secreted proteins (secretome) can be analyzed by 2D. Proteins are separated by their isoelectric point (pI) and molecular weight ranges[184]

Furthermore, studies on tissue secretomes more closely resemble the *in vivo* situation than cell culture workflows. To date, only a few human arterial secretomes (pathological and nondiseased tissue) have been subject to analysis. In this sense, in the present work, we propose, on the one hand, the study of proteomics in the secretome of patients with arteriosclerosis and of certain inflammatory adipocytokines. On the other hand, we propose the use of proteomics to identify early biomarkers of carotid atherosclerosis.

1.11. Proteomics

The molecular mechanisms of atherosclerosis are not completely understood. In this regard, "omics"-based approaches (such as genomics, transcriptomics, proteomics, and metabolomics) have enabled the overall characterization, at the molecular level, of complex global biological systems and their changes in pathological processes. The study of cardiovascular diseases has been frequently approached through proteomic analysis. In this context, proteomics has emerged as a useful tool for analysing the proteins involved in the pathogenesis of diseases such as atherosclerosis[185,186].

Due to easier access to the carotid artery, proteomic studies have been performed not only in the circulation but also within plaques[187] as well as in the secretome of surgically removed plaques[188].

Proteomics is the science that dynamically studies all body proteins in concrete time and environmental conditions, and it encompasses the set of techniques that enable proteome analyses[189]. Proteins are the final effectors of the processes that occur in cells, so studying proteomics is the most appropriate way to analyse biological processes that occur in an organism as well as to understand the molecular mechanisms underlying these processes.

An analysis of an organism's proteome has traditionally been based on separation of proteins by two-dimensional electrophoresis (2-DE) and their identification by mass spectrometry (MS). This type of study is now known as classical proteomics. The appearance of the electrospray ionization (ESI) 14 method, which allows the identification of MS proteins immediately following liquid chromatography separation (LC–MS), has recently opened a new field in proteomics known as second-generation proteomics or "shotgun proteomics".

Carotid atheroma plaques have been studied using 2-DE[190–192], antibody microarrays[193] and Western arrays[194]. A 2-DE proteomics study of atherosclerotic plaque that compared plaque composition with the composition of healthy regions of the same artery observed a presence of a high number of varied proteins[195].

In the search for potential biomarkers, several proteomics study designs have sought to identify new plasma biomarkers for atherosclerosis and its clinical manifestations[196,197].

Ideally, putative biomarkers must satisfy several criteria[198], and the most important among them are the following: diagnostic specificity and sensitivity, ability to differentiate between stroke subtypes, correlation between biomarker concentrations and a certain outcome, prospective validation, incremental value to that of the existing markers, clinical usefulness, and cost-effectiveness.

1.12. Proteomic Analysis Procedure

Sample Preparation

Sample preparation is a critical step for a valid proteomic analysis. Samples that can be studied are cells, tissues or fluids. If we want to obtain a valid proteome, it is necessary to break down the tissue and/or break down the cells. An exception is fluids.

Cell rupture can be performed by physical methods: osmotic shock, mechanical breakage, freezing/thawing, sounding, and chemicals, which are based on the use of detergents. One of the most commonly used is sodium dodecyl sulphate (SDS). Figure 17 displays the process of sample preparation.

Proteins are extracted from biological samples and then digested and ionized prior to introduction to the mass spectrometer. Each MS scan results in a mass spectrum, measuring m/z values and peak intensities. Based on observed spectral information, database searching is typically employed to identify the peptides most likely responsible for high-abundance peaks. Finally, peptide information is rolled up to the protein level, and protein abundance is quantified using either peak intensities or spectral counts (Figure 18)[199]

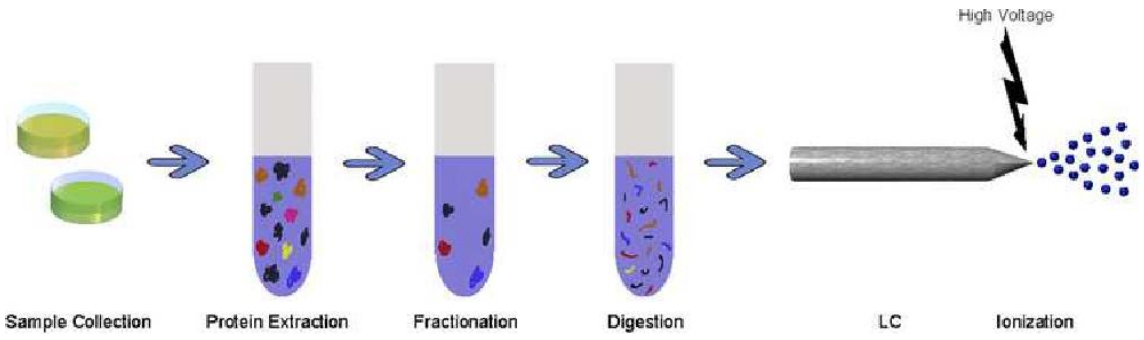

Figure 17. Sample preparation. Complex biological samples are first processed to extract proteins. Proteins are typically fractionated to eliminate high-abundance proteins or other proteins that are not of interest. The remaining proteins are then digested into peptides, which are commonly introduced to a liquid chromatography (LC) column for separation. Upon eluting fromthe LC column, peptides are ionized[199]

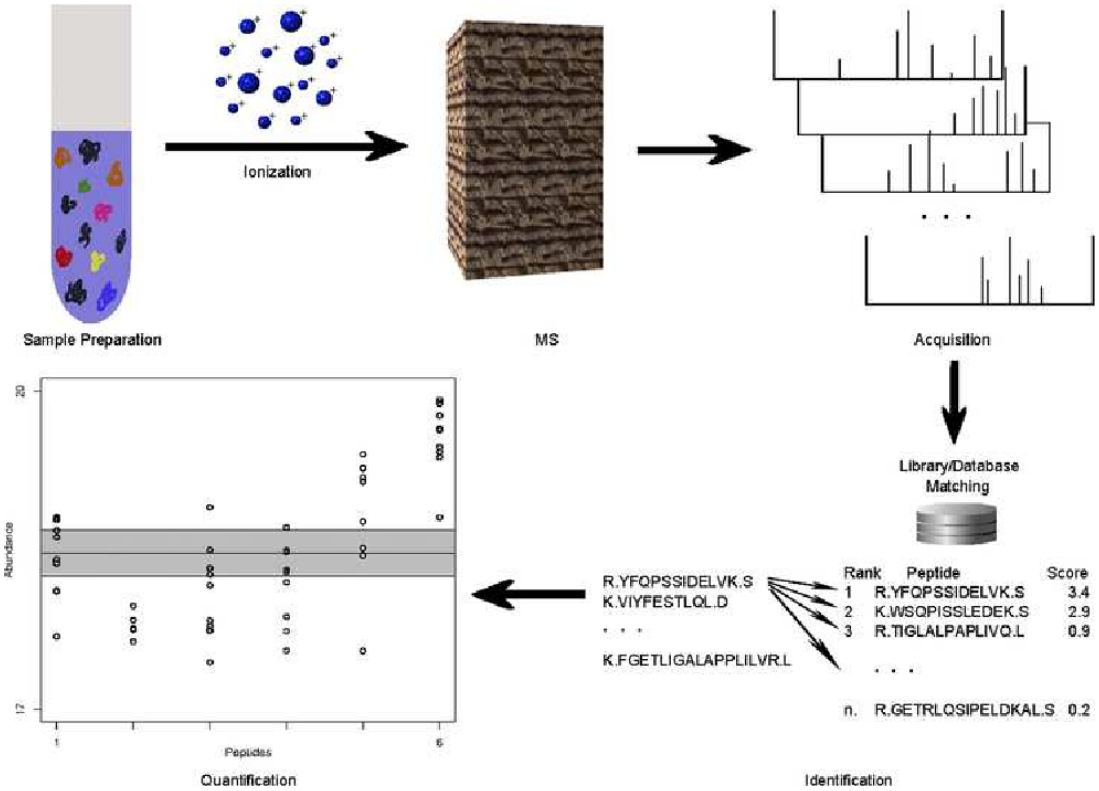

Figure 18. Overview of LC-MS-based proteomics[199]

Protein Separation Techniques

The two most frequently used techniques to perform the separation of proteins are two-dimensional electrophoresis (2-DE) and liquid two-dimensional chromatography (LC).

- 2-DE was the first technique used to separate protein complexes, which were subsequently identified in MALDI-TOF-type mass spectrometers. It is based on conventional gel electrophoresis polyacrylamide or SDS–PAGE, in which proteins are separated by their molecular mass in the presence of the SDS

detergent. To this type of electrophoresis that constitutes the second dimension is added a previous additional electrophoresis, the first-dimension electrophoresis, based on the isoelectric point called isoelectric focusing (IEF). The result obtained is a map of protein spots in two dimensions: isoelectric point and molecular mass.

- Chromatography is a separation technique that distributes the components of a mixture in two phases: one stationary and the other mobile. The most commonly used chromatographic technique in proteomics is liquid chromatography (LC). In chromatographic methods that are used in proteomics, a porous matrix is used as the stationary phase. There are different types of LC depending on the physical and chemical properties of the stationary phase.

Mass spectrometry is an analytical technique used to determine the molecular mass of a compound using instruments that can measure the mass/charge (m/z) ratio of ions under vacuum conditions. These instruments are called mass spectrometers. A mass spectrometer consists of an ion source responsible for ionizing peptides, the mass analyser and the detector responsible for recording *m/z* values and intensities, respectively, for each ion species. Each MS scan results in a mass spectrum, and a single sample may be subjected to thousands of scans (Figure 19).

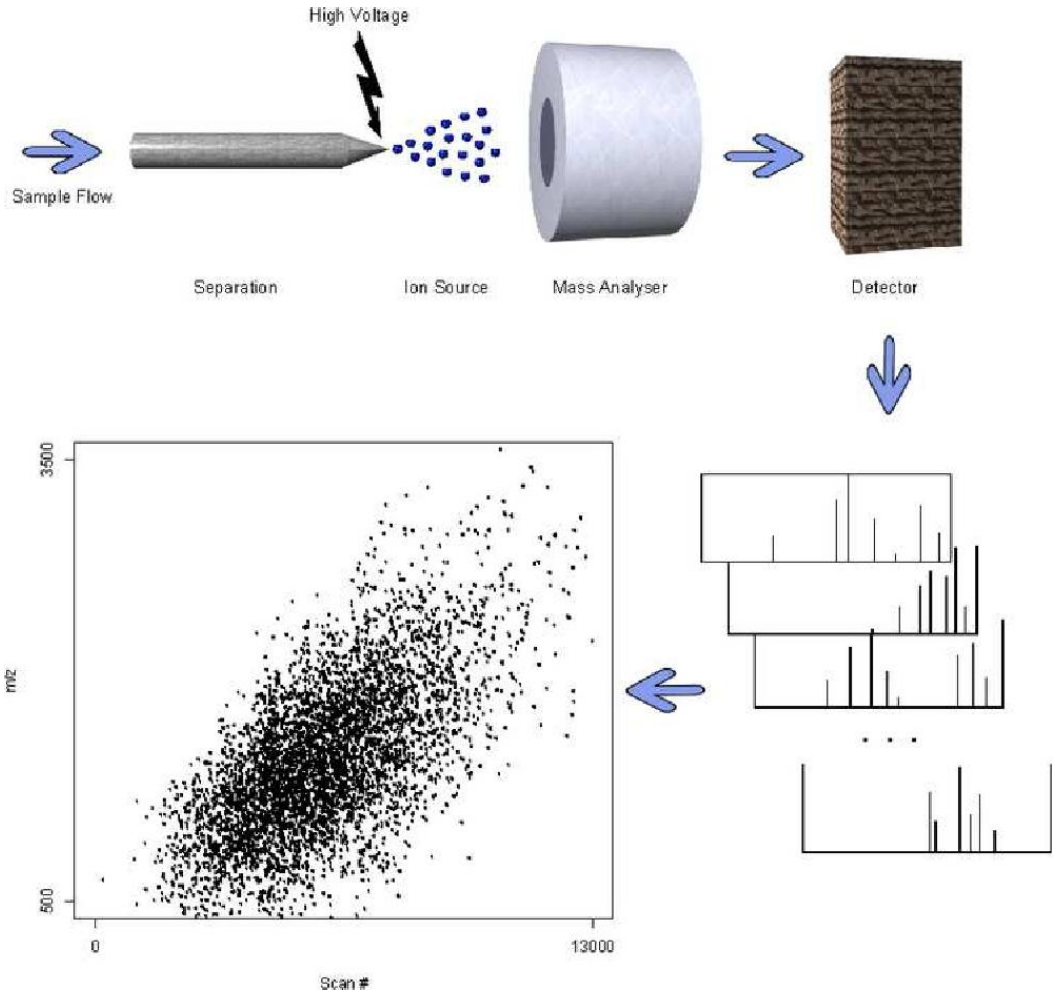

Figure 19. Mass spectrometer consists in Ion source, the mass analyzer and the detector presenting the basic process of mass spectrometry.

Process of mass spectrometry is composed of data acquisition, protein identification and protein quantization[199]

1.13. Biomarkers in Carotid Atherosclerosis

One of the latest reviews of serum biomarkers in carotid atherosclerosis tried to define available serum biomarkers (Table 3) with clinical implications in asymptomatic carotid atherosclerotic stenosis patients, focusing on those that may predict carotid plaque presence, instability, and symptom development[200].

Classification	Biomarkers
Inflammatory	Hs-CRP, PTX-3, SAA, IL-6, IL1beta, TNF – alfa, MCP-1, suPAR
Endothelial and cell adhesion	VCAM-1, ICAM-1, L-selectin, E-selectin, endothelial MP
Matrix degrading and proteolysis	MMP-1, MMP-2, MMP-7, MMP-9, TIMP-1
Lipid	LDL, sdLDL, ox-LDL, HDL, TRL, Lp-PLA2, Lp(a), ApoA-I, ApoB, ApoE
Metabolic	Adipokines (resistin, adiponectin, FABP4), homocysteine, OPG
Hematologic	RDW, WBC count, neutrophil count. T lymphocytes, monocytes
Angiogenic and neovascularization	VEGF
Thrombosis-related	PAI-1
Other	miRNA

Table 3. Classification of biomarkers. *Apo*, Apolipoprotein; *HDL*, high-density lipoprotein; *hs-CRP*, high-sensitivity C-reactive protein; *ICAM*, intercellular adhesion molecule; *IL*, interleukin; *LDL*, low-density lipoprotein; *LP(a)*, lipoprotein (a); *Lp-PLA2*, lipoprotein phospholipase A2; *MCP*, monocyte chemotactic protein; *miRNA*, micro-RNA; *MMP*, matrix metalloproteinase; *MP*, microparticles; *OPG*, osteoprotegerin; *ox-LDL*, oxidized low-density lipoprotein; *PTX*, pentraxin; *RDW*, red blood cell distribution width; *SAA*, serum amyloid-A protein; *sdLDL*, small and dense LDL cholesterol particles; *suPAR*, plasma-soluble urokinase plasminogen activator receptor; *TIMP*, tissue inhibitors of metalloproteinase; *TNF*, tumor necrosis factor; *TRL*, triglyceride-rich lipoprotein; *VCAM*, vascular cell adhesion molecule; *VEGF*, vascular endothelial growth factor; *WBC*, white blood cell[200]

Lipid-Related and Lipoproteins Biomarkers

Atherosclerotic plaques are lipid-enriched, and their lipid content strongly determines plaque vulnerability and the rate of stenosis progression. The lipids accumulated in the carotid lesion may be of intracellular origin or the consequence of extracellular accumulation of modified lipoproteins, mainly aggregated and fused LDL[201]. Extracellular cholesterol crystals accumulated in a carotid plaque promote inflammation and erosion of the fibrous cap, eventually leading to plaque vulnerability[202].

The size and biological properties of HDL and LDL are highly influenced by their lipid and apolipoprotein (apo) composition and their content in hydrolytic enzymes. The plasma levels of these molecules have been suggested as putative biomarkers of ischaemic stroke. Lipid-related biomarkers are suggested to be mainly involved in atherothrombotic stroke. Among apolipoproteins, the main candidates are the apoB/apoA-I ratio, apoE, and apoJ, also known as clusterin[203,204].

Some enzymes associated with LDL and HDL have been proposed as putative ischaemic stroke biomarkers, mainly platelet-activating factor acetylhydrolases (PAF-AH), also known as lipoprotein-associated phospholipase A2, and paraoxonase-1 (PON1). High serum PAF-AH mass levels have been described specifically in atherothrombotic stroke[205], and its increased concentrations are correlated with plaque instability in both symptomatic and asymptomatic patients with carotid artery stenosis[206].

Low levels of PON1 activity increase ischaemic stroke risk[207], and they are positively correlated with HDLc [208] and have unfavourable stroke outcome [209].

Small dense low-density lipoprotein cholesterol (SdLDL-C) levels are associated with ischaemic stroke[210].

The inflammatory properties of modified LDLs are a main topic in atherosclerosis research. The plasma levels of ox-LDL are particularly increased in large artery atherosclerosis and are related to poor functional outcome within 1 year after stroke onset (Ishigaki et al., 2009). High levels of ox-LDL in carotid plaque are correlated with unstable plaque[211,212] and are particularly increased in plaques from symptomatic patients (Sigala et al., 2010). Apart from ox-LDL, the presence of a circulating form of modified LDL with inflammatory properties, called electronegative LDL (LDL(-)), has been described. LDL (-) is an LDL subfraction with a high negative charge, and it constitutes approximately 3-5% of the total LDL in healthy subjects. An increased proportion of LDL(-) was found in pathologies associated with cardiovascular risk, in patients with acute myocardial infarction[214], and even in the presence of subclinical atherosclerosis, wherein a correlation was found with the extent of carotid stenosis[215]. In

arterial wall cells, LDL(-) induces the release of several inflammatory mediators[216,217] that are putative biomarkers for ischaemic stroke.

Inflammatory Biomarkers

Inflammatory biomarkers have been studied extensively within the context of stroke (Table 3). Cytokines, metalloproteinases, adhesion molecules, and cell receptors, among other factors, may be increased in the circulation and may be targeted for the diagnosis and assessment of the prognosis of atherothrombotic stroke[200].

C-reactive protein (CRP) is considered one of the most significant biomarkers of inflammation, and the measurement of both CRP and high-sensitivity CRP (hs-CRP) is widely used in clinical practice for vascular disease stratification[218]. Hs-CRP is associated with the presence of unstable carotid artery stenosis[219].

Pentraxin-3 (PTX3) is another acute phase protein associated with the presence of atherosclerotic plaque[220]. Serum amyloid-A protein (SAA) is an acute phase apolipoprotein (Apo) related to high-density lipoprotein (HDL). SAA is significantly associated with progressive atherosclerosis measured by ultrasound examination[221]. Interleukin 6 (IL-6) is a master proinflammatory and procoagulant cytokine. IL-6 may reflect local inflammatory activity because it is upregulated in patients with plaque instability features on MR[222].

Proinflammatory chemerin, leptin, and resistin are considered adipokines that influence vascular wall function. The association of circulating adipokines with cerebrovascular symptomatology and carotid plaque vulnerability was investigated. The results showed that low levels of chemerin and elevated levels of restinin were related to plaque instability, the risk of stroke, and the severity of carotid artery disease[223]. Adiponectin, an anti-inflammatory and vasculoprotective adipokine, may act as a novel prognostic biomarker for atherosclerosis in stroke[224,225]. Visfatin has both a pro- and antiatherogenic effect, and at high concentrations it leads to plaque destabilization[226]. Osteoprotegerin (OPG) is a secretory glycoprotein that belongs to the tumour necrosis factor receptor family. At high concentrations, it has been correlated with cardiovascular and vascular disease, and it contributes to atherosclerotic plaque stability[218] Hs-CRP is associated with the presence of unstable carotid artery stenosis[219].

However, to date, no specific inflammatory biomarker or algorithmic combination biomarkers for carotid arteriosclerosis have been accepted in clinical practice.

MicroRNAs

Other biomarkers that are gaining interest in their potential role in the diagnosis and monitoring of several diseases are microRNAs (miRNAs). miRNAs are short, single-stranded, nonprotein-coding RNA sequences that function as key posttranscriptional regulators. miRNAs are stable and detectable in peripheral blood. They appear to play a role in atherosclerotic plaque formation and stability[227] and in stroke[228]. MiR-21 is one of the most validated miRNAs in carotid atherosclerosis. It is upregulated in the serum from patients with ischaemic stroke and asymptomatic atherosclerosis[229] and in human atherosclerotic plaques[230]

HYPOTHESES AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE
Ajla Alibalic

2.1. HYPOTHESES:

2.1.1. Hypothesis Study 1.

The protein secretion profile of carotid atherosclerotic plaque is different than that of nonatherosclerotic mammary secretomes. The differential protein levels could be used as biomarkers of carotid atherosclerotic plaque and would improve the knowledge of the atherosclerotic process.

2.1.2. Hypothesis Study 2.

The adipo/cytokine levels in the secretomes of unstable carotid atherosclerotic plaques differ from those in nonatherosclerotic mammary secretomes, with a possible role in the stability of atherosclerotic plaques.

2.1.3. Hypothesis Sub-Study 3.

The levels of secretome and circulating adipo/cytokines are related to cardiovascular risk factors.

2.2. OBJECTIVES:

2.2.1. Objectives Study 1.

Main objective:

To identify potential candidate biomarkers for carotid atherosclerosis in secretome samples of patients with unstable carotid plaque.

Secondary objectives:

To analyse the protein secretion profile of carotid atherosclerotic plaque compared with nonatherosclerotic mammary secretomes.

To evaluate their potential use as atherosclerotic biomarkers, we studied the functional pathways in which these secreted proteins are involved.

2.2.2. Objectives Study 2.

Main objective:

To study the possible role of some adipo/cytokines in the stability of atherosclerotic plaques.

Secondary objectives:

To analyse the adipo/cytokine levels in the secretomes of unstable carotid atherosclerotic plaques compared with nonatherosclerotic mammary secretomes.

To compare circulating levels of adipo/cytokines between patients suffering from atherosclerosis and a group of healthy subjects.

2.2.3. Objectives Sub-Study 3.

Main objective:

To analyse the levels of several pro- and anti-inflammatory adipo/cytokines in serum and unstable plaque secretome according to the presence of obesity, arterial hypertension, diabetes mellitus, dyslipidaemia and smoker status.

Secondary objectives:

To analyse the relationship between levels of adipo/cytokines and cholesterol LDL levels.

MATERIAL AND METHODS

UNIVERSITAT ROVIRA I VIRGILI
PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE
Ajla Alibalic

3.1. Study 1

Title: Proteomic Profile of Unstable Atheroma Plaque: Increased Neutrophil Defensin 1, Clusterin, and Apolipoprotein E Levels in Carotid Secretome

We analyzed the protein secretion profile of carotid atherosclerotic plaque and non-atherosclerotic mammary secretomes. To evaluate their potential use as atherosclerotic biomarkers, we also studied the functional pathways in which these secreted proteins are involved.

Subjects/Samples

Human carotid atherosclerotic plaques (CAPs) were obtained from patients (men, n = 12) who underwent carotid endarterectomy at the Angiology and Vascular Surgery Unit of the Hospital Universitari Joan XXIII (Tarragona, Spain). Patients with cerebrovascular ischemia and internal carotid artery stenosis >75% were included, diagnosed by color Doppler assisted duplex imaging and arteriography. The CAP diagnosis was made by an experienced pathologist following the American Heart Association (AHA) guidelines [231]. Mammary arteries were used as non-atherosclerotic control arteries (MA). Segments of mammary arteries (men, n = 10) were obtained during coronary revascularization surgery at the Cardiovascular Surgery Department of the Germans Trias i Pujol Hospital (Badalona, Spain). Patients who had an acute illness, acute or chronic inflammatory or infective diseases, or malignant neoplastic disease were excluded.

Blood samples were obtained from each individual immediately before surgery and after overnight fasting. Serum was obtained by standard protocols and preserved at −80 °C until use.

Clinical and Biochemical Assessments

A complete anthropometric, physical examination and biochemical analysis was carried out on each patient. Body height and weight were measured with the patient standing in light clothes and shoeless. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m2). Laboratory studies included glucose, insulin, glycated hemoglobin (HbA1c), total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides, all of which were analyzed using a conventional automated analyzer. Insulin resistance (IR) was estimated using the homeostasis model assessment of IR (HOMA2-IR) [232].

Tissue processing and secretome preparation

Tissue samples were transported from the surgical room to the laboratory in phosphate-buffered saline (PBS) at room temperature. Immediately upon arrival, the tissue was transferred to a Petri dish and washed with phosphate-buffered saline (PBS). Samples were then cut into similar-sized pieces about 3-5 mm in length and transferred to a 12-well tissue culture plate containing 2 mL/well of protein-free Roswell Park Memorial Institute medium (RPMI) (RPMI-1640, Gibco, Invitrogen, NY) supplemented with penicillin (100 U/ mL), streptomycin (100 μg/mL), and 50 mM hydroxyethyl piperazineethanesulfonic acid (HEPES). These procedures were all carried out under a laminar flow hood using sterile equipment. After 24 h of incubation at 37 °C and 5% of CO2, the media containing the secreted proteins, the so-called secretome, were collected, aliquoted, and stored at −80 °C until analysis. Additionally, a section of each atherosclerotic plaque was placed in formol 10% and further studied by an experienced pathologist from the Hospital Universitari Joan XXIII (Tarragona) following the AHA guidelines[231]

Protein Preparation

Secretome samples were concentrated by ultrafiltration using 3 kDa Amicon Ultra 0.5 mL filters from Millipore in accordance with the manufacturer's instructions. The protein concentration was determined by Bradford's method. A total of 200 µg total protein per sample was run on a self-poured stacking SDS-PAGE gel (12% resolving gel and 4% stacking gel) at 20 mA/gel. The electrophoresis was stopped when the front dye had barelypassed from the stacking gel (4% acrylamide) into the resolving gel (12% acrylamide) and before the protein mixture had separated into discrete bands in the gel. In this way, all proteins were concentrated in a single band, which removed sample contaminants and made reproducibility for comparison easier. This single concentrated band obtained for every sample was stained using Coomassie Brilliant Blue G-250, excised, cut into small pieces, and stored at 4 °C in ultrapure water.

Protein Digestion

Protein digestion was performed according to Shevchenko et al. with minor variations[233]. Gel pieces were destained using 75% acetonitrile, 25% 0.5 M triethylammonium bicarbonate pH 7.9 solutions. Then, they were dehydrated by successive washes of 50 mM triethylammonium bicarbonate pH 7.9 solution and acetonitrile and vacuum-dried. Subsequently, proteins were reduced using 5 mM tris(2-carboxyethyl)phosphine (TCEP) in 50 mM triethylammonium bicarbonate pH 7.9 for 1h at 60 °C and alkylated with 3.81 mM Iodoacetamide (IAA) in the same buffer for 30 min at room temperature in the dark. For digestion, samples were incubated with 15.4 ng/µL sequencing-grade trypsin in 50 mM triethylammonium bicarbonate at pH 7.9 overnight at 37 °C. After digestion, the peptides were extracted from the gel by elution in a mixture of 50% acetonitrile, 5% formic acid. Tryptic peptides were dried by SpeedVac and resuspended in 30 µL TEAB 0.5M, pH 7.9. iTRAQ 8-plex Labeling and Purification Digested samples were labeled using iTRAQ 8-plex reagents (ABSCIEX). To accommodate all of the samples of the study, three iTRAQ 8-plex reagent kits are necessary, and for this reason samples were equally distributed in three subgroups (A,B and C) containing both CAP and MA samples to minimize technical variability (Table 4).

iTRAQ groups	labeling	Sample type
A	113	CAPb
	114	MA
	115	CAP
	116	MA
	117	CAP
	118	cAP
	119	MA
	121	CAP
B	113	CAPb
	114	MA
	115	MA
	116	CAP
	117	MA
	118	MA
	119	CAP
	121	MA
C	113	CAPb
	114	CAP
	115	CAP
	116	CAP
	117	CAP
	118	MA
	119	MA
	121	Ma

Table 4. Sample Labeling and Distribution in Three iTRAQ Groups (A,B and C).
CAP, carotid atherosclerotic plaque; MA, control mammary artery. B, this sample was added in all groups to normalize the quatification results that are expressed as ratios of the different labeling tags versus tag 113.

Hereby, one CAP sample (labeled with 113- tag) was used as a normalizer sample for the three iTRAQ groups and to cover all of the proteins present in CAP arteries. Quantification results are expressed as ratios of the different labeling tags versus tag 113, and these ratios were used for statistical purposes.

The iTRAQ labeling reaction was performed according to manufacturer's instructions, incubated at room temperature for 120 min, and stopped by adding water. After peptides were labeled, they were purified by using a SCX column (Strata SCX 55 μm, 70 Å, Phenomenex). Then, samples were desalted and concentrated using a C18 Sep-Pak column (Waters, Bedford, MA) previous to nanoLC−MS/MS analysis.

Nano LC Chromatography and Mass Spectrometry

Peptides were separated onto a C-18 reversed-phase (RP) nanocolumn (75 μm I.D.; 15 cm long; 3 μm particle diameter, Nikkyo Technos, Japan) coupled to a trap nanocolumn (100 μm I.D.; 2 cm long; 5 μm particle diameter, Thermo Fisher Scientific, San Jose, CA).

The three iTRAQ groups (A, B and C) were analyzed by triplicate so that a higher number of covered proteins and single peptides could be quantified. 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 analyses a flow rate of 300 nL/min was used to elute peptides for real time ionization and peptide fragmentation on an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution = 30 000 FHMW) followed by MS/MS scan (R = 7500 FHMW) from the ten 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 spectra were analyzed using Mascot search engine node (Matrix Science; version 2.4.1.0). Mascot was set up to search Swissprot 2012_03.fastadatabase (v 2.4, 535 248 entries), restricting for human taxonomy (20 255 sequences) and assuming trypsin digestion. Two missed cleavages were allowed and an error of 0.80 Da for fragment ion mass and 10.0 ppm for a parent ion. Oxidation of methionine and acetylation of N-termini was specified as variable modifications, whereas iTRAQ 8-plex and carbamidomethylation of cysteine were set as static modifications. 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 [234] For proteins identified with only one peptide, the fragmentation spectra were visually verified. The analysis carried out by triplicate was considered as replicates on Proteome Discover software and finally a PD Excel report was generated for each iTRAQ group (see Supporting Information of first study original publication).

Quantitative Proteome Analysis

Protein quantification was done by calculating the ratios obtained between each iTRAQ mass tag of each unique peptide from a given protein against tag 113. The quantification is the average value of the ratios obtained for the unique peptides for each protein and was normalized based on protein median.

These ratios were exported to an Excel spreadsheet for statistical analysis (Supporting Information of first study original publication).

ELISA Assays Defrosted secretome samples were centrifuged at 3000 rpm and 4 °C for 15 min. Then, they were analyzed by enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions. Neutrophil defensin 1 (EIAab, Wuhan, China), apopoliprotein E (AssayPro, St. Charles, MO), clusterin (RayBiotech, Norcross, GA), and zinc-alpha-2-glycoprotein (BlueGene Biotech., Shangai, China) were determined in secretome samples.

Statistical Analysis

The statistical analysis was performed on Mass Profiler Professional software v.12.1 (Agilent Technologies). For statistical calculations, only these proteins that were quantified in >70% samples under almost one condition (CAP and MA) were considered. Differences between groups were calculated using a Student's t test and to avoid false positives, a multiple testing correction using a Benjamini−Hochberg method was used. p values <0.05 and fold change >1.5 were selected as cutoff values. Principal component analysis (PCA) and hierarchical clustering analysis were performed using Mass Profile Professional software v.12.1 (Agilent Technologies). Pathway analysis was performed using The ConsensusPathDB-human platform. Other statistical calculations were performed using the SPSS software (version 20.0; SPSS, Chicago, IL).

3.2. Study 2

Title: Adipo/cytokines in atherosclerotic secretomes: increased visfatin levels in unstable carotid plaque

Subjects/Samples

The study was approved by the institutional review board "Comitè d'Ètica d'Investigació Clínica, Hospital Universitari de Sant Joan de Reus" (10-04-29/4proj3). All participants gave written informed consent for participation in medical research.

Human unstable carotid atherosclerotic plaques were obtained from patients (men, n = 18) who underwent carotid endarterectomy at the Angiology and Vascular Surgery Unit of the Hospital Universitari Joan XXIII (Tarragona, Spain). Patients with cerebrovascular ischemia and internal carotid artery stenosis >75 % were included, diagnosed by colour Doppler assisted duplex investigation and arteriography. The diagnosis of unstable carotid atherosclerotic plaques was made by an experienced pathologist following the American Heart Association (AHA) guidelines [231].

Mammary arteries were used as non-atherosclerotic control arteries. Segments of mammary arteries (men, n = 13) were obtained during coronary revascularisation surgery at the Cardiovascular Surgery Department of the Hospital Germans Trias i Pujol (Badalona, Spain). Patients who had an acute illness, acute or chronic inflammatory or infective diseases, or malignant neoplastic disease were excluded.

We also recruited serum of healthy men cohort (n = 16), whose medical history included no cardiovascular event. Subjects who had an acute illness, acute or chronic inflammatory or infective diseases, or malignant neoplastic disease were excluded.

All subjects recruited were male. Blood samples were obtained from each individual immediately before surgery and after overnight fasting. Serum was obtained by standard protocols and preserved at −80 °C until use.

Clinical and biochemical assessments

A complete anthropometric, biochemical, and physical examination was carried out on each patient. Body height and weight were measured with the patient standing in light clothes and shoeless. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m2). Laboratory studies included glucose, insulin, glycated haemoglobin (HbA1c), total cholesterol, high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides, all of which were analysed using a conventional automated analyser. Insulin resistance (IR) was estimated using the homeostatic model assessment of IR (HOMA2-IR)[232]

Arterial tissue culture – obtaining the secretome

Tissue simples obtaining in this study is identical to study 1. Additionally, a section of each atherosclerotic plaque was placed in phormol 10 % and further studied by an experienced pathologist from the Hospital Universitari Joan XXIII (Tarragona) following the AHA guidelines[231]

Measurements of adipo/cytokines levels

Defrosted secretome samples were centrifuged at 1200 rpm and 4 °C for 15 min. Then, they were analysed by enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions. Adiponectin (EMD Millipore, St. Charles, MI, USA), visfatin (AdipoGen, San Diego, CA, USA), lipocalin-2 (R&D Systems Inc, Minneapolis, USA), resistin (Biovendor, Modrice, Czech Republic), interleukin (IL)-6 (R&D Systems Inc, Minneapolis, USA) and tumor necrosis factor receptor 2 (TNFR2) (BioSource Europe, Nivelles, Belgium) were determined in secretome samples. Only visfatin was determined in both secretome and serum samples. The adiponectin assay sensitivity was 0.2 ng/ml, and intraassay and inter-assay coefficients of variation (CV) were 3.4 and 5.7, respectively. The visfatin assay sensitivity was 30 pg/ml, and intra-assay and inter-assay CV were 5.63 and 5.92, respectively. The lipocalin-2 assay sensitivity was 0.012 ng/ml, and intra-assay and inter-assay CV were 3.7 and 6.5, respectively. The resistin assay sensitivity was 0.012 ng/ml, and intra-assay and inter-assay CV were 5.9 and 7.6, respectively. The IL-6 assay sensitivity was 0.039 pg/mL,

and intra-assay and inter-assay CV were 7.4 and 7.8, respectively. Finally, sTNF-RII assay sensitivity was 0.1 ng/ml, and intra-assay and inter-assay CV were 4.9 and 7.9, respectively. In order to normalize adipo/cytokine measurements, total protein concentration was assessed using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Statistical analysis

All the values reported are expressed as mean ± standard deviation (SD) and were analysed using the Windows SPSS/PC+ statistical package (version 22.0; SPSS, Chicago, IL, USA). Differences between groups were calculated using Student's t test or one-way ANOVA analysis. The strength of association between variables was calculated using Pearson's method for parametric variables and the Spearman Rho correlation test for non-parametric contrasts. P values <0.05 were considered to be statistically significant.

3.3. Sub-Study 3

Title: Circulating and secretory levels of adipo/cytokines in relation to cardiovascular risk factors

Material and methods

This is a substudy from study number 2. So, the patients and methods in this study are identical to study 2. A new complementary statistical study has been carried out.

Statistical study

The data were analyzed using the SPSS/PC+ for Windows statistical package (version 23.0; SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess the distribution of variables. All the values reported are expressed as mean ± standard deviation (SD). Differences between groups were calculated using Student's t test analysis and the strength of association between variables was calculated using Pearson's method because variables were parametric. P values <0.05 were considered to be statistically significant.

RESULTS

UNIVERSITAT ROVIRA I VIRGILI
PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE
Ajla Alibalic

4.1. Study 1

Title: Proteomic Profile of Unstable Atheroma Plaque: Increased Neutrophil Defensin 1, Clusterin, and Apolipoprotein E Levels in Carotid Secretome

Baseline characteristics of subjects

The clinical characteristics and biochemical measurements of the population studied are shown in Table 5. Patients were classified according to the samples obtained: carotid atherosclerotic plaques (CAP) samples from patients undergoing endarterectomy (n=12) and mammary artery (MA) samples from patients undergoing cardiac bypass (n=10). They were all men. The analyses indicated that patients from the CAP and MA groups were of similar ages. Biochemical parameters showed no significant differences between these groups. The 80% of MA patients and the 83% of CAP patients received lipid-lowering therapy. As expected, hypolipemiant treatment did not show significant differences in both groups (p=0.840).

	CAP group (n=12) Mean ± SD	MA group (n=10) Mean ± SD	p
Age (years)	68,83 ± 6,39	67,00 ± 9,99	0.456
BMI (kg/m^2)	28,06 ± 3,26	30,64 ± 1,27	0.065
Glucose (mg/dl)	129,17 ± 49,75	118,88 ± 45,64	0.678
HbA1c (%)	6,39 ± 1,07	7,03 ± 1,38	0.385
Insulin (mUI/L)	8,29 ± 4,95	10,47 ± 6,89	0.755
HOMA2-IR	1,01 ± 0,67	1,59 ± 0,97	0.950
Triglycerides (mg/dL)	112,00 ± 44,67	113,50 ± 27,97	0.571
Cholesterol (mg/dl)	122,10 ± 36,22	130,07 ± 24,96	0.427
HDL-C (mg/dL)	29,08 ± 7,20	24,33 ± 4,76	0.125
LDL-C (mg/dL)	70,73 ± 27,49	78,56 ± 19,48	0.437

Table 5. Clinical baseline characteristics of the cohort studied
CAP, carotid atherosclerotic plaque; MA, control mammary artery; BMI, body mass index; HbA1c, glycosylated haemoglobin; HOMA2-IR, homeostatic model assessment 2-insulin resistance; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein. Data are expressed as mean ± SD. $p<0.05$ are considered statistically significant. HOMA-2 is calculated using the HOMA Calculator version 2.2.2 (http://www.dtu.ox.ac.uk).

Protein identification and quantification

In order to assess potential biomarkers for atheromatous plaque progression, we focused on the carotid atherosclerotic secretome and compared it to changes in the mammary secretome. Using a nontargeted proteomic approach, we identified and quantified a total of 162 proteins in the human arterial secretome (Table 6).

RESULTS

Swiss-Prot ID	Protein Name	Coverage %	Unique Peptides*	Peptides	PSMs	MW [kDa]	calc. pI
P01834	Ig kappa chain C region	80.19	5	5	119	11.6	5.87
P68871	Hemoglobin subunit beta	65.99	5	8	440	16.0	7.28
P0CG05	Ig lambda-2 chain C regions	62.26	3	5	55	11.3	7.24
Q01995	Transgelin	55.72	11	11	64	22.6	8.84
P0CG04	Ig lambda-1 chain C regions	46.23	1	3	43	11.3	7.87
P02042	Hemoglobin subunit delta	44.22	3	6	222	16.0	8.05
P02768	Serum albumin	42.53	28	28	793	69.3	6.28
P08670	Vimentin	42.49	16	19	183	53.6	5.12
P69905	Hemoglobin subunit alpha	35.21	5	5	146	15.2	8.68
P02647	Apolipoprotein A-I	33.71	9	9	49	30.8	5.76
P0CG48	Polyubiquitin-C	32.85	2	2	8	77.0	7.66
P02675	Fibrinogen beta chain	31.98	12	12	175	55.9	8.27
P60709	Actin. cytoplasmic 1	28.80	1	9	103	41.7	5.48
P63261	Actin. cytoplasmic 2	28.80	1	9	101	41.8	5.48
P02649	Apolipoprotein E	28.39	8	8	30	36.1	5.73
P32119	Peroxiredoxin-2	27.78	4	5	27	21.9	5.97
P01860	Ig gamma-3 chain C region	27.59	2	8	162	41.3	7.9
Q96KK5	Histone H2A type 1-H	27.34	3	3	11	13.9	10.89
P01598	Ig kappa chain V-I region EU	26.85	2	2	6	11.8	8.44
P02679	Fibrinogen gamma chain	26.71	11	11	75	51.5	5.62
P00738	Haptoglobin	26.35	10	10	40	45.2	6.58
P01765	Ig heavy chain V-III region TIL	26.09	2	2	6	12.3	9.13
P02787	Serotransferrin	25.50	16	16	125	77.0	7.12
P01766	Ig heavy chain V-III region BRO	25.00	2	2	30	13.2	6.57
P01620	Ig kappa chain V-III region SIE	24.77	2	2	14	11.8	8.48
P01857	Ig gamma-1 chain C region	23.94	2	7	157	36.1	8.19
P02743	Serum amyloid P-component	23.77	5	5	18	25.4	6.54
P01625	Ig kappa chain V-IV region Len	21.93	2	2	2	12.6	7.93
P09493	Tropomyosin alpha-1 chain	21.48	3	8	25	32.7	4.74
P60174	Triosephosphate isomerase	21.33	5	5	9	30.8	5.92
P01871	Ig mu chain C region	19.91	8	8	37	49.3	6.77
P07951	Tropomyosin beta chain	19.72	2	7	27	32.8	4.7
P63267	Actin. gamma-enteric smooth muscle	19.41	1	7	78	41.8	5.48
P00338	L-lactate dehydrogenase A chain	19.28	4	6	28	36.7	8.27
P59665	Neutrophil defensin 1	19.15	2	2	10	10.2	6.99
P18206	Vinculin	19.14	18	18	51	123.7	5.66
P01859	Ig gamma-2 chain C region	19.02	2	6	142	35.9	7.59
P01700	Ig lambda chain V-I region HA	18.75	2	2	11	11.9	8.91

P00915	Carbonic anhydrase 1	18.01	5	5	54	28.9	7.12
P01876	Ig alpha-1 chain C region	17.85	2	5	61	37.6	6.51
P01717	Ig lambda chain V-IV region Hil	17.76	1	1	1	11.5	6.51
P01617	Ig kappa chain V-II region TEW	17.70	1	2	6	12.3	6
P62805	Histone H4	17.48	2	2	2	11.4	11.36
P05452	Tetranectin	17.33	3	3	3	22.5	5.67
P01593	Ig kappa chain V-I region AG	16.67	1	1	6	12.0	5.99
P02792	Ferritin light chain	16.57	3	3	13	20.0	5.78
P21333	Filamin-A	15.72	30	30	139	280.6	6.06
P06310	Ig kappa chain V-II region	15.04	1	2	6	14.7	9.25
P01877	Ig alpha-2 chain C region	15.00	1	4	50	36.5	6.1
P06733	Alpha-enolase	14.75	6	6	33	47.1	7.39
P02671	Fibrinogen alpha chain	14.67	12	12	119	94.9	6.01
Q06830	Peroxiredoxin-1	14.57	2	3	14	22.1	8.13
P01023	Alpha-2-macroglobulin	14.18	18	18	101	163.2	6.46
O43707	Alpha-actinin-4	13.83	5	10	33	104.8	5.44
P01009	Alpha-1-antitrypsin	13.64	6	6	66	46.7	5.59
P01024	Complement C3	13.47	19	19	78	187.0	6.4
P02545	Prelamin-A/C	13.25	8	8	30	74.1	7.02
P01011	Alpha-1-antichymotrypsin	13.24	5	5	41	47.6	5.52
P06396	Gelsolin	12.15	9	9	44	85.6	6.28
P08294	Extracellular superoxide dismutase [Cu-Zn]	12.08	2	2	3	25.8	6.61
P62942	Peptidyl-prolyl cis-trans isomerase FKBP1A	12.04	1	1	1	11.9	8.16
P02760	Protein AMBP	11.93	3	3	10	39.0	6.25
P12814	Alpha-actinin-1	11.88	3	8	25	103	5.41
P51884	Lumican	11.83	4	4	24	38.4	6.61
P05109	Protein S100-A8	11.83	1	1	3	10.8	7.03
P37802	Transgelin-2	11.56	2	2	5	22.4	8.25
P07737	Profilin-1	11.43	1	1	4	15.0	8.27
P30086	Phosphatidylethanolamine-binding protein 1	10.70	1	1	3	21.0	7.53
P07195	L-lactate dehydrogenase B chain	10.48	1	3	5	36.6	6.05
P04075	Fructose-bisphosphate aldolase A	10.44	4	4	19	39.4	8.09
P10909	Clusterin	9.80	3	3	16	52.5	6.27
P02144	Myoglobin	9.74	1	1	6	17.2	7.68
P01008	Antithrombin-III	9.70	4	4	9	52.6	6.71
P02652	Apolipoprotein A-II	9.00	1	1	1	11.2	6.62
P07339	Cathepsin D	8.98	3	3	5	44.5	6.54
P06703	Protein S100-A6	8.89	1	1	11	10.2	5.48
P02766	Transthyretin	8.84	1	1	3	15.9	5.76
P04406	Glyceraldehyde-3-phosphate dehydrogenase]	8.66	2	2	11	36.0	8.46

P60660	Myosin light polypeptide 6	8.61	1	1	3	16.9	4.65
P31949	Protein S100-A11	8.57	1	1	10	11.7	7.12
P02763	Alpha-1-acid glycoprotein 1	8.46	2	2	2	23.5	5.02
P35542	Serum amyloid A-4 protein	8.46	1	1	2	14.7	9.07
P01605	Ig kappa chain V-I region Lay	8.33	1	1	5	11.8	7.96
P00325	Alcohol dehydrogenase 1B	8.27	3	3	6	39.8	8.29
P04433	Ig kappa chain V-III region VG (Fragment)	7.83	1	1	7	12.6	4.96
P04083	Annexin A1	7.8	2	2	2	38.7	7.02
P36955	Pigment epithelium-derived factor	7.66	3	3	6	46.3	6.38
P17661	Desmin	7.66	1	4	21	53.5	5.27
P52565	Rho GDP-dissociation inhibitor 1	7.35	1	1	1	23.2	5.11
P14618	Pyruvate kinase isozymes M1/M2	7.34	3	3	11	57.9	7.84
P06888	Ig lambda chain V-I region EPS	7.34	1	1	11	11.4	9.29
P23528	Cofilin-1	7.23	1	1	1	18.5	8.09
O60814	Histone H2B type 1-K	7.14	1	1	2	13.9	10.32
P07355	Annexin A2	7.08	2	2	4	38.6	7.75
P0C0L4	Complement C4-A	7.00	10	10	19	192.7	7.08
P00558	Phosphoglycerate kinase 1	6.71	2	2	7	44.6	8.1
P13797	Plastin-3	6.67	3	4	9	70.8	5.6
P12883	Myosin-7	6.67	11	11	18	223	5.8
P00441	Superoxide dismutase [Cu-Zn]	6.49	1	1	6	15.9	6.13
P02790	Hemopexin	6.28	3	3	13	51.6	7.02
P06702	Protein S100-A9	6.14	1	1	2	13.2	6.13
P61626	Lysozyme C	6.08	1	1	7	16.5	9.16
P23284	Peptidyl-prolyl cis-trans isomerase B	6.02	1	1	1	23.7	9.41
P08758	Annexin A5	5.94	2	2	9	35.9	5.05
P78417	Glutathione S-transferase omega-1	5.81	1	1	1	27.5	6.6
P08107	Heat shock 70 kDa protein 1A/1B	5.77	3	3	12	70.0	5.66
P63104	14-3-3 protein zeta/delta	5.71	1	1	2	27.7	4.79
P06732	Creatine kinase M-type	5.51	1	1	3	43.1	7.25
P02794	Ferritin heavy chain	4.92	1	1	2	21.2	5.55
P08571	Monocyte differentiation antigen CD14	4.8	1	1	1	40.1	6.23
P00450	Ceruloplasmin	4.79	4	4	12	122.1	5.72
Q6NZI2	Polymerase I and transcript release factor	4.62	1	1	1	43.4	5.6
P04004	Vitronectin	4.6	2	2	2	54.3	5.8
P05155	Plasma protease C1 inhibitor	4.6	2	2	3	55.1	6.55
P02751	Fibronectin	4.44	7	7	28	262.5	5.71
P29401	Transketolase	4.33	2	2	2	67.8	7.66
P62258	14-3-3 protein epsilon	4.31	1	1	2	29.2	4.74
P04264	Keratin. type II cytoskeletal 1	4.04	3	3	6	66.0	8.12

P25311	Zinc-alpha-2-glycoprotein	4.03	1	1	3	34.2	6.05
P68366	Tubulin alpha-4A chain	4.02	1	1	3	49.9	5.06
P00918	Carbonic anhydrase 2	3.85	1	1	1	29.2	7.4
P08603	Complement factor H	3.82	4	4	8	139.0	6.61
P02774	Vitamin D-binding protein	3.8	1	1	1	52.9	5.54
Q01518	Adenylyl cyclase-associated protein 1	3.79	1	1	2	51.9	8.06
Q13642	Four and a half LIM domains protein 1	3.72	1	1	2	36.2	8.97
P08238	Heat shock protein HSP 90-beta	3.59	2	2	8	83.2	5.03
P26038	Moesin	3.47	2	2	2	67.8	6.4
P07451	Carbonic anhydrase 3	3.46	1	1	1	29.5	7.34
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	3.4	2	2	5	101.3	6.79
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	3.33	3	3	5	103.3	6.98
P13796	Plastin-2	3.03	1	2	9	70.2	5.43
P51911	Calponin-1	3.03	1	1	3	33.1	9.07
P40925	Malate dehydrogenase. cytoplasmic	2.99	1	1	2	36.4	7.36
P40121	Macrophage-capping protein	2.87	1	1	4	38.5	6.19
A6NIK2	Leucine-rich repeat-containing protein 10B	2.74	1	1	1	32.7	7.36
P20774	Mimecan	2.68	1	1	1	33.9	5.63
P02749	Beta-2-glycoprotein 1	2.61	1	1	5	38.3	7.97
P55058	Phospholipid transfer protein	2.43	1	1	1	54.7	7.01
P37837	Transaldolase	2.37	1	1	1	37.5	6.81
P18428	Lipopolysaccharide-binding protein	2.29	1	1	1	53.3	6.7
P06727	Apolipoprotein A-IV	2.27	1	1	2	45.4	5.38
P04217	Alpha-1B-glycoprotein	2.22	1	1	1	54.2	5.86
P00488	Coagulation factor XIII A chain	2.19	1	1	1	83.2	6.09
P16930	Fumarylacetoacetase	1.91	1	1	1	46.3	6.95
P02765	Alpha-2-HS-glycoprotein	1.91	1	1	2	39.3	5.72
P01042	Kininogen-1	1.86	1	1	3	71.9	6.81
P04003	C4b-binding protein alpha chain	1.84	1	1	1	67.0	7.3
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	1.83	1	1	1	54.6	5.07
P00751	Complement factor B	1.83	1	1	4	85.5	7.06
P07602	Proactivator polypeptide	1.72	1	1	2	58.1	5.17
P04040	Catalase	1.71	1	1	1	59.7	7.39
P02748	Complement component C9	1.61	1	1	5	63.1	5.59
Q05682	Caldesmon	1.51	1	1	1	93.2	5.66
O75083	WD repeat-containing protein 1	1.32	1	1	2	66.2	6.65
P00747	Plasminogen	1.23	1	1	1	90.5	7.24
Q8N436	Inactive carboxypeptidase-like protein X2	1.19	1	1	2	85.8	6.87
P00734	Prothrombin	1.13	1	1	2	70.0	5.9
Q9Y490	Talin-1	0.94	2	2	3	269.6	6.07

P35579	Myosin-9	0.82	1	1	1	226.4	5.6
P04114	Apolipoprotein B-100	0.77	3	3	8	515.3	7.05
P01031	Complement C5	0.6	1	1	3	188.2	6.52
P07996	Thrombospondin-1	0.6	1	1	1	129.3	4.94

Table 6. Protein identified in human carotid plaque secretome. The proteins included in this table met all identification criteria laid out in the Methods section. Proteins are arranged according to the % coverage of target protein by identified peptides. *Unique peptide: the number of identified unique peptides subjected to quantification for the given protein. PSM, peptide spectrum match; MW, molecular weight; calc. pI, predicted isoelectric point.

The analyses of the three ITRAQ groups (A, B and C) were prepared in triplicate so that a higher number of covered proteins and single peptides could be quantified (Figure 20).

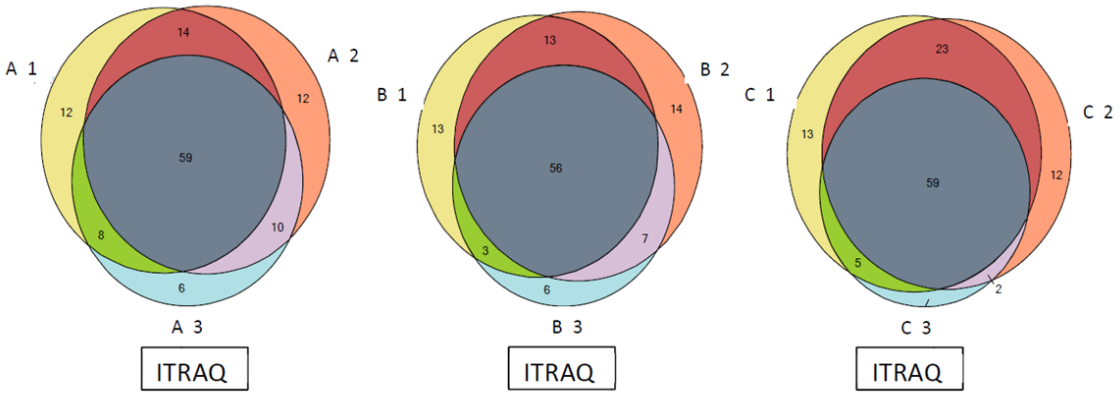

Figure 20. Analysis of the three ITRAQ groups (A, B and C) prepared in triplicate so that a higher number of covered proteins and unique peptides were quantified.

To study changes in the whole proteome, multivariate statistics (hierarchical clustering analyses and principal component analysis (PCA)) were applied. Figure 21 (A and B) shows that we were able to discriminate samples from each group, which suggests that atheromatous plaque progression has a specific secretome.

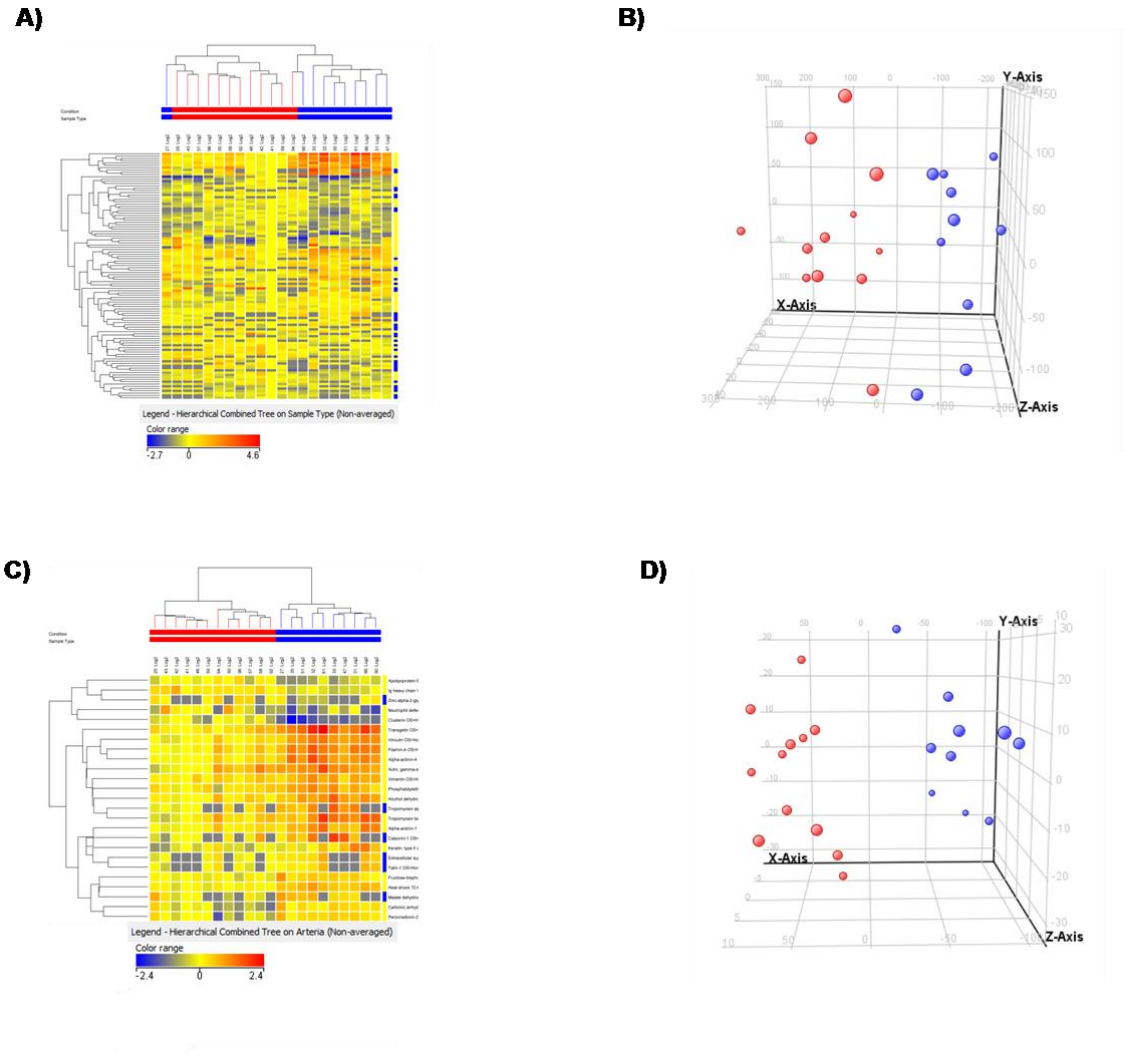

Figure 21. Proteomic analysis in CAP and MA secretome samples. Heat map representation of hierarchical clustering of molecular features found in each sample of two groups (**A** represents quantified proteins and **C** statistically significant proteins). The scale from -2.4 blue (low abundance) to +2.4 red (high abundance) represents this normalized abundance in arbitrary units. The CAP group is represented as red samples and MA secretomes as blue samples. Tridimensional principal component analysis (PCA) was used before (**B**) and after (**D**) Student's unpaired t-test. The CAP group is represented as red spots and the MA group as blue spots. CAP, carotid atherosclerotic plaque; MA, mammary artery secretome.

Subsequently, we used univariate statistics to define specific potential biomarkers. We found that 25 proteins exhibit statistically significant differences in secretome levels between carotid atherosclerotic plaque and non-atherosclerotic mammary artery (Table 7). The table shows the role that each protein might play in atherosclerosis according to the literature. Of all the differentially expressed proteins, four had significantly

increased levels in CAP secretomes: neutrophil defensin 1, apolipoprotein E, clusterin and zinc-alpha-2-glycoprotein. Multivariate statistics of the 25 proteins that exhibit differences (Figure 21C and 21D) also showed good clusterization and sample discrimination for each group, which suggests that atheromatous plaque progression has a specific secretome.

Swiss-Prot ID	Protein	Gene name	Role in atherosclerosis[b]	Ratio	P-value	Effect
P51911	Calponin-1	CNN1	SMC differentiation	0.30	0.0024	decreased
P09493	Tropomyosin-1 alpha	TPM1	SMC differentiation	0.35	<0.001	decreased
P00325	Alcohol dehydrogenase 1B	ADH1B	-	0.41	<0.001	decreased
Q01995	Transgelin	TAGLN	SMC differentiation	0.21	<0.001	decreased
P07951	Tropomyosin beta chain	TMP2	SMC differentiation	0.31	<0.001	decreased
P21333	Filamin-A	FLNA	Focal adhesion	0.31	<0.001	decreased
P30086	Phosphatidylethanolamine-binding protein 1	PEBP1	-	0.50	0.0013	decreased
P63267	Actin, gamma-enteric smooth muscle	ACTG2	Focal adhesion	0.40	0.0036	decreased
P18206	Vinculin	VCL	Focal adhesion	0.33	<0.001	decreased
P08670	Vimentin	VIM	Focal adhesion	0.58	0.0027	decreased
O43707	Alpha-actinin-4	ACTN4	Focal adhesion	0.30	<0.001	decreased
P12814	Alpha-actinin-1	ACTN1	Focal adhesion	0.42	<0.001	decreased
P08107	Heat shock protein-70 1A	HSPA1A	Inflammation	0.48	<0.001	decreased
P04075	Fructose-bisphosphate aldolase A	ALDOA	-	0.59	0.0018	decreased
P00915	Carbonic anhydrase 1	CA1	Endothelial dysfunction	0.49	0.0013	decreased
P32119	Peroxiredoxin-2	PRDX2	Antioxidant enzyme	0.51	0.0014	decreased
P40925	Malate dehydrogenase	MDH1	-	0.56	0.0091	decreased
P04264	Keratin, type II cytoskeletal 1	KRT1	-	0.47	0.0091	decreased
P08294	Extracellular superoxide dismutase	SOD3	Antioxidant enzyme	0.58	<0.001	decreased
Q9Y490	Talin-1	TLN1	Focal adhesion	0.63	0.0099	decreased
P59665	Neutrophil defensin 1	DEFA1	Leukocyte migration	1.91	0.0366	increased
P01766	Ig heavy chain V-III region BRO	-	-	1.71	<0.001	increased
P02649	Apolipoprotein E	APOE	Lipid transport	1.61	0.0018	increased
P10909	Clusterin	CLU	Lipid transport	2.28	<0.001	increased
P25311	Zinc-alpha-2-glycoprotein	AZGP1	Endothelial dysfunction	1.66	0.0099	increased

Table 7. Secretome proteins differ significantly between CAP and MA group[a].

[a]Student's unpaired t test was used. To avoid false positives, a multiple testing correction using a Benjamini-Hochberg method was also used. P value <0.05 and fold change >1.5 were selected as cutoff values. [b]Role in atherosclerosis refers to data reported in the literature.

Validation by ELISA assay

ELISA assays were applied to secretome samples in order to validate the differential release observed by iTRAQ labelling spectrometry. For the verification, we selected proteins with an increase profile in CAP secretomes. We confirmed that neutrophil defensin 1, apolipoprotein E and clusterin levels were significantly increased in CAP secretomes than in mammary artery group (Figure 22A, 22B and 22C). Conversely, the levels of zinc-alpha-2-glycoprotein showed no significant differences between the two secretome groups analysed (Figure 22D).

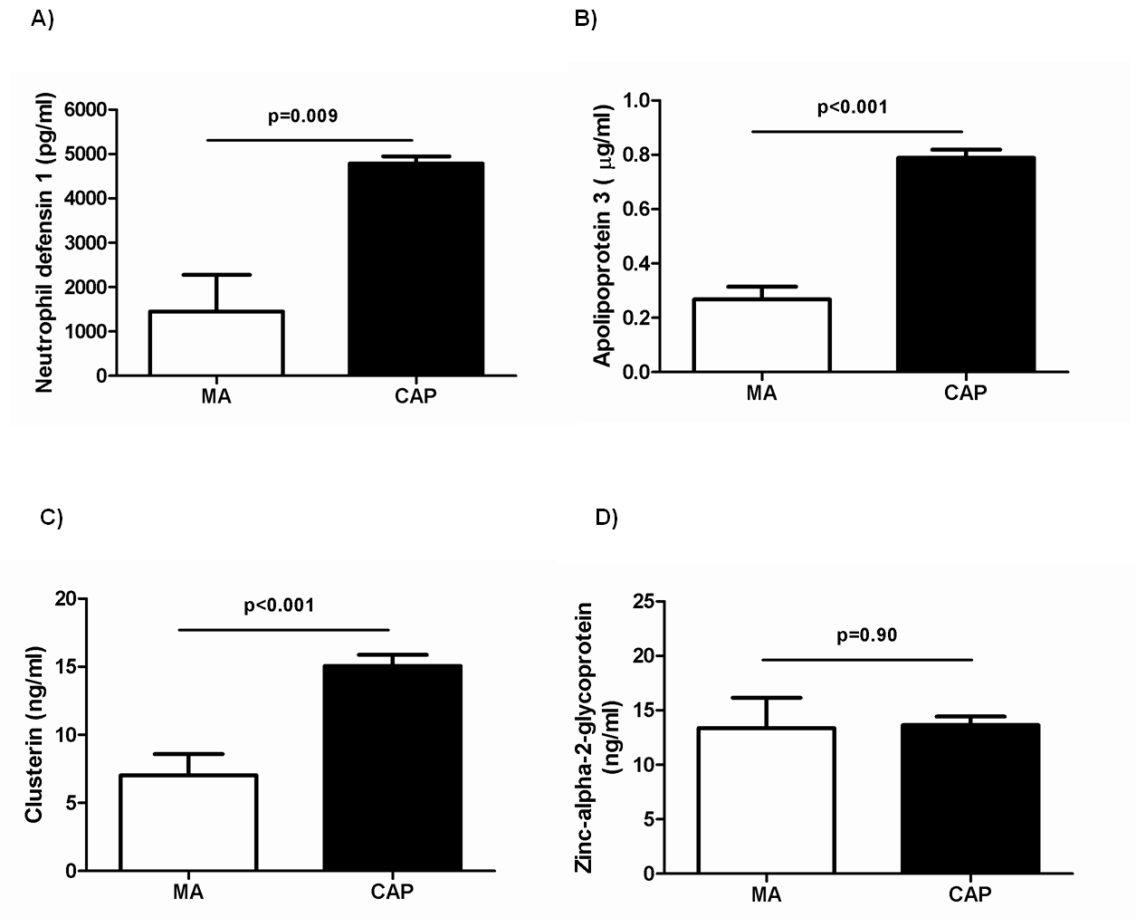

Figure 22. Protein validation by ELISA assays. Neutrophil defensin 1 (**A**), apolipoprotein E (**B**), clusterin (**C**) and zinc-alpha-2-glycoprotein (**D**) levels in secretome samples

Pathway analysis

To determine whether differentially expressed proteins belong to specific pathways, we conducted pathway analysis on proteomic data. The ConsensusPathDB-human Platform, which integrates interaction networks in the *Homo sapiens* proteome, was used to calculate the pathway impact. Briefly, this platform collates pathways from several public databases of protein interactions, signaling and metabolic pathways, and gene regulation in humans. We applied our analysis to the following databases: KEGG, Reactome, Netpath, Biocarta, HumanCyc and the pathway interaction database (PID), Signalink, Inoh, Wikipathways, Pharmgkb, Humancyc and Ehmn. The use of multiple databases enhances coverage and therefore reduces bias. Only pathways showing two proteins or more in the over-representation analyses and a p value cutoff <0.05 were taken into account (Figure 23). We used this tool for proteins that exhibit statistically significant differences in secretome levels. A list of 10 related pathways was generated. Interestingly, differentially secreted proteins are involved in pathways such as focal adhesion and leukocyte transendothelial migration.

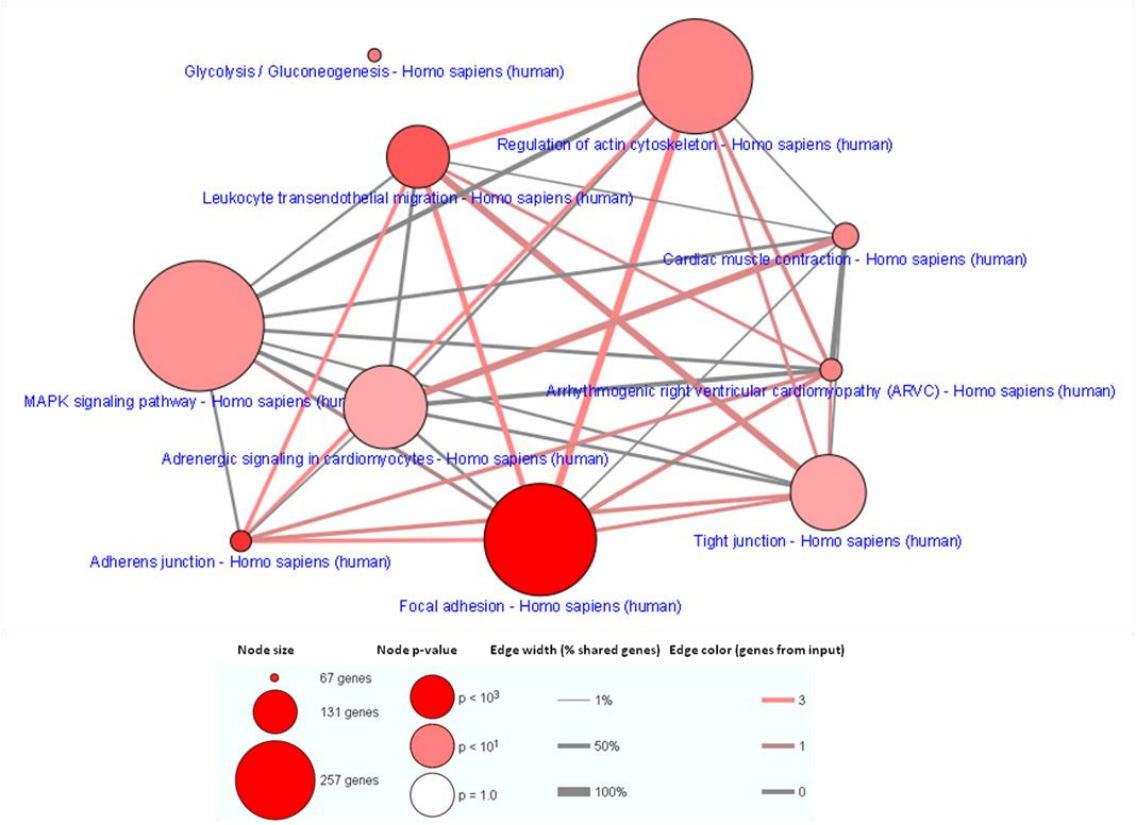

Figure 23. The ConsensusPathDB-human platform integrating interaction networks in *Homo sapiens* proteome was used to calculate pathway impact. Pathway analysis was conducted on proteins that exhibit statistically significant differences in secretome levels. Minimum overlap with input protein = 2 and p-value cutoff < 0.05.

4.2. Study 2

Title: Adipo/cytokines in atherosclerotic secretomes: increased visfatin levels in unstable carotid plaque

Characteristics of the population studied

The general characteristics and biochemical measurements of the population studied are shown in Table 8. Subjects were classified according to the samples obtained: serum group of healthy subjects (n=16), non-atherosclerotic mammary artery samples from patients undergoing coronary artery bypass (n=13) and unstable carotid atherosclerotic plaque samples from patients undergoing endarterectomy (n=18). The three groups studied had similar body mass index (BMI)s and they were all men. Anthropometrical and biochemical parameters showed no significant differences between non-atherosclerotic mammary artery and unstable carotid atherosclerotic plaque groups. As expected, carotid atherosclerotic plaque and mammary artery patients showed significant lower lipid profile because these subjects were taking lipid-lowering drugs. Table 7 also shows that the levels of glucose and HbA1c were significantly higher in the carotid atherosclerotic plaque and mammary artery group than in serum group of healthy subjects.

	Serum group of healthy subjects **(n=16) Mean ± SD**	Coronary patients with non-atherosclerotic mammary artery **(n=13) Mean ± SD**	Unstable carotid atherosclerotic plaque group **(n=18) Mean ± SD**
Age (years)	52.47 ± 13.25	65.08 ± 10.48	69.17 ± 7.44*
BMI (kg/m^2)	32.19 ± 11.76	29.39 ± 3.36	27.74 ± 3.13
Glucose (mg/dl)	91.31 ± 14.24	129.19 ± 55.44$^{\#}$	123.56 ± 45.37*
HbA1c (%)	4.97 ± 0.39	6.81 ± 1.39$^{\#}$	6.29 ± 1.07*
Insulin (mUI/L)	12.76 ± 16.19	11.76 ± 7.15	7.21 ± 4.93
HOMA2-IR	1.62 ± 1.95	1.59 ± 0.97	1.01 ± 0.67

Triglycerides (mg/dL)	115.02 ± 71.38	110.33 ± 27.84	103.00 ± 40.61
Cholesterol (mg/dl)	192.33 ± 37.81	128.34 ± 23.92#	118.81 ± 34.54*

Table 8. Anthropometric measurements and metabolic analysis of the population studied.

Adipo/cytokine levels in the secretome

To study the local role of adipo/cytokines in atherosclerosis, we evaluated the presence of adiponectin, visfatin, lipocalin-2, resistin, IL-6 and TNFR2 in secretomes of the unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery tissue cultures (Table 9). Of all the molecules analysed, visfatin was the only adipo/cytokine that was differently expressed in secretome samples. Specifically, visfatin levels were significantly higher in the unstable carotid atherosclerotic plaque than in non-atherosclerotic mammary artery secretomes (p=0.021). Conversely, the levels of adiponectin and IL-6 showed no significant differences between the two secretome groups analysed. Finally, the levels of lipocalin-2, resistin and TNFR2 were almost undetectable in the secretome samples. No significant correlations between adipo/cytokines were found.

	Unstable carotid atherosclerotic plaque group **(n=18)** **Mean ± SD**	Coronary patients with non-atherosclerotic mammary artery **(n=13)** **Mean ± SD**
Visfatin (ng/µg total protein)	0.100 ± 0.017	0.046 ± 0.012*
Adiponectin (µg/µg total protein)	0.311 ± 0.039	0.369 ± 0.096
IL-6 (pg/µg total protein)	0.048 ± 0.012	0.039 ± 0.008
Lipocalin-2 (ng/µg total protein)	0.009 ± 0.002	0.008 ± 0.001

Resistin (ng/µg total protein)	0.001 ± 0.001	0.001 ± 0.001
TNFR2 (ng/µg total protein)	0.007 ± 0.002	0.005 ± 0.001

Table 9. Adipo/cytokine levels in secretome samples. IL-6, interleukin 6; TNFR2, tumor necrosis factor receptor 2. Data are expressed as mean ± SD. $p<0.05$ are considered statistically significant. *refer to the statistically significant differences between unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery group.

Circulating Visfatin and adipo/cytokines levels in serum

As only differences *in sit*u visfatin levels were observed and in order to study whether these differences were only a local effect or if they were also reflected in serum, we measured visfatin circulating levels in the group of patients suffering from atherosclerosis and in a serum group of healthy subjects (n=16). Figure 24 shows that there were no differences between unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery group. However, visfatin serum concentration was higher in both unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery groups than in the serum cohort of healthy subjects (p=0.037 and p=0.001; respectively). This difference remained significant after adjusting for age, BMI and glucose metabolism.

Then, we analysed the circulating levels of two adipo/cytokines with different profile, pro- and anti-inflammatory (IL-6 and adiponectin, respectively). We found that adiponectin circulating levels were significantly higher in the serum group of healthy subjects (29.20±8.42) than unstable carotid atherosclerotic plaque group (11.23±1.69, p=0.025) and non-atherosclerotic mammary artery patients (9.26±2.35, p=0.031). However, we observed no differences in the circulating levels of IL-6 between groups. No significant correlations between these adipo/cytokines and visfatin were found.

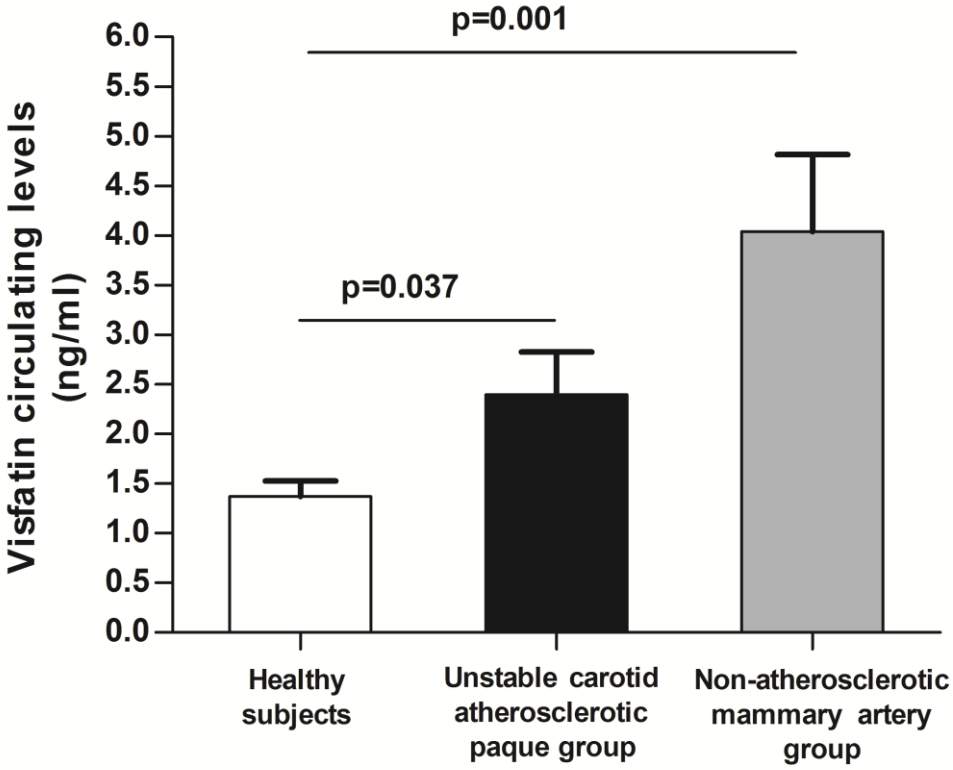

Figure 24. Visfatin serum levels in different groups: unstable carotid atherosclerotic plaque group (n=18), coronary patients with non-atherosclerotic mammary artery (n=13) and serum cohort of healthy subjects (n=16). $p<0.05$ were considered statistically significant.

4.3. Sub-study 3

Title: Secretome and circulating levels of adipo/cytokines in relation with cardiovascular risk factors

The aim of this substudy was to analyse the levels of several pro- and anti-inflammatory adipo/cytokines in serum and unstable plaque secretome according to the presence of obesity, arterial hypertension, diabetes mellitus, and dyslipidemia and being smoker. Also, we analyzed the characteristics of the population, classifying patients who met the LDL cholesterol goals according to the guidelines.

Secretome and circulating levels of adipo/cytokines in relation to the presence of obesity, arterial hypertension, diabetes mellitus, dyslipidemia and smoking

When we analyzed the secretome and circulating levels of adipo/cytokines in relation to the presence of cardiovascular risk factors, we found no differences between levels of adipo/cytokines in the unstable plaque secretomes and circulating levels, in relation to the presence of arterial hypertension, dyslipidemia, diabetes mellitus and tobacco use. We only found significative low secretome adiponectin levels in patients with obesity in relation to patients without obesity ($p=0.026$).

Then, we classified the Study 2 cohort according levels of LDL-cholesterol as recommended the ESC guidelines in very-high cardiovascular risk.
Given that the ESC guideline recommendation of 2016 on LDL cholesterol levels patients with very high cardiovascular risk was under 70 mg/dl, we first comparedgroups of patients who met the recommendations or not. The clinical characteristics and biochemical measurements according this classification are shown in Table 10. Only the 55.5% of patients were well controlled with the hipolipemant treatment. Anthropometrical and biochemical parameters showed no significant differences between both groups, except for HOMA2, total cholesterol and LDL Cholesterol.

Variables	LDL<70 Mean (SD) n = 10	LDL>70 Mean (SD) n = 8	p-value
Age (years)	68.60 (6.86)	67.25 (9.130)	0.725
Sex N (%)	10 (55.9)	8(40.0)	0.106
BMI (kg/m2)	29.67 (3.20)	27.55 (3.48)	0.352
HBP	1.00	0.83 (0.40)	0.447
SBP (mmHg)	137.85 (14.20)	151.60 (15.88)	0.146
DBP (mmHg)	71.37(11.93)	80.25(6.20)	0.083
Insulin (mUI/L)	10.07 (5.97)	7.68 (5.51)	0.475
HOMA2	1.61 (1.01)	2.82(2.79)	*0.016
Smoking	0.83(0.98)	1.20 (0.83)	0.527
Biochemical parameters - Mean (SD)			
Glucose (mg/dl)	130 (40.22)	123 (61.36)	0.798
HbA1C (%)	6.64 (1.21)	6.56(1.22)	0.895
Creatinine (mg/dl)	0.95(0.22)	1.07(0.39)	0.431
Total-cholesterol (mg/dl)	102.05(15.37)	149.91(24.39)	0.000
HDL Cholesterol (mg/dL)	25.70(4.24)	29.75(8.77)	0.216
LDL Cholesterol (mg/dL)	55.75(9.48)	95.50(20.74)	*0.001
AST (U/L)	22.87(8.65)	22.50 (12.04)	0.947
ALT (U/L)	25.80 (12.21)	29.37 (22.36)	0.671
GGT (U/L)	35.80 (36.08)	24.75 (13.19)	0.425
AP (U/L)	65.20 (23.28)	53.37 (15.10)	0.233
Total billirrubin (mg/dl)	0.41 (0.17)	0.50 (0.30)	0.451
Ferritin (ng/ml)	120.20 (103.95)	145.56 (97.95)	0.637
Adiponectina instable (pg/ml)	0,10 (0.04)	0.08 (0.01)	1
Adiponectina plasma(pg/ml)	12,22(7.84)	8.15 (6.67)	0.178
IL6 instable (pg/ml)	0,012 (0.006)	0.01 (0.003)	0.291
IL6 plasma (pg/ml)	1,6416(1.14)	2.23 (0.84)	0.054

Table 10. Baseline characteristics of subjects classified according to recommendations of the ESC guidelines of 2016 on LDL-cholesterol levels in very-high cardiovascular risk (<70 mg/dl).

Then, we did the same analysis according ESC guideline recommendation of 2021 where recommendation of on LDL cholesterol levels in patients with very high cardiovascular risk was 55 mg/dl, in this sense, we compared groups of patients who met the recommendations or not. Also, in this subclassification, only the 55.5% of patients were well controlled with the hipolipemant treatment. The clinical characteristics and biochemical measurements according this classification are shown in Table 11. There existed significant differences between groups regarding DBP, HbA1C, total cholesterol, LDL Cholesterol and AP.

Variables	**LDL<55 Mean (SD) n = 10**	**LDL>55 Mean (SD) n = 8**	**p-value**
Age (years)	64.25 (9.17)	69.07 (7.29)	0.285
BMI (kg/m2)	31.32 (5.48)	28.20 (2.75)	0.225
HBP	1 .00	0.83 (0.40)	0.447
SBP (mmHg)	138 (18.05)	146.37 (15.14)	0.415
DBP (mmHg)	68.50 (5.97)	78.25 (10.42)	*0.045
Insulin (mUI/L)	10.50 (6.28)	8.71 (5.75)	0.617
HOMA2	1.41 (0.85)	2.35 (2.24)	0.430
Smoking	0.75 (0.95)	1.14 (0.89)	0.513
Biochemical parameters - Mean (SD)			
Glucose (mg/dl)	107.00 (19.64)	133 (53.95)	0.367
HbA1C (%)	5.85 (0.28)	6.82 (1.25)	*0.017
Creatinine (mg/dl)	0.91 (0.08)	1.03 (0.34)	0.249
Total-cholesterol (mg/dl)	89.30 (13.91)	133.04 (27.65)	*0.008
HDL Cholesterol (mg/dL)	26.25 (4.34)	27.85 (7.38)	0.688
LDL Cholesterol (mg/dL)	46.50 (8.21)	81.10 (23.06)	*0.011
AST (U/L)	28.50 (9.46)	20.40 (9.39)	0.171
ALT (U/L)	32.00 (15.57)	26.07 (17.67)	0.554
GGT (U/L)	39.00 (39.47)	28.57 (25.52)	0.645
AP (U/L)	83.25 (23.21)	53.28 (14.38)	*0.005
Total billirrubin (mg/dl)	0.40 (0.08)	0.47 (0.27)	0.641
Ferritin (ng/ml)	191.00 (135.07)	109.28 (81.39)	0.160
Adiponectina instable (pg/ml)	0.08 (0.01)	0.10 (0.03)	1
Adiponectina plasma (pg/ml)	12.58 (7.63)	9.81 (7.53)	0.45
IL6 instable (pg/ml)	0.009 (0.007)	0.01 (0.003)	0.734
IL6 plasma (pg/ml)	1.65 (0.24)	1.97 (1.12)	0.705

Table 11. Baseline characteristics of subjects classified according to recommendations of the ESC guidelines of 2021on LDL-cholesterol levels in very-high cardiovascular risk (<55 mg/dl).

Correlations of plasma levels of adipo/cytokines with age, glycidic and lipid metabolism parameters

The literature has described levels of adipo/cytokines in plasma deregulated in patients with dyslipidemia, diabetes and obesity, so, we have studied whether there is a correlation between the circulating levels of adipo/cytokines with the parameters of glycemic and lipid metabolism. Plasma levels of IL-6 do not correlate with any of the metabolic parameters described (data not shown). However, there is a negative correlation between plasma adiponectin levels and diastolic blood pressure (rho=-0.54, p=0.025).

Finally, when we studied the correlation between circulating levels of the diferent adypo/citokines, we found a negative correlation between plasma levels of IL-6 and of adiponectin (Figure 25).

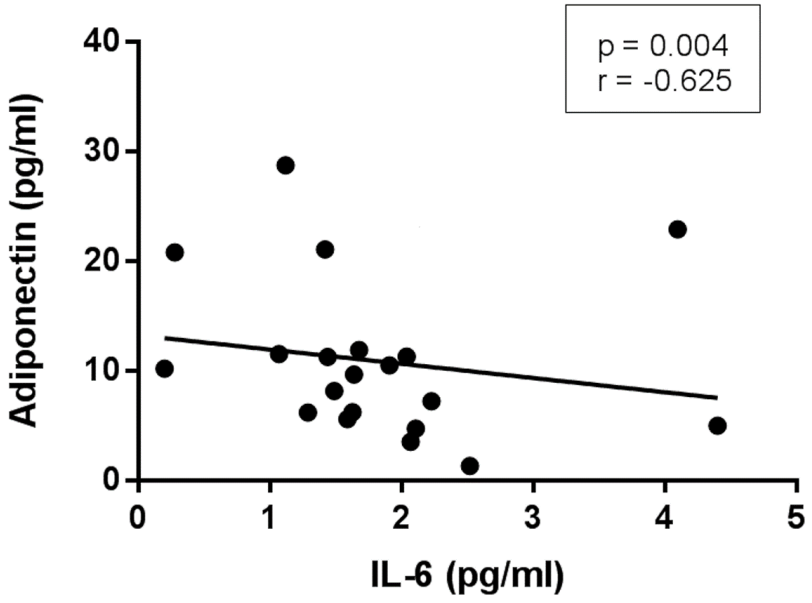

Figure 25. Correlation between IL-6 and adiponectin circulating levels.

Correlations between levels of adipo/cytokines in unstable plaque secretome with parameters of glycemic, lipid metabolism

When we studied the correlations between secretome levels of adipo/cytokines with parameters of glycemic, lipid metabolism, regarding visfatin, lipocaline and resistin we were not able to find any correlation (data not shown).

However, adiponectin levels in unstable plaque secretome were positively correlated with total bilirubin (rho=0.076, p=0.047).

Adipo/cytokine levels according to lipid control

In the present study, we also wanted to analyze the levels of adipo/cytokines in unstable plaque secretomes and circulating levels classifying the cohort in patients with lipid control adequate for their cardiovascular risk (LDL<70 mg/dl) or inadequate (LDL>70 mg/dl), according to ESC guidelines of 2016 on LDL-cholesterol levels in very-high cardiovascular risk and also according ESC guidelines of 2021.

We found no differences with respect to the circulating or secretome levels of adipo/cytokines regarding the levels of LDL.

RESULTS

DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE
Ajla Alibalic

5.1. Study 1

Title: Proteomic Profile of Unstable Atheroma Plaque: Increased Neutrophil Defensin 1, Clusterin, and Apolipoprotein E Levels in the Carotid Secretome

Despite intensive research on unstable atherosclerotic plaques, there is still no clear understanding of the molecular and pathophysiological processes responsible for plaque rupture and its consequences[190,235]. The aim of our studies was the identification of biological markers that would help to identify "vulnerable patients" and predict rupture events in time to avoid a fatal endpoint and to improve the knowledge of atherosclerosis development.

Several proteomic approaches have been used to study carotid tissue to understand the mechanisms and progression of atherosclerosis. In the search for biomarkers related to cardiovascular risk development and atherosclerosis itself, proteomic approaches have provided a large amount of data notably contributing, with no doubt, to identifying potential markers and, moreover, to better understanding the atherosclerotic process[190,235–237].

However, these studies have identified only a small number of proteins in relatively few samples and thus cannot address the interindividual variation of candidate biomarkers[192,238].

Given the complexity of atherosclerotic disease, with multiple processes implicated (e.g., angiogenesis, cell proliferation and cell death) and involving various systems, such as coagulation or immune response, it is rather unlikely that a single protein marker could be responsible for the progression and fate of atherosclerotic plaques. In this regard, and in agreement with other researchers, we think that it should be more appropriate to use a multipanel of proteins as a marker of atherosclerosis, although the selection of the best-performing panel is not easy[237].

Classically, proteomic approaches have focused on analysing differential protein expression patterns between total extracts of tissue specimens with unstable

(haemorrhagic/thrombotic) vs. stable (fibrous) plaques. Until 2016, the human artery secretome had not been extensively analysed, so the novelty of our first study was to analyse the proteomic changes that occur in the secretomes of carotid atherosclerotic plaque (CAP) and nonatherosclerotic mammary arteries (MAs). To date, there is no unique approach regarding the experimental design in terms of tissue sections analysed, the source of atherosclerotic arteries themselves or the chosen methodology, but this has been clearly improving with time[237].

Multivariate statistics of our study revealed overall changes in 162 proteins. Furthermore, when univariate statistics were applied, 25 proteins emerged as potential biomarkers of carotid atherosclerotic plaque. The functional analysis performed in our work corroborated the results of other studies and revealed that selected proteins were involved in pathways related to vascular disease, such as focal adhesion and leukocyte transendothelial migration[188]. Our findings showed the potential role of secretome evaluation in carotid atherosclerosis as a means to determine new possible biomarkers and further study the progression and physiology of the disease.

Of the 25 proteins that exhibited significant differences in secretome levels between CAP and nonatherosclerotic MA, we first focus on significantly **decreased** proteins in carotid atherosclerotic plaque secretomes. We classified the decreased proteins in CAP secretomes into different groups based on their potential role in atherosclerosis, as reported in the literature. Although we found that a significant number of the proteins selected were involved in the focal adhesion pathway, these proteins are indirectly related to the extracellular matrix and could be implicated in the remodelling of atherosclerotic plaque maturation, such as proteoglycans[239]. Proteoglycans interact with a large number of components in vascular disease and impact their atherogenecity[240]. The alterations described could indicate tissue remodelling with a loss of elasticity in the artery. Moreover, we observed proteins involved in smooth muscle cell differentiation, contraction, proliferation, migration and focal adhesion pathways, which corroborates the leading role that vascular smooth muscle cells play in plaque progression[241,242]. In humans, smooth muscle cells (SMCs) are the main cell type in the artery medial layer, in preatherosclerotic

diffuse thickening of the intima, and in all stages of atherosclerotic lesion development. SMCs secrete the proteoglycans responsible for the initial binding and retention of atherogenic lipoproteins in the artery intima, with this retention driving foam cell formation and subsequent stages of atherosclerosis[243]. Pathologic intimal thickening is an important stage of atherosclerosis that leads to atheroma. The number of intimal SMCs does not change from diffuse intimal thickening to pathologic intimal thickening[244]. We also found differences in proteins that were involved in protecting against oxidized stress. One of them, extracellular superoxide dismutase (ecSOD), is an antioxidant enzyme in vascular tissues. It plays a major role in modulating blood pressure and could prevent endothelial dysfunction[245–247]. Another antioxidant enzyme was peroxiredoxin 2 (Prdx2), which was also decreased, and it regulates proinflammatory responses, vascular remodelling, and overall oxidative stress[248–250]. In this sense, Park et al. showed that Prdx 2 deficiency in apolipoprotein E-deficient (ApoE) mice accelerates atherosclerosis by increasing the infiltration of immune cells into plaques[251.] Recent studies have reported Prdx2 as a potential negative regulator of inflammatory vascular diseases, and it has been identified as a protein that is increased in patients with ruptured abdominal aortic aneurysm compared with patients with nonruptured abdominal aortic aneurysm[252]. Moreover, we also observed decreased levels of carbonic anhydrase 1 (CA1) in CAP secretomes. Aamand et al.[253] showed that CA1 plays a role in the generation of vasoactive nitric oxide (NO), which suggests that low levels of CA1 induce impaired NO production and, subsequently, endothelial dysfunction. Vascular calcification is an important pathogenic process in atherosclerosis. Most aortic aneurysms and aortic dissections are caused by atherosclerosis and are related to vascular calcification[254]. CA1 plays important roles in promoting biocalcification. Ando et al. examined abdominal aortic aneurysm using proteomics and detected an abundant CA1 autoantigen, suggesting an important role for CA1 in the formation of this lesion[255,256], demonstrating that CA1 plays an important role in atherosclerosis calcification in human and mouse model studies. Moreover, induction of rat VSMCs, which represent 70% of atherosclerotic plaque cells, with

β-GP led to massive calcium deposition, significantly increased expression of ossification-related genes and increased CA1 expression levels[256].

Finally, in accordance with Martin-Ventura JL et al.[257], we also detected a decrease in heat shock protein-70 1A (Hsp70 1A) levels in CAP secretomes. Hsp70 is a chaperone with anti-inflammatory and anti-apoptotic properties that improve the viability of stressed vascular smooth muscle cells[258]. Although some studies have reported the vasoprotective role of intracellular HSP70, the evidence regarding extracellular HSP70 is contradictory. Under stressful conditions, HSP70 can be released into the extracellular medium and act as an inflammatory mediator [259]

On the other hand, the **increased** proteins in CAP secretomes were neutrophil defensin 1, apolipoprotein E, clusterin and zinc-alpha-2-glycoprotein. Regarding neutrophil defensin 1, some studies have revealed that polymorphonuclear neutrophils (PMNs) participate in the development of atherosclerotic lesions[260–262]. Activated PMNs were shown to produce and release reactive oxygen species, inflammatory leukotrienes and proteolytic lysosomal enzymes, directly inducing vascular damage. High circulating levels of PMN-platelet aggregates have been reported in patients with clinical atherosclerosis, and recent studies suggest that these aggregates may play a role in the vascular response to injury[263]. During inflammation, large amounts of intracellular proteins, such as neutrophil defensins, are released from the activated PMN[264,265]. These proteins have been found in human atherosclerotic arteries[266]. Apart from its role in the inflammation underlying atherosclerosis, this protein inhibits LDL metabolism and fibrinolysis and promotes Lp(a) binding[267]. Defensin 1 binds to apolipoproteins enriched in LDL. This interaction facilitates clearance of LDL particles in the liver via the LDL receptor. Paulin et al. identified a nonredundant mechanism by which defensin allows for reduction of LDL-cholesterol, a process that may be therapeutically instructed to lower cardiovascular risk[268]

In addition, some authors[269] have reported that neutrophil defensins can induce leukocyte transendothelial migration and increase foam cell formation. Moreover, Higazi M et al.[270] suggested that α-defensin-1 has competing effects on

atherogenesis. Accelerated hepatic uptake and clearance of α-defensin-1/LDL complexes from the blood reduces their probability of depositing in the vasculature. On the other hand, α-defensin-1/LDL complexes have a greater intrinsic propensity to deposit and remain in vascular cells and the matrix[271], where they stimulate macrophage recruitment, the generation of cathepsins B and S, the formation of foam cells and increased endothelial permeability to LDL[272]. In this regard, according to our results, neutrophil defensin 1 is another key molecule in the pathogenesis of atherosclerosis.

Our study identified zinc-alpha-2-glycoprotein (ZAG) as a new adipokine that has not yet been studied in atheroma plaques. In agreement with our findings, several studies have shown that serum ZAG levels are upregulated in a variety of pathological processes involving endothelial dysfunction[273–276]. However, the immunoassay data did not show profiles similar to those obtained in the spectrometry experiments. This protein has been quantified by mass spectrometry using only one unique peptide. In this sense, the literature purports that this sequence could be easily modified[277,278], and these changes could affect the quantification by mass spectrometry or ELISA detection. Otherwise, the quantitative changes detected by mass spectrometry referred to a very minor difference. However, several recent studies have reported that ZAG plays an important role in lipid metabolism, in reducing obesity and improving insulin sensitivity, both in experimental animal models and in human studies. ZAG expression in human adipose tissue was positively associated with adiponectin expression[279], and ZAG anti-inflammatory activity[280,281]. ZAG prevents atherosclerosis by inhibiting inflammation. Furthermore, ZAG is not only a marker of cardiovascular risk but also a causal risk factor for disease progression. Lower levels of ZAG are correlated with more severe coronary estenosis[282].

Regarding the role of clusterin in atherosclerotic lesions, other authors studying atheroma plaques have reported results similar to ours[283,284]. Indeed, clusterin is upregulated in a wide variety of clinical situations, including ageing, diabetes and atherosclerosis[285,286]. Although the role of clusterin in atherosclerosis remains

unknown, several studies have shown that clusterin distribution in the human aorta is increased as this disease progresses[284,287]. Clusterin was markedly induced in the media and neointima after vascular injury. Furthermore, decreased clusterin expression reduced the proliferation of vascular smooth muscle cells[288]. The vascular role of clusterin is enhanced in vascular smooth muscle cells (VSMCs)[289]. Kim et al. performed adenoviral delivery of the clusterin gene for its overexpression *in vivo*. They reported that CLU overexpression attenuates the expression of proinflammatory chemokines, cell adhesion molecules and matrix degrading endopeptidases stimulated by TNF-α, which are therapeutic targets for the development of neointimal hyperplasia after injury[290]. This demonstrates that upregulation of CLU might play a protective role during the prevention and treatment of vascular diseases such as atherosclerosis and restenosis[291].

Apolipoprotein E (apoE), a fascinating multifunctional polymorphic apoprotein, influences cardiovascular and neurologic health and disease through common polymorphisms[292,293]. Human apoE plays an important role in the metabolism of lipids, including cholesterol, and promotes the clearance of atherogenic lipoproteins such as very-low-density lipoprotein (VLDL) and chylomicron remnants from the circulation[294,295]. Isoelectric focusing identified 3 isoforms termed apoE2, apoE3 and apoE4 that have a significant impact on the conventional clinical laboratory profile and influence cardiovascular health[295].

Liver-produced circulating apo E facilitates clearance of the remnants of the triglyceride-rich lipoproteins (chylomicrons and VLDL), receiving apoE from HDL. ApoE4 leaves HDL more readily, enhancing clearance of remnants whose cholesterol downregulates hepatic LDL receptor expression and thus increases the plasma LDL concentration[295]. Macrophage-produced apoE plays an important antiatherogenic role by promoting cholesterol efflux from cells in the arterial wall[296]. Regulation of apoE expression from macrophages is under the control of several signalling pathways and is stimulated by several molecules, such as apolipoprotein A-I, HDL, ATP binding cassette transporter (ABCA1), protein kinase A, intracellular calcium and the microtubular network[297].

Recent new data on adipose-produced apoE point to a novel metabolic role for apoE in obesity[298]. Manifest atherosclerosis and subclinical atherosclerosis are consistently associated with apoE4. Relative to carriers of apoE3, carriers of apoE4 have a 46% increase and carriers of apoE2 have a 26% decrease in risk except, strangely, for persons of Mongolian ancestry[299]. In older Europeans, apoE4 also conferred a higher risk[300]. Carotid intima-media thickness is greater in apoE4 carriers and is more extensive with concomitant diabetes[301]. Although apoE4 promoted cardiovascular disease in diabetic subjects, there was no protection from the apoE2 gene[302]. A large Danish study over 25 years revealed that higher concentrations of apoE were associated with cardiovascular and cancer mortality, while lower concentrations were associated with dementia mortality[303]

In addition, the function of apoE is linked to both proinflammatory and anti-inflammatory cytokines[304]. ApoE influences inflammation[305]. Therefore, under pathological conditions, the expression of apoE could be upregulated by macrophages in the arterial wall. In our study, one explanation for the high levels of apoE in carotid atherosclerotic secretomes could be that this molecule may have a protective role.

Finally, other authors have studied atheroma plaques from the proteomics point of view. First, Eslava-Alcon et al. proposed a panel of 76 proteins identified in at least two proteomic studies analysing unstable atherosclerotic plaques that could be used as a prognostic signature of plaque instability[237]. Others have studied endothelial progenitor cells (EPCs), which constitute a promising alternative in cardiovascular regenerative medicine due to their assigned role in angiogenesis and vascular repair. In response to injury, EPCs promote vascular remodelling by replacing damaged endothelial cells and/or by secreting angiogenic factors over the damaged tissue. In this sense, Vega et al. evaluated the initial response of early EPCs (eEPCs) from healthy individuals after direct contact with the factors released by carotid arteries complicated with atherosclerotic plaques (AP). They found that the AP secretome stimulated eEPC proliferation and mobilization *ex vivo*, and this increase was accompanied by augmented permeability, cell contraction and an increase in cell–cell adhesion. Furthermore, a comparative mass spectrometry analysis of control

versus stimulated eEPCs revealed a differential expression of proteins in the AP-treated cells, mostly involved in cell migration, proliferation and vascular remodelling. Some of these protein changes were also detected in the eEPCs isolated from atherosclerotic patients compared with the eEPCs from healthy donors, in agreement with our study. The authors have particularly focused on those approaches directly analysing either the atherosclerotic tissue or its secretome, taking into account that the secretome ultimately represents the first factors that plaques send to the bloodstream as a sign of damage[306].

We should note the following drawbacks of our study. First, we used mammary arteries as controls as previous studies have shown a lower incidence of atherosclerosis in these arteries. Although nondiseased carotid arteries would be the best choice, they were unfortunately not available. Second, our results were obtained in homogeneous groups of men. Therefore, they cannot be extrapolated to other population groups with mixed sexes or associated metabolic diseases. Third, our patients were in treatment. Although we cannot exclude the influence of drugs on our results, this study design provided information on the real clinical situation during necessary lipid-lowering therapy. Finally, another limitation of the study was the sample size due to the difficulty in obtaining human artery samples. Nevertheless, our highly sensitive method demonstrates a very wide spectrum of proteins in CAP and MA secretomes, which was verified by ELISA. Further studies to validate our findings would be useful.

5.2. Study 2

Title: Adipo/cytokines in atherosclerotic secretomes: Increased visfatin levels in unstable carotid plaque

Despite multidisciplinary investigations, the local action of adipo/cytokines expressed in the secretomes of atherosclerotic plaques is not yet well understood. The aim of our second study was to analyse the presence of several adipo/cytokines with different pro- and anti-inflammatory profiles in the secretome of an unstable carotid atherosclerotic plaque and to compare it with the adipo/cytokines in a nonatherosclerotic mammary artery. The main finding was that visfatin levels were significantly higher in unstable carotid atherosclerotic plaques than in nonatherosclerotic mammary artery secretomes, suggesting a possible connection between high levels of visfatin and unstable carotid atherosclerotic plaques.

Visfatin is an adipokine produced in adipose tissue, bone marrow, skeletal muscle, and liver with a physiological role that is not completely understood[307–309.] It was first identified as a pre-B-cell colony-enhancing factor but was shown to possess enzymatic functions in nicotinamide adenine dinucleotide biosynthesis, with ubiquitous expression in skeletal muscles, liver, cardiomyocytes, and brain cells. Visfatin exists in intracellular (iNAMPT) and extracellular (eNAMPT) forms. Intracellularly, visfatin/iNAMPT plays a regulatory role in NAD+ biosynthesis and thereby affects many NAD-dependent proteins, such as sirtuins and PARPs. Extracellular visfatin is associated with many hormone-like signalling pathways and activates some intracellular signalling cascades. Importantly, eNAMPT has been associated with several cardiovasculo-metabolic disorders[310]. In the context of metabolic diseases, most studies have focused on increased circulating levels and adipose tissue expression of visfatin[311,312]. Additionally, visfatin was initially proposed as a clinical marker of atherosclerosis, endothelial dysfunction and vascular damage[313]. Visfatin is an active player that promotes vascular inflammation, atherosclerosis development and progression, and plaque destabilization[313–315]. Visfatin exerts its proliferative, proinflammatory, and proangiogenic effects by stimulating molecular signalling pathways such as

phosphatidylinositol 3-kinases (PI3K), nuclear factor-Kb (NF-kB), signal transducer and activator of transcription (STAT3) and extracellular signal-regulated kinases (ERKs)[316–319].

Regarding the **local effect** of visfatin on atherosclerotic lesions and its role in plaque destabilization, other authors directly studying atheroma plaques have reported results similar to ours[320–322]. Two studies with the most similarities to our second work reported that visfatin should be regarded as an inflammatory mediator localized to foam cell macrophages within unstable atherosclerotic lesions, which potentially plays a role in plaque destabilization[320,323,324]. Vascular endothelial growth factor (VEGF) and metalloproteinases (MMPs) provide some insights to partly explain these mechanisms. For example, MMP may explain visfatin-induced plaque destabilization[325]. Similarly, VEGF may explain the final steps that lead to visfatin- induced angiogenesis[326–328].

Moreover, Zhou et al. reported that visfatin induces cholesterol accumulation in macrophages and accelerates the process of atherosclerosis[321]. Increased levels of visfatin were observed in ischaemic stroke patients[308,329], particularly in patients with high-grade carotid atherosclerosis[329]. The induction of visfatin in plaques was higher in symptomatic than in asymptomatic patients[320,330]. In the same study, Dahl et al. reported that visfatin had a combined ability to increase TNF-α as well as to respond with increased expression upon TNF-α stimulation. Therefore, this bidirectional interaction between TNF-α and visfatin could represent a pathogenic loop in unstable atherosclerotic lesions[320,330]. Another study further demonstrated that the regulation of visfatin in macrophages is related to proatherogenic stimuli, including hypoxia, TNF-α and ox-LDL[331]. Other possible direct mechanisms of visfatin on atherosclerosis have been reported: promotion of smooth muscle cell proliferation, alteration of the expression and activity of matrix metalloproteinases, greater atherosclerotic plaque vulnerability and impairment of endothelial vasodilatory responses[332–335]. In the present study, we only included patients with cerebrovascular ischaemia and unstable carotid atherosclerotic plaque. Therefore, we could not compare visfatin levels between stable and unstable carotid plaque secretomes. Although the exact biological mechanisms involving visfatin in the pathogenesis of atherosclerosis are not well established, visfatin

appears to be an active factor in the development and progression of atherosclerosis through its effects on cytokine and chemokine secretion, macrophage survival, leukocyte recruitment by endothelial cells, vascular smooth muscle inflammation and plaque destabilization[313,321,336,337]

Regarding **circulating levels**, we found higher visfatin serum concentrations in patients with carotid atherosclerosis and coronary patients with nonatherosclerotic mammary arteries who underwent coronary revascularization surgery. In our study, mammary arteries were used as control arteries, as previous studies have shown a lower incidence of atherosclerosis[338,339]. However, it is important to remark that although mammary artery patients have a nondiseased arterial secretome, they have atherosclerotic coronary disease. Likewise, in recent years, several studies have established positive associations between enhanced circulating visfatin levels and atherogenic inflammatory diseases, which suggests a possible role of visfatin in atherosclerosis pathogenesis[313,315,340–342]. Specifically, visfatin was associated with infarct-related artery occlusion, and an association with coronary artery disease was found[343,344]. On the other hand, some authors claim that high visfatin levels, instead of depicting changes in the atherosclerotic process, are more likely to reflect changes in systemic inflammation in patients with cardiovascular disease[313,345]. Although our local and systemic results reinforce the first hypothesis, additional human studies are needed if these data are to be clarified.

Regarding the other adipo/cytokines, the levels of lipocalin-2 and TNFR2 were almost undetectable in the secretome samples. Although resistin is positively correlated with stroke severity[346], ischaemic stroke risk, and poor atherothrombotic stroke prognosis[347,348], the levels of resistin were almost undetectable in the secretome samples in our study. In addition, the levels of adiponectin and IL-6 showed no differences between the two secretome groups analysed. Several studies have described a protective role of adiponectin in cardiovascular diseases[349,350]. Adiponectin levels have been found to be inversely associated with inflammatory markers[351,352]. Regarding atherosclerotic neurologic disease, all stroke subtypes show decreased adiponectin levels[353–355].

Notably, although we did not find differences between the secretome groups, we found higher serum levels of adiponectin in control individuals than in both unstable carotid atherosclerosis and nonatherosclerotic mammary artery patients. Further studies are needed to assess whether adiponectin can have a direct effect *in situ* by inhibiting the formation of an atherosclerotic plaque.

The results of our second study require the following observations. First, we used mammary arteries as control arteries, as previous studies have shown a lower incidence of atherosclerosis. Nonatherosclerotic carotid arteries would be the best choice, but they are unfortunately not available. Second, this study was cross-sectional, so it allowed us to detect correlations but not to formulate predictions. Future prospective studies are necessary to more thoroughly elucidate the association between some molecules, such as visfatin, and atherosclerosis and their potential role as new therapeutic approaches and biomarkers of unstable vs. stable plaques. As this study was conducted by only including unstable plaques, we could only suggest doing further research to confirm this hypothesis.

5.3. Sub - study 3

Title: Levels of secretome and circulating adipo/cytokines in relation to cardiovascular risk factors

The aim of this substudy was to analyse the levels of several pro- and anti-inflammatory adipo/cytokines in serum and in unstable plaque secretome according to the presence of obesity, arterial hypertension, diabetes mellitus, dyslipidaemia and tobacco consumption.

In our study, we found no differences between the levels of most adipo/cytokines in unstable plaque secretomes and circulating concentrations in relation to the presence of arterial hypertension, dyslipidaemia, diabetes mellitus and smoking. However, we found significantly lower plasma adiponectin secretome levels in patients with obesity, as occurs with its circulating levels in obese patients[356–359]. The plasma concentration of

adiponectin has been described to be negatively correlated with body mass index, body fat percentage, fasting insulin concentration and plasma triglycerides but positively correlated with the plasma content of HDL cholesterol[360]. Additionally, surgical treatment of obesity induces an increase in plasma levels of adiponectin, which correlates with weight loss[361]. An experimental model of obesity has shown that low plasma levels of adiponectin induce insulin resistance and atherosclerosis[362].

On the other hand, we found an inverse correlation between adiponectin and IL-6 serum levels, as expected. Adiponectin is an anti-inflammatory molecule, whereas IL-6 is a proinflammatory molecule. Mounting experimental data have revealed that adiponectin exhibits beneficial effects on energy homeostasis and cardiovascular functions that are attributed to its direct modulation of a proinflammatory factor, interleukin-6[363]. On the other hand, an inverse correlation between adiponectin and diastolic blood pressure was demonstrated in our study, indicating that this molecule contributes to the modulation of blood pressure according to some *in vitro* and *in vivo* experiments[364]. Moreover, adiponectin levels are also decreased in people with hypertension. Individuals with low levels of adiponectin present less endothelium-dependent vasodilation, which could explain one of the mechanisms involved in arterial hypertension associated with central obesity[365].

On the other hand, our patients have very high cardiovascular risk and, in this line, were treated with lipid-lowering drugs. However, only 55.5% of them reached the recommended LDL cholesterol levels according to the clinical guidelines (2016; 2021). This means that we should have a more aggressive approach to LDL cholesterol treatment. Most likely, the attitude “lower is best” should be our guide in designing personalized medical treatment for dyslipidaemia. Clinical trials and meta-analyses have established that lowering LDL-C, particularly by statin therapy, reduces the progression of coronary atherosclerosis and the risk of coronary events[366,367].

Finally, adipo/cytokines may affect plasma levels of cholesterol and lipoproteins by modulating the synthesis and secretion of apolipoproteins, lipolytic enzyme activities, or the expression of lipoprotein receptors[368,369].

We wanted to study the relationship between the levels of adipo/cytokines and LDL. However, we could not find any differences between the levels of circulating

adipo/cytokines or secretome levels of adipo/cytokines and the levels of LDL, probably due to limitations in the sample size.

Some limitations should be considered for this substudy, so our results need to be interpreted carefully. First, the research presented has a limited number of patients. Second, we investigated a cohort of men with unstable carotid atherosclerosis who underwent surgical treatment. Because of these limitations, the data cannot be extrapolated to all patients with carotid atherosclerosis or women. Additionally, our research did not include cohort validation, so these results must be validated in large population-based studies.

CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE
Ajla Alibalic

6.1. Study 1

1. Secretome analysis plays an important role in identifying new possible biomarkers in carotid atherosclerosis and in studying the progression and physiology of the disease.
2. By means of iTRAQ labelling spectrometry, some proteomic changes were detected in the secretomes of carotid atherosclerotic plaques compared with nonatherosclerotic mammary arteries.
3. Some proteins involved in focal adhesion, oxidative stress, inflammation, and endothelial dysfunction, among others, were differentially identified in the secretomes of carotid atherosclerotic plaques.
4. The increased proteins in CAP secretomes were neutrophil defensin 1, apolipoprotein E, clusterin and zinc-alpha-2-glycoprotein.
5. Prospective studies are needed to confirm which profile of secreted proteins could be useful targets for diagnosing and treating carotid atherosclerosis.

6.2. Study 2

1. Of the adipo/cytokines analysed in secretomes, visfatin was the only cytokine that was increased in unstable carotid artery plaques compared with nonatherosclerotic mammary artery secretomes.
2. Regarding visfatin serum levels, there were no differences between the unstable carotid atherosclerotic plaque and nonatherosclerotic mammary artery groups. However, visfatin circulating levels in patients with atherosclerosis were increased compared with those in a serum cohort of healthy subjects, suggesting a possible role of visfatin in atherogenic inflammatory diseases.
3. In the studies published to date, the role of visfatin and its precise effect on atherosclerotic plaques remain unclear. The exact and precise mechanisms underlying the biological effects of visfatin require further investigation.

6.3. Sub-study 3

1. Despite the fact that the patients in substudy 3 had a very high cardiovascular risk and were treated with lipid-lowering drugs, only 55.5% of them reached the recommended LDL cholesterol levels according to the clinical guidelines.
2. Circulating adipo/cytokines did not distinguish patients with carotid atherosclerosis and different clinical manifestations of metabolic syndrome.
3. Low adiponectin secretome levels were found in patients with carotid atherosclerosis and obesity.

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UNIVERSITAT ROVIRA I VIRGILI
PROTHEOMIC AND ADIPO/CYTOKINE BIOMARKER ANALYSIS OF UNSTABLE CAROTID ATHEROMA PLAQUE
Ajla Alibalic

ANNEX

UNIVERSITAT ROVIRA I VIRGILI
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Proteomic Profile of Unstable Atheroma Plaque: Increased Neutrophil Defensin 1, Clusterin, and Apolipoprotein E Levels in Carotid Secretome

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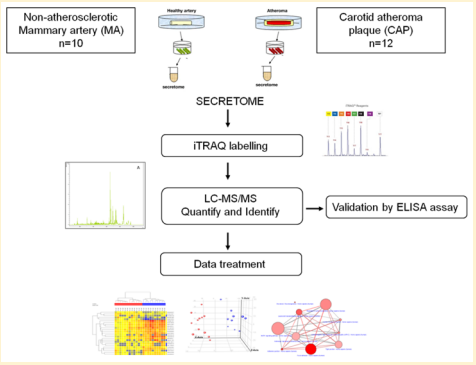
Supporting Information

ABSTRACT: Because of the clinical significance of carotid atherosclerosis, the search for novel biomarkers has become a priority. The aim of the present study was to compare the protein secretion profile of the carotid atherosclerotic plaque (CAP, n = 12) and nonatherosclerotic mammary artery (MA, n = 10) secretomes. We used a nontargeted proteomic approach that incorporated tandem immunoaffinity depletion, iTRAQ labeling, and nanoflow liquid chromatography coupled to high-resolution mass spectrometry. In total, 162 proteins were quantified, of which 25 showed statistically significant differences in secretome levels between carotid atherosclerotic plaque and nondiseased mammary artery. We found increased levels of neutrophil defensin 1, apolipoprotein E, clusterin, and zinc-alpha-2-glycoprotein in CAP secretomes. Results were validated by ELISA assays. Also, differentially secreted proteins are involved in pathways such as focal adhesion and leukocyte transendothelial migration. In conclusion, this study provides a subset of identified proteins that are differently expressed in secretomes of clinical significance.



KEYWORDS: *proteomic, atheroma plaque, secretome, atherosclerosis*

INTRODUCTION

Atherosclerosis, the underlying cause of most clinical cardiovascular events, is a chronic and progressive inflammatory disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Its prevalence is assumed to have risen along with the worldwide increase in obesity and diabetes.[1,2] One of the major clinical manifestations of this inflammatory disease is carotid atherosclerosis, which is prevalent and often clinically silent; however, on many occasions, embolisms can cause acute temporary occlusion of the cerebral circulation, resulting in a transient ischemic attack or stroke.[3] With the increasing incidence of atherothrombosis, due to carotid atheroma plaque rupture, the search for novel therapeutic approaches and biomarkers is a priority. Moreover, new biomarkers should have the potential to improve risk-stratification, diagnosis or treatment. Although this pathology has been extensively studied, its molecular mechanisms are not completely understood.

In this regard, the "omics"-based approaches (such as genomics, transcriptomics, proteomics, and metabolomics) have enabled us to make an overall characterization, at the molecular level, of complex global biological systems and their changes in pathological processes. In this context, proteomics has emerged as a useful tool for analyzing the proteins involved in the pathogenesis of such diseases as atherosclerosis.[4,5] In the search for potential biomarkers, several proteomic study designs have sought to identify new plasma biomarkers for atherosclerosis and its clinical manifestations.[6,7] The results of these studies are often based on relatively few samples and so are unable to address the interindividual variation of candidate biomarkers. Moreover, at the methodological level, direct plasma analysis by proteomic techniques is limited by challenges such as interference from

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highly abundant proteins (e.g., albumin and immunoglobulins), which restricts the number of proteins identified/quantified. In this regard, analysis of the secretome has emerged as a new strategy for studying the atheroma plaque in humans.[8,9] The secretome is the subset of proteins released by a cell or tissue under certain conditions and shows a narrower dynamic range of proteins than serum or plasma, which means less complexity. Furthermore, studies on tissue secretomes more closely resemble the in vivo situation than cell culture workflows. To date, only a few human arterial secretomes (pathological and nondiseased tissue) have been subject to analysis.

The main objective of this study was to identify potential candidate biomarkers for carotid atherosclerosis. Specifically, we analyzed the protein secretion profile of carotid atherosclerotic plaque and nonatherosclerotic mammary secretomes. To evaluate their potential use as atherosclerotic biomarkers, we also studied the functional pathways in which these secreted proteins are involved.

EXPERIMENTAL SECTION

Subjects/Samples

Human carotid atherosclerotic plaques (CAPs) were obtained from patients (men, n = 12) who underwent carotid endarterectomy at the Angiology and Vascular Surgery Unit of the Hospital Universitari Joan XXIII (Tarragona, Spain). Patients with cerebrovascular ischemia and internal carotid artery stenosis >75% were included, diagnosed by color Doppler-assisted duplex imaging and arteriography. The CAP diagnosis was made by an experienced pathologist following the American Heart Association (AHA) guidelines.[10] Mammary arteries were used as nonatherosclerotic control arteries (MA). Segments of mammary arteries (men, n = 10) were obtained during coronary revascularization surgery at the Cardiovascular Surgery Department of the Germans Trias i Pujol Hospital (Badalona, Spain). Patients who had an acute illness, acute or chronic inflammatory or infective diseases, or malignant neoplastic disease were excluded.

Blood samples were obtained from each individual immediately before surgery and after overnight fasting. Serum was obtained by standard protocols and preserved at −80 °C until use.

The institutional review board approved the study. All participants gave written informed consent for participation in medical research.

Clinical and Biochemical Assessments

A complete anthropometric, physical examination and biochemical analysis was carried out on each patient. Body height and weight were measured with the patient standing in light clothes and shoeless. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2). Laboratory studies included glucose, insulin, glycated hemoglobin (HbA1c), total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides, all of which were analyzed using a conventional automated analyzer. Insulin resistance (IR) was estimated using the homeostasis model assessment of IR (HOMA2-IR).[11]

Tissue Processing and Secretome Preparation

Tissue samples were transported from the surgery to the laboratory in phosphate-buffered saline (PBS) at room temperature. Immediately upon arrival, the tissue was transferred to a Petri dish and washed with PBS. Samples were then cut into similar-sized pieces about 3−5 mm in length and transferred to a 12-well tissue culture plate containing 2 mL/well of protein-free Roswell Park Memorial Institute medium (RPMI) (RPMI-1640, Gibco, Invitrogen, NY) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 50 mM HEPES. These procedures were all carried out under a laminar flow hood using sterile equipment. After 24 h of incubation at 37 °C and 5% of CO_2, the media containing the secreted proteins, the so-called secretome, were collected, aliquoted, and stored at −80 °C until analysis. Additionally, a section of each atherosclerotic plaque was placed in formol 10% and further studied by an experienced pathologist from the Hospital Universitari Joan XXIII (Tarragona) following the AHA guidelines.[10]

Protein Preparation

Secretome samples were concentrated by ultrafiltration using 3 kDa Amicon Ultra 0.5 mL filters from Millipore in accordance with the manufacturer's instructions. The protein concentration was determined by Bradford's method. A total of 200 μg total protein per sample was run on a self-poured stacking SDS-PAGE gel (12% resolving gel and 4% stacking gel) at 20 mA/gel. The electrophoresis was stopped when the front dye had barely passed from the stacking gel (4% acrylamide) into the resolving gel (12% acrylamide) and before the protein mixture had separated into discrete bands in the gel. In this way, all proteins were concentrated in a single band, which removed sample contaminants and made reproducibility for comparison easier. This single concentrated band obtained for every sample was stained using Coomassie Brilliant Blue G-250, excised, cut into small pieces, and stored at 4 °C in ultrapure water.

Protein Digestion

Protein digestion was performed according to Shevchenko et al. with minor variations.[12] Gel pieces were destained using 75% acetonitrile, 25% 0.5 M triethylammonium bicarbonate pH 7.9 solution. Then, they were dehydrated by successive washes of 50 mM triethylammonium bicarbonate pH 7.9 solution and acetonitrile and vacuum-dried. Subsequently, proteins were reduced using 5 mM tris(2-carboxyethyl)phosphine (TCEP) in 50 mM triethylammonium bicarbonate pH 7.9 for 1h at 60 °C and alkylated with 3.81 mM Iodoacetamide (IAA) in the same buffer for 30 min at room temperature in the dark. For digestion, samples were incubated with 15.4 ng/μL sequencing-grade trypsin in 50 mM triethylammonium bicarbonate at pH 7.9 overnight at 37 °C. After digestion, the peptides were extracted from the gel by elution in a mixture of 50% acetonitrile, 5% formic acid. Tryptic peptides were dried by SpeedVac and resuspended in 30 μL TEAB 0.5M, pH 7.9.

iTRAQ 8plex Labeling and Purification

Digested samples were labeled using iTRAQ 8-plex reagents (AB SCIEX). To accommodate all of the samples of the study, three iTRAQ 8-plex reagent kits are necessary, and for this reason samples were equally distributed in three subgroups (A−C) containing both CAP and MA samples to minimize technical variability (Table 1). Hereby, one CAP sample (labeled with 113-tag) was used as a normalizer sample for the three iTRAQ groups and to cover all of the proteins present in CAP arteries. Quantification results are expressed as ratios of the different labeling tags versus tag 113, and these ratios were used for statistical purposes.

The iTRAQ labeling reaction was performed according to manufacturer's instructions, incubated at room temperature for 120 min, and stopped by adding water. After peptides were

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Table 1. Sample Labeling and Distribution in Three iTRAQ Groups (A−C)[a]

iTRAQ groups	labeling	sample type
A	113	CAP[b]
	114	MA
	115	CAP
	116	MA
	117	CAP
	118	CAP
	119	MA
	121	CAP
B	113	CAP[b]
	114	MA
	115	MA
	116	CAP
	117	MA
	118	MA
	119	CAP
	121	MA
C	113	CAP[b]
	114	CAP
	115	CAP
	116	CAP
	117	CAP
	118	MA
	119	MA
	121	MA

[a]CAP, carotid atherosclerotic plaque; MA, control mammary artery. [b]This sample was added in all groups to normalize the quantification results that are expressed as ratios of the different labeling tags versus tag 113.

labeled, they were purified by using a SCX column (Strata SCX 55 μm, 70 Å, Phenomenex). Then, samples were desalted and concentrated using a C18 Sep-Pak column (Waters, Bedford, MA) previous to nanoLC−MS/MS analysis.

Nano LC Chromatography and Mass Spectrometry

Peptides were separated onto a C-18 reversed-phase (RP) nanocolumn (75 μm I.D.; 15 cm long; 3 μm particle diameter, Nikkyo Technos, Japan) coupled to a trap nanocolumn (100 μm I.D.; 2 cm long; 5 μm particle diameter, Thermo Fisher Scientific, San Jose, CA).

The three iTRAQ groups (A−C) were analyzed by triplicate so that a higher number of covered proteins and single peptides could be quantified. 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 analyses a flow rate of 300 nL/min was used to elute peptides for real time ionization and peptide fragmentation on an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution = 30 000 FHMW) followed by MS/MS scan (R = 7500 FHMW) from the ten 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 spectra were analyzed using Mascot search engine node (Matrix Science; version 2.4.1.0). Mascot was set up to search Swissprot 2012_03.fastadatabase (v 2.4, 535 248 entries), restricting for human taxonomy (20 255 sequences) and assuming trypsin digestion. Two missed cleavages were allowed and an error of 0.80 Da for fragment ion mass and 10.0 ppm for a parent ion. Oxidation of methionine and acetylation of N-termini was specified as variable modifications, whereas iTRAQ 8-plex and carbamidomethylation of cysteine were set as static modifications. 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.[9] For proteins identified with only one peptide, the fragmentation spectra were visually verified. The analysis carried out by triplicate was considered as replicates on Proteome Discover software and finally a PD Excel report was generated for each iTRAQ group (Supporting Information).

Quantitative Proteome Analysis

Protein quantification was done by calculating the ratios obtained between each iTRAQ mass tag of each unique peptide from a given protein against tag 113. The quantification is the average value of the ratios obtained for the unique peptides for each protein and was normalized based on protein median. These ratios were exported to an Excel spreadsheet for statistical analysis (Supporting Information).

ELISA Assays

Defrosted secretome samples were centrifuged at 3000 rpm and 4 °C for 15 min. Then, they were analyzed by enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions. Neutrophil defensin 1 (EIAab, Wuhan, China), apopoliprotein E (AssayPro, St. Charles, MO), clusterin (RayBiotech, Norcross, GA), and zinc-alpha-2-glycoprotein (BlueGene Biotech., Shangai, China) were determined in secretome samples.

Statistical Analysis

The statistical analysis was performed on Mass Profiler Professional software v.12.1 (Agilent Technologies). For statistical calculations, only these proteins that were quantified in >70% samples under almost one condition (CAP and MA) were considered. Differences between groups were calculated using a Student's t test and to avoid false positives, a multiple testing correction using a Benjamini−Hochberg method was used. p values <0.05 and fold change >1.5 were selected as cutoff values. Principal component analysis (PCA) and hierarchical clustering analysis were performed using Mass Profile Professional software v.12.1 (Agilent Technologies). Pathway analysis was performed using The ConsensusPathDB-human platform. Other statistical calculations were performed using the SPSS software (version 20.0; SPSS, Chicago, IL).

RESULTS

Baseline Characteristics of Subjects

The clinical characteristics and biochemical measurements of the population studied are shown in Table 2. Patients were classified according to the samples obtained: CAP samples from patients undergoing endarterectomy (n = 12) and MA samples from patients undergoing cardiac bypass (n = 10). They were all men.

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Table 2. Clinical Baseline Characteristics of the Cohort Studied[a]

	CAP group (n = 12) mean ± SD	MA group (n = 10) mean ± SD
age (years)	68.83 ± 6.39	67.00 ± 9.99
BMI (kg/m^2)	28.06 ± 3.26	30.64 ± 1.27
glucose (mg/dL)	129.17 ± 49.75	118.88 ± 45.64
HbA1c (%)	6.39 ± 1.07	7.03 ± 1.38
insulin (mUI/L)	8.29 ± 4.95	10.47 ± 6.89
HOMA2-IR	1.01 ± 0.67	1.59 ± 0.97
triglycerides (mg/dL)	112.00 ± 44.67	113.50 ± 27.97
cholesterol (mg/dl)	122.10 ± 36.22	130.07 ± 24.96
HDL-C (mg/dL)	29.08 ± 7.20	24.33 ± 4.76
LDL-C (mg/dL)	70.73 ± 27.49	78.56 ± 19.48

[a]CAP, carotid atherosclerotic plaque; MA, control mammary artery; BMI, body mass index; HbA1c, glycosylated hemoglobin; HOMA2-IR, homeostatic model assessment 2-insulin resistance; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein. Data are expressed as mean ± SD $p < 0.05$ are considered statistically significant. HOMA-2 is calculated using the HOMA Calculator version 2.2.2 (http://www.dtu.ox.ac.uk).

The analyses indicated that patients from the CAP and MA groups are of similar ages. Biochemical parameters showed no significant differences between these groups. 80% of MA patients and 83% of CAP patients received lipid-lowering therapy. As expected, hypolipemiant treatment did not show significant differences in both groups (p = 0.840).

Protein Identification and Quantification

To assess potential biomarkers for atheromatous plaque progression, we focused on the carotid atherosclerotic secretome and compared it with changes in the mammary secretome. Using a nontargeted proteomic approach, we identified and quantified a total of 162 proteins in the human arterial secretome (Table 3). The analyses of the three ITRAQ groups (A−C) were prepared in triplicate so that a higher number of covered proteins and single peptides could be quantified (Figure 1). To study changes in the whole proteome, we applied multivariate statistics (hierarchical clustering analyses and principal component analysis (PCA)). Figure 2A,B shows that we were able discriminate samples from each group, which suggests that atheromatous plaque progression has a specific secretome. Subsequently, we used univariate statistics to define specific potential biomarkers. We found that 25 proteins exhibit statistically significant differences in secretome levels between carotid atherosclerotic plaque and nonatherosclerotic mammary artery (Table 4). The Table shows the role that each protein might play in atherosclerosis according to the literature. Of all the differentially expressed proteins, four had significantly increased levels in CAP secretomes: neutrophil defensin 1, apolipoprotein E, clusterin, and zinc-alpha-2-glycoprotein. Multivariate statistics of the 25 proteins that exhibit differences (Figure 2C,D) also showed good clusterization and sample discrimination for each group, which suggests that atheromatous plaque progression has a specific secretome.

Validation by ELISA Assay

ELISA assays were applied to secretome samples to validate the differential release observed by iTRAQ labeling spectrometry. For the verification, we selected proteins with an increase profile in CAP secretomes. We confirmed that neutrophil defensin 1, apolipoprotein E, and clusterin levels were significantly increased in CAP secretomes than in mammary artery group (Figure 3A−C). Conversely, the levels of zinc-alpha-2-glycoprotein showed no significant differences between the two secretome groups analyzed (Figure 3D).

Pathway Analysis

To determine whether differentially expressed proteins belong to specific pathways, we conducted pathway analysis on proteomic data. The ConsensusPathDB-human Platform, which integrates interaction networks in the *Homo sapiens* proteome, was used to calculate the pathway impact. In brief, this platform collates pathways from several public databases of protein interactions, signaling and metabolic pathways, and gene regulation in humans. We applied our analysis to the following databases: KEGG, Reactome, Netpath, Biocarta, HumanCyc and the pathway interaction database (PID), Signalink, Inoh, Wikipathways, Pharmgkb, Humancyc, and Ehmn. The use of multiple databases enhances coverage and therefore reduces bias. Only pathways showing two proteins or more in the over-representation analyses and a p-value cutoff <0.05 were taken into account (Figure 4). We used this tool for proteins that exhibit statistically significant differences in secretome levels. A list of 10 related pathways was generated. Interestingly, differentially secreted proteins are involved in pathways such as focal adhesion and leukocyte transendothelial migration.

DISCUSSION

Several proteomic approaches have been used to study carotid tissue and understand the mechanisms and progression of atherosclerosis. These studies have identified only a small number of proteins in relatively few samples and so cannot address the interindividual variation of candidate biomarkers.[13,14] To date, the human artery secretome has not been extensively analyzed so the novelty of this study lies in the fact that we analyzed the proteomic changes that occur in secretomes of carotid atherosclerotic plaque (CAP) and nonatherosclerotic mammary artery (MA). Multivariate statistics revealed overall changes in 162 proteins. Furthermore, when univariate statistics were applied, 25 proteins emerged as potential biomarkers of carotid atherosclerotic plaque. The functional analysis performed in our study corroborated the results of recent studies and revealed that selected proteins were involved in pathways related to vascular disease such as focal adhesion and leukocyte transendothelial migration.[15] Our findings show the potential role of secretome evaluation in carotid atherosclerosis, as a means to determine new possible biomarkers and further study the progression and physiology of the disease.

Of the 25 proteins selected, we will first focus on significantly decreased proteins in carotid atherosclerotic plaque secretomes. We classified the decreased proteins in CAP secretomes into different groups on the basis of their potential role in atherosclerosis, as reported in the literature. Although we found that a significant number of the proteins selected were involved in focal adhesion pathway, these proteins are indirectly related to extracellular matrix and they could be implicated in remodelling of atherosclerotic plaque maturation such as proteoglycans.[16] The alterations described could indicate tissue remodelling with a loss of elasticity in the artery. Moreover, we observed proteins involved in smooth muscle cell differentiation, contraction, proliferation, migration, and focal adhesion pathways, which corroborates that vascular smooth muscle cells play a leading role in plaque progression.[17,18] We also found proteins that were involved in protecting against oxidized stress. One of

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Table 3. Protein Identified in Human Carotid Plaque Secretome[a]

Swiss-Prot ID	protein name	coverage %	unique peptides[b]	peptides	PSMs	MW (kDa)	calcd pI
P01834	Ig kappa chain C region	80.19	5	5	119	11.6	5.87
P68871	hemoglobin subunit beta	65.99	5	8	440	16.0	7.28
P0CG05	Ig lambda-2 chain C regions	62.26	3	5	55	11.3	7.24
Q01995	transgelin	55.72	11	11	64	22.6	8.84
P0CG04	Ig lambda-1 chain C regions	46.23	1	3	43	11.3	7.87
P02042	hemoglobin subunit delta	44.22	3	6	222	16.0	8.05
P02768	serum albumin	42.53	28	28	793	69.3	6.28
P08670	vimentin	42.49	16	19	183	53.6	5.12
P69905	hemoglobin subunit alpha	35.21	5	5	146	15.2	8.68
P02647	apolipoprotein A-I	33.71	9	9	49	30.8	5.76
P0CG48	polyubiquitin-C	32.85	2	2	8	77.0	7.66
P02675	fibrinogen beta chain	31.98	12	12	175	55.9	8.27
P60709	actin. cytoplasmic 1	28.80	1	9	103	41.7	5.48
P63261	actin. cytoplasmic 2	28.80	1	9	101	41.8	5.48
P02649	apolipoprotein e	28.39	8	8	30	36.1	5.73
P32119	peroxiredoxin-2	27.78	4	5	27	21.9	5.97
P01860	Ig gamma-3 chain C region	27.59	2	8	162	41.3	7.9
Q96KK5	histone H2A type 1-H	27.34	3	3	11	13.9	10.89
P01598	Ig kappa chain V−I region EU	26.85	2	2	6	11.8	8.44
P02679	fibrinogen gamma chain	26.71	11	11	75	51.5	5.62
P00738	haptoglobin	26.35	10	10	40	45.2	6.58
P01765	Ig heavy chain V−III region TIL	26.09	2	2	6	12.3	9.13
P02787	serotransferrin	25.50	16	16	125	77.0	7.12
P01766	Ig heavy chain V−III region BRO	25.00	2	2	30	13.2	6.57
P01620	Ig kappa chain V−III region SIE	24.77	2	2	14	11.8	8.48
P01857	Ig gamma-1 chain C region	23.94	2	7	157	36.1	8.19
P02743	serum amyloid P-component	23.77	5	5	18	25.4	6.54
P01625	Ig kappa chain V−IV region Len	21.93	2	2	2	12.6	7.93
P09493	tropomyosin alpha-1 chain	21.48	3	8	25	32.7	4.74
P60174	triosephosphate isomerase	21.33	5	5	9	30.8	5.92
P01871	Ig mu chain C region	19.91	8	8	37	49.3	6.77
P07951	tropomyosin beta chain	19.72	2	7	27	32.8	4.7
P63267	actin. gamma-enteric smooth muscle	19.41	1	7	78	41.8	5.48
P00338	L-lactate dehydrogenase A chain	19.28	4	6	28	36.7	8.27
P59665	neutrophil defensin 1	19.15	2	2	10	10.2	6.99
P18206	vinculin	19.14	18	18	51	123.7	5.66
P01859	Ig gamma-2 chain C region	19.02	2	6	142	35.9	7.59
P01700	Ig lambda chain V−I region HA	18.75	2	2	11	11.9	8.91
P00915	carbonic anhydrase 1	18.01	5	5	54	28.9	7.12
P01876	Ig alpha-1 chain C region	17.85	2	5	61	37.6	6.51
P01717	Ig lambda chain V−IV region Hil	17.76	1	1	1	11.5	6.51
P01617	Ig kappa chain V−II region TEW	17.70	1	2	6	12.3	6
P62805	histone H4	17.48	2	2	2	11.4	11.36
P05452	tetranectin	17.33	3	3	3	22.5	5.67
P01593	Ig kappa chain V−I region AG	16.67	1	1	6	12.0	5.99
P02792	ferritin light chain	16.57	3	3	13	20.0	5.78
P21333	filamin-A	15.72	30	30	139	280.6	6.06
P06310	Ig kappa chain V−II region	15.04	1	2	6	14.7	9.25
P01877	Ig alpha-2 chain C region	15.00	1	4	50	36.5	6.1
P06733	alpha-enolase	14.75	6	6	33	47.1	7.39
P02671	fibrinogen alpha chain	14.67	12	12	119	94.9	6.01
Q06830	peroxiredoxin-1	14.57	2	3	14	22.1	8.13
P01023	alpha-2-macroglobulin	14.18	18	18	101	163.2	6.46
O43707	alpha-actinin-4	13.83	5	10	33	104.8	5.44
P01009	alpha-1-antitrypsin	13.64	6	6	66	46.7	5.59
P01024	complement C3	13.47	19	19	78	187.0	6.4
P02545	prelamin-A/C	13.25	8	8	30	74.1	7.02
P01011	alpha-1-antichymotrypsin	13.24	5	5	41	47.6	5.52
P06396	gelsolin	12.15	9	9	44	85.6	6.28
P08294	extracellular superoxide dismutase [Cu−Zn]	12.08	2	2	3	25.8	6.61
P62942	peptidyl-prolyl cis−trans isomerase FKBP1A	12.04	1	1	1	11.9	8.16

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Table 3. continued

Swiss-Prot ID	protein name	coverage %	unique peptides[b]	peptides	PSMs	MW (kDa)	calcd pI
P02760	protein AMBP	11.93	3	3	10	39.0	6.25
P12814	alpha-actinin-1	11.88	3	8	25	103	5.41
P51884	Lumican	11.83	4	4	24	38.4	6.61
P05109	protein S100-A8	11.83	1	1	3	10.8	7.03
P37802	transgelin-2	11.56	2	2	5	22.4	8.25
P07737	profilin-1	11.43	1	1	4	15.0	8.27
P30086	phosphatidylethanolamine-binding protein 1	10.70	1	1	3	21.0	7.53
P07195	L-lactate dehydrogenase B chain	10.48	1	3	5	36.6	6.05
P04075	fructose-bisphosphate aldolase A	10.44	4	4	19	39.4	8.09
P10909	clusterin	9.80	3	3	16	52.5	6.27
P02144	myoglobin	9.74	1	1	6	17.2	7.68
P01008	antithrombin-III	9.70	4	4	9	52.6	6.71
P02652	apolipoprotein A-II	9.00	1	1	1	11.2	6.62
P07339	cathepsin D	8.98	3	3	5	44.5	6.54
P06703	protein S100-A6	8.89	1	1	11	10.2	5.48
P02766	transthyretin	8.84	1	1	3	15.9	5.76
P04406	glyceraldehyde-3-phosphate dehydrogenase	8.66	2	2	11	36.0	8.46
P60660	myosin light polypeptide 6	8.61	1	1	3	16.9	4.65
P31949	protein S100-A11	8.57	1	1	10	11.7	7.12
P02763	alpha-1-acid glycoprotein 1	8.46	2	2	2	23.5	5.02
P35542	serum amyloid A-4 protein	8.46	1	1	2	14.7	9.07
P01605	Ig kappa chain V−I region Lay	8.33	1	1	5	11.8	7.96
P00325	alcohol dehydrogenase 1B	8.27	3	3	6	39.8	8.29
P04433	Ig kappa chain V−III region VG (Fragment)	7.83	1	1	7	12.6	4.96
P04083	annexin A1	7.8	2	2	2	38.7	7.02
P36955	pigment epithelium-derived factor	7.66	3	3	6	46.3	6.38
P17661	desmin	7.66	1	4	21	53.5	5.27
P52565	Rho GDP-dissociation inhibitor 1	7.35	1	1	1	23.2	5.11
P14618	pyruvate kinase isozymes M1/M2	7.34	3	3	11	57.9	7.84
P06888	Ig lambda chain V−I region EPS	7.34	1	1	11	11.4	9.29
P23528	cofilin-1	7.23	1	1	1	18.5	8.09
O60814	histone H2B type 1-K	7.14	1	1	2	13.9	10.32
P07355	annexin A2	7.08	2	2	4	38.6	7.75
P0C0L4	complement C4-A	7.00	10	10	19	192.7	7.08
P00558	phosphoglycerate kinase 1	6.71	2	2	7	44.6	8.1
P13797	plastin-3	6.67	3	4	9	70.8	5.6
P12883	myosin-7	6.67	11	11	18	223	5.8
P00441	superoxide dismutase [Cu−Zn]	6.49	1	1	6	15.9	6.13
P02790	hemopexin	6.28	3	3	13	51.6	7.02
P06702	protein S100-A9	6.14	1	1	2	13.2	6.13
P61626	lysozyme C	6.08	1	1	7	16.5	9.16
P23284	peptidyl-prolyl cis−trans isomerase B	6.02	1	1	1	23.7	9.41
P08758	annexin A5	5.94	2	2	9	35.9	5.05
P78417	glutathione S-transferase omega-1	5.81	1	1	1	27.5	6.6
P08107	heat shock 70 kDa protein 1A/1B	5.77	3	3	12	70.0	5.66
P63104	14-3-3 protein zeta/delta	5.71	1	1	2	27.7	4.79
P06732	creatine kinase M-type	5.51	1	1	3	43.1	7.25
P02794	ferritin heavy chain	4.92	1	1	2	21.2	5.55
P08571	monocyte differentiation antigen CD14	4.8	1	1	1	40.1	6.23
P00450	ceruloplasmin	4.79	4	4	12	122.1	5.72
Q6NZI2	polymerase I and transcript release factor	4.62	1	1	1	43.4	5.6
P04004	vitronectin	4.6	2	2	2	54.3	5.8
P05155	plasma protease C1 inhibitor	4.6	2	2	3	55.1	6.55
P02751	fibronectin	4.44	7	7	28	262.5	5.71
P29401	transketolase	4.33	2	2	2	67.8	7.66
P62258	14-3-3 protein epsilon	4.31	1	1	2	29.2	4.74
P04264	keratin. type II cytoskeletal 1	4.04	3	3	6	66.0	8.12
P25311	zinc-alpha-2-glycoprotein	4.03	1	1	3	34.2	6.05
P68366	tubulin alpha-4A chain	4.02	1	1	3	49.9	5.06
P00918	carbonic anhydrase 2	3.85	1	1	1	29.2	7.4
P08603	complement factor H	3.82	4	4	8	139.0	6.61

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Table 3. continued

Swiss-Prot ID	protein name	coverage %	unique peptides[b]	peptides	PSMs	MW (kDa)	calcd pI
P02774	vitamin D-binding protein	3.8	1	1	1	52.9	5.54
Q01518	adenylyl cyclase-associated protein 1	3.79	1	1	2	51.9	8.06
Q13642	four and a half LIM domains protein 1	3.72	1	1	2	36.2	8.97
P08238	heat shock protein HSP 90-beta	3.59	2	2	8	83.2	5.03
P26038	moesin	3.47	2	2	2	67.8	6.4
P07451	carbonic anhydrase 3	3.46	1	1	1	29.5	7.34
P19827	interalpha-trypsin inhibitor heavy chain H1	3.4	2	2	5	101.3	6.79
Q14624	interalpha-trypsin inhibitor heavy chain H4	3.33	3	3	5	103.3	6.98
P13796	plastin-2	3.03	1	2	9	70.2	5.43
P51911	calponin-1	3.03	1	1	3	33.1	9.07
P40925	malate dehydrogenase. cytoplasmic	2.99	1	1	2	36.4	7.36
P40121	macrophage-capping protein	2.87	1	1	4	38.5	6.19
A6NIK2	leucine-rich repeat-containing protein 10B	2.74	1	1	1	32.7	7.36
P20774	mimecan	2.68	1	1	1	33.9	5.63
P02749	beta-2-glycoprotein 1	2.61	1	1	5	38.3	7.97
P55058	phospholipid transfer protein	2.43	1	1	1	54.7	7.01
P37837	transaldolase	2.37	1	1	1	37.5	6.81
P18428	lipopolysaccharide-binding protein	2.29	1	1	1	53.3	6.7
P06727	apolipoprotein A-IV	2.27	1	1	2	45.4	5.38
P04217	alpha-1B-glycoprotein	2.22	1	1	1	54.2	5.86
P00488	coagulation factor XIII A chain	2.19	1	1	1	83.2	6.09
P16930	fumarylacetoacetase	1.91	1	1	1	46.3	6.95
P02765	alpha-2-HS-glycoprotein	1.91	1	1	2	39.3	5.72
P01042	kininogen-1	1.86	1	1	3	71.9	6.81
P04003	C4b-binding protein alpha chain	1.84	1	1	1	67.0	7.3
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	1.83	1	1	1	54.6	5.07
P00751	complement factor B	1.83	1	1	4	85.5	7.06
P07602	proactivator polypeptide	1.72	1	1	2	58.1	5.17
P04040	catalase	1.71	1	1	1	59.7	7.39
P02748	complement component C9	1.61	1	1	5	63.1	5.59
Q05682	caldesmon	1.51	1	1	1	93.2	5.66
O75083	WD repeat-containing protein 1	1.32	1	1	2	66.2	6.65
P00747	plasminogen	1.23	1	1	1	90.5	7.24
Q8N436	inactive carboxypeptidase-like protein X2	1.19	1	1	2	85.8	6.87
P00734	prothrombin	1.13	1	1	2	70.0	5.9
Q9Y490	talin-1	0.94	2	2	3	269.6	6.07
P35579	myosin-9	0.82	1	1	1	226.4	5.6
P04114	apolipoprotein B-100	0.77	3	3	8	515.3	7.05
P01031	complement C5	0.6	1	1	3	188.2	6.52
P07996	thrombospondin-1	0.6	1	1	1	129.3	4.94

[a]Proteins included in this Table met all identification criteria laid out in the Experimental Methods. Proteins are arranged according to the % coverage of target protein by identified peptides. [b]Unique peptide: the number of identified unique peptides subjected to quantification for the given protein. PSM, peptide spectrum match; MW, molecular weight; calcd pI, predicted isoelectric point.

them, extracellular superoxide dismutase (ecSOD), is an antioxidant enzyme in vascular tissues. It is known that it plays a major role in modulating blood pressure and could prevent endothelial dysfunction.[19,20] Another antioxidant enzyme was peroxiredoxin 2 (Prdx2), which regulates proinflammatory responses, vascular remodelling, and overall oxidative stress.[21,22] A recent study showed that Prdx 2 deficiency in apolipoprotein E-deficient (ApoE/) mice accelerates atherosclerosis by increasing the infiltration of immune cells into plaques.[23] We also observed decreased levels of carbonic anhydrase 1 (CA1) in CAP secretomes. Aamand et al.[24] showed that CA1 plays a role in the generation of vasoactive nitric oxide (NO), which suggests that low levels of CA1 induced impaired NO production and, subsequently, endothelial dysfunction. Finally, in agreement with Martin-Ventura et al.,[25] we also detected a decrease in heat shock protein-70 1A (Hsp70 1A) levels in CAP secretomes. Hsp70 is a chaperone with antiinflammatory and antiapoptotic properties that improve the viability of stressed vascular smooth muscle cells.[26]

The increased proteins in CAP secretomes were neutrophil defensin 1, apolipoprotein E, clusterin, and zinc-alpha-2-glycoprotein. As far as neutrophil defensin 1 is concerned, recent studies have revealed that polymorphonuclear neutrophils participate in the development of atherosclerotic lesions.[27−29] During inflammation, large amounts of intracellular proteins such as neutrophil defensins are released from the activated polymorphonuclear neutrophils.[30,31] These proteins have been found in human atherosclerotic arteries.[32,33] Apart from its role in the inflammation underlying atherosclerosis, this protein inhibits LDL metabolism and fibrinolysis and promotes Lp(a) binding.[34] In addition, some authors[35] have reported that neutrophil defensins could induce leukocyte transendothelial migration and increased foam cell formation. In this regard,

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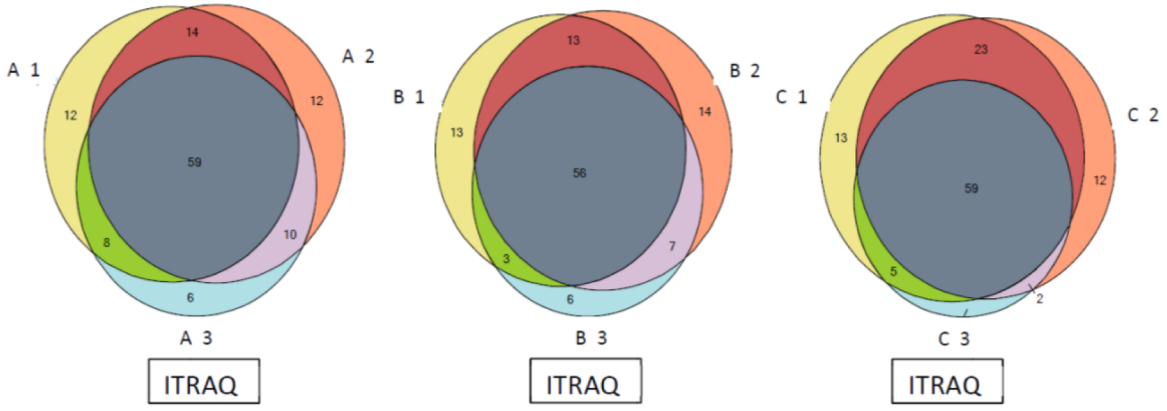

Figure 1. Analysis of the three ITRAQ groups (A–C) prepared in triplicate so that a higher number of covered proteins and unique peptides were quantified.

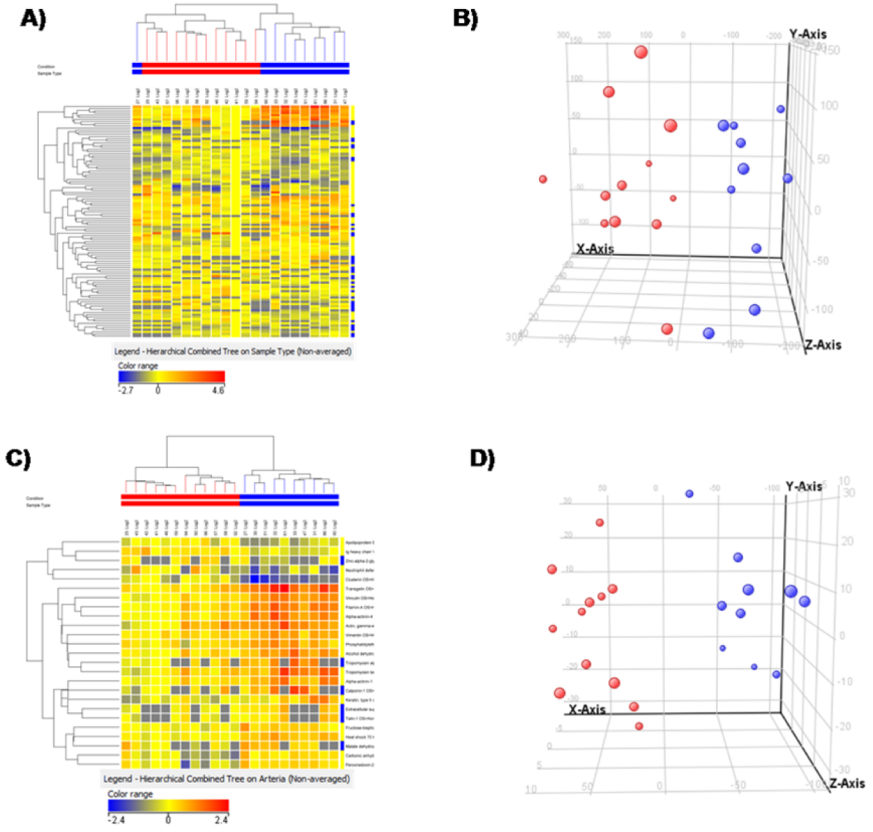

Figure 2. Proteomic analysis in CAP and MA secretome samples. Heat map representation of hierarchical clustering of molecular features found in each sample of two groups. (Panel A represents quantified proteins and Panel C shows statistically significant proteins.) The scale from −2.4 blue (low abundance) to +2.4 red (high abundance) represents this normalized abundance in arbitrary units. The CAP group is represented as red samples and MA secretomes are represented as blue samples. Tridimensional principal component analysis (PCA) was used before (B) and after (D) Student's unpaired *t* test. The CAP group is represented as red spots and the MA group is represented as blue spots. CAP, carotid atherosclerotic plaque; MA, mammary artery secretome.

according to our results, neutrophil defensin 1 is another key molecule in the pathogenesis of atherosclerosis.

Zinc-alpha-2-glycoprotein (ZAG) is a newly identified adipokine, and it has not yet been studied on atheroma plaque. In agreement with our findings, several studies have shown that serum ZAG levels are upregulated in a variety of pathological processes involving endothelial dysfunction;[36−38] however, immunoassay data did not show similar profiles as the ones obtained in spectrometry experiments. This protein has been quantified by mass spectrometry using only one unique peptide. In this sense, it has been described in the literature that this sequence could be easily modified,[39,40] and these changes could affect the quantification by mass spectrometry or ELISA detection. Otherwise, the quantitative changes detected by mass spectrometry referred to a very minor difference.

As far as the role of clusterin in atherosclerotic lesions is concerned, other authors studying the atheroma plaque have reported similar results to ours.[41,42] Indeed, clusterin is upregulated in a wide variety of clinical situations including aging, diabetes, and atherosclerosis.[43,44] Although the role of

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Table 4. Secretome proteins differ significantly between CAP and MA group[a]

Swiss-Prot ID	protein	gene name	role in atherosclerosis[b]	ratio	*P* value	effect
P51911	calponin-1	CNN1	SMC differentiation	0.30	0.0024	decreased
P09493	tropomyosin-1 alpha	TPM1	SMC differentiation	0.35	<0.001	decreased
P00325	alcohol dehydrogenase 1B	ADH1B		0.41	<0.001	decreased
Q01995	transgelin	TAGLN	SMC differentiation	0.21	<0.001	decreased
P07951	tropomyosin beta chain	TMP2	SMC differentiation	0.31	<0.001	decreased
P21333	filamin-A	FLNA	focal adhesion	0.31	<0.001	decreased
P30086	phosphatidylethanolamine-binding protein 1	PEBP1		0.50	0.0013	decreased
P63267	actin, gamma-enteric smooth muscle	ACTG2	focal adhesion	0.40	0.0036	decreased
P18206	vinculin	VCL	focal adhesion	0.33	<0.001	decreased
P08670	vimentin	VIM	focal adhesion	0.58	0.0027	decreased
O43707	alpha-actinin-4	ACTN4	focal adhesion	0.30	<0.001	decreased
P12814	alpha-actinin-1	ACTN1	focal adhesion	0.42	<0.001	decreased
P08107	heat shock protein-70 1A	HSPA1A	inflammation	0.48	<0.001	decreased
P04075	fructose-bisphosphate aldolase A	ALDOA		0.59	0.0018	decreased
P00915	carbonic anhydrase 1	CA1	endothelial dysfunction	0.49	0.0013	decreased
P32119	peroxiredoxin-2	PRDX2	antioxidant enzyme	0.51	0.0014	decreased
P40925	malate dehydrogenase	MDH1		0.56	0.0091	decreased
P04264	keratin, type II cytoskeletal 1	KRT1		0.47	0.0091	decreased
P08294	extracellular superoxide dismutase	SOD3	antioxidant enzyme	0.58	<0.001	decreased
Q9Y490	talin-1	TLN1	focal adhesion	0.63	0.0099	decreased
P59665	neutrophil defensin 1	DEFA1	leukocyte migration	1.91	0.0366	increased
P01766	Ig heavy chain V−III region BRO			1.71	<0.001	increased
P02649	apolipoprotein E	APOE	lipid transport	1.61	0.0018	increased
P10909	clusterin	CLU	lipid transport	2.28	<0.001	increased
P25311	zinc-alpha-2-glycoprotein	AZGP1	endothelial dysfunction	1.66	0.0099	increased

[a]Student's unpaired *t* test was used. To avoid false positives, a multiple testing correction using a Benjamini−Hochberg method was also used. *P* value <0.05 and fold change >1.5 were selected as cutoff values. [b]Role in atherosclerosis refers to data reported in the literature.

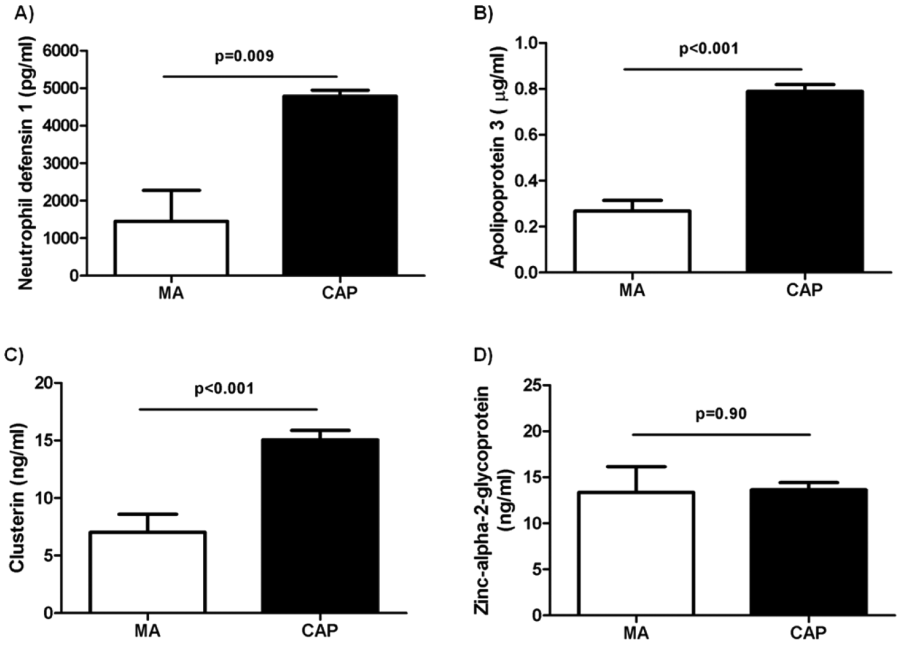

Figure 3. Protein validation by ELISA assays. Neutrophil defensin 1 (A), apolipoprotein E (B), clusterin (C), and zinc-alpha-2-glycoprotein (D) levels in secretome samples.

clusterin in atherosclerosis remains unknown, several studies have shown that clusterin distribution in human aorta is increased as this disease progress.[42,45]

Human apolipoprotein E (apoE) plays an important role in the metabolism of lipids, including cholesterol, and promotes the clearance of atherogenic lipoproteins such as very low density lipoprotein (VLDL) and chylomicron remnants from the circulation.[46] In addition, the expression of apoE in macrophages plays an important antiatherogenic role by promoting cholesterol efflux from cells in the arterial wall.[47] Recent studies have indicated that the regulation of apoE expression from macrophages is under the control of several signaling pathways. ApoE secretion was stimulated by several molecules such as apolipoprotein A-I, HDL, ATP binding cassette transporter

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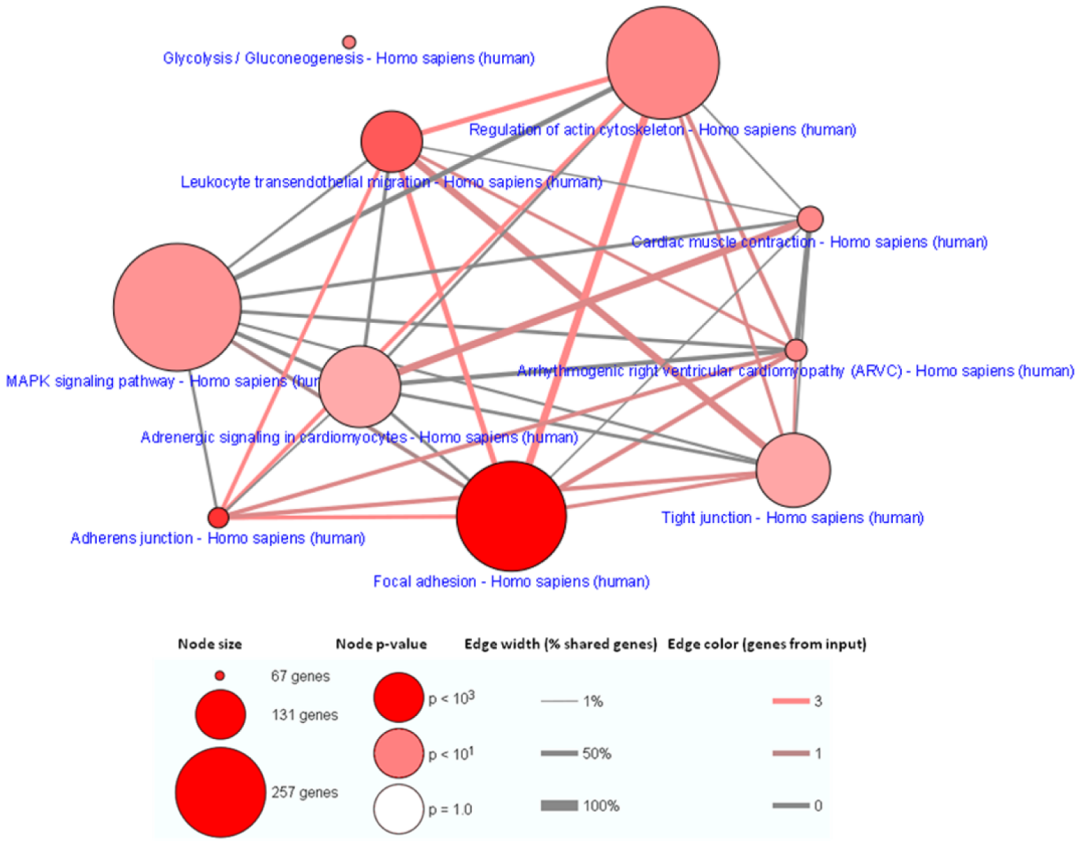

Figure 4. ConsensusPathDB-human platform integrating interaction networks in *Homo sapiens* proteome was used to calculate pathway impact. Pathway analysis was conducted on proteins that exhibit statistically significant differences in secretome levels. Minimum overlap with input protein = 2 and *p*-value cutoff <0.05.

(ABCA1), protein kinase A, intracellular calcium, and microtubular network.[48] In addition, the function of apoE is linked with both proinflammatory and antiinflammatory cytokines.[49] Therefore, under pathological conditions, the expression of apoE could be upregulated by macrophages in the arterial wall. In our study, one explanation for high levels of apoE in carotid atherosclerotic secretomes finding could be that this molecule may act as a protective factor.

We should point out the following drawbacks of our study. First, we used mammary arteries as controls because previous studies have shown a lower incidence of atherosclerosis. Although nondiseased carotid arteries would be the best choice, unfortunately they were not available. Second, our results were obtained in homogeneous groups of men. Therefore, they cannot be extrapolated to other population groups with mixed genders or associated metabolic diseases. Third, our patients were in treatment. Although we cannot exclude the influence of drugs on our results, this study design provided information on real clinical situation during necessary lipid lowering therapy. Finally, another limitation of the study was the sample size due to the difficulty in obtaining human artery samples. Despite that, our highly sensitive method evidences a very wide spectrum of proteins in CAP and MA secretomes that was verified by ELISA assays. Further studies would be useful to validate our findings.

CONCLUSIONS

Our results indicate the potential role of secretome analysis in carotid atherosclerosis as a means to identify new possible biomarkers and further study the progression and physiology of the disease. By means of iTRAQ labeling spectrometry, some proteins involved in focal adhesion, oxidative stress, inflammation, and endothelial dysfunction, among others, were differentially identified. Prospective studies are needed to confirm what profile of secreted proteins could be useful targets for diagnosing and treating carotid atherosclerosis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00936.

List of proteins identified/quantified in human secretome of the carotid atherosclerotic plaque and non-atherosclerotic mammary artery. Proteome Discover report Excel files containing the identified proteins for each iTRAQ group (A) and its relative quantification (XLSX)

List of proteins identified/quantified in human secretome of the carotid atherosclerotic plaque and non-atherosclerotic mammary artery. Proteome Discover report Excel files containing the identified proteins for each iTRAQ group (B) and its relative quantification (XLSX)

List of proteins identified/quantified in human secretome of the carotid atherosclerotic plaque and non-atherosclerotic mammary artery. Proteome Discover report Excel files containing the identified proteins for each iTRAQ group (C) and its relative quantification (XLSX)

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Author Contributions

G.A. and T.A. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BMI, body mass index; CVD, cardiovascular disease; HbA1c, glycosylated hemoglobin; HDL-C, high density lipoprotein; HOMA2-IR, homeostasis model assessment of insulin resistance; CAP, carotid atherosclerotic plaque; MA, mammary artery; LDL-C, low density lipoprotein; PBS, phosphate-buffered saline; RPMI, protein-free Roswell Park Memorial Institute medium

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RESEARCH ARTICLE Open Access

Adipo/cytokines in atherosclerotic secretomes: increased visfatin levels in unstable carotid plaque

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Abstract

Background: Novel pro-inflammatory and anti-inflammatory derivatives from adipose tissue, known as adipokines, act as metabolic factors. The aim of this study was to analyse the secreted expression of different adipo/cytokines in secretomes of unstable carotid atherosclerotic plaque versus non-atherosclerotic mammary artery.

Methods: We evaluated the secretion levels of adiponectin, visfatin, lipocalin-2, resistin, IL-6 and TNFR2 by ELISA in human secretomes from cultured unstable carotid atherosclerotic plaque ($n = 18$) and non-atherosclerotic mammary artery ($n = 13$). We also measured visfatin serum levels in patients suffering from atherosclerosis and in a serum cohort of healthy subjects ($n = 16$).

Results: We found that visfatin levels were significantly increased in unstable carotid atherosclerotic plaque secretome than in non-atherosclerotic mammary artery secretome. No differences were found with regard the other adipo/cytokines studied. Regarding visfatin circulating levels, there were no differences between unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery group. However, these visfatin levels were increased in comparison to serum cohort of healthy subjects.

Conclusions: Of all the adipo/cytokines analysed, only visfatin showed increased levels in secretomes of unstable carotid atherosclerotic plaque. Additional human studies are needed to clarify the possible role of visfatin as prognostic factor of unstable carotid atherosclerotic plaque.

Keywords: Atheroma plaque, Secretome, Visfatin, Atherosclerosis, Adipo/cytokines

Background

Carotid artery stenosis as a causative factor of ischemic strokes or transient ischemic attacks constitutes a major therapeutic target. Since obesity is considered a risk factor associated to atherosclerosis, a lot of research over recent years has tried to gain greater insights into the link between atherosclerosis and adipose tissue that has been described as an endocrine organ that secretes a wide variety of proteins called adipokines [1–3]. Currently, it is well known that adipokines play a relevant role in the pathophysiology of cardiovascular diseases (CVDs) [4–6]. These molecules can act as enzymes, hormones or growth factors in the modulation of insulin resistance and the metabolism of fats and glucose, and, therefore, have an indirect effect on atherosclerosis [7]. To note, visceral fat accumulation associated with adipokine dysregulation affects on both atherosclerotic plaque development and plaque disruption. When the advanced plaque becomes unstable, rupture can occur and may be provided by the adipokine-induced prothrombotic and inflammatory state [8, 9]. During the last century, the epidemic of obesity and CVDs has lead to intense research into the role of adipokines in obesity and atherosclerosis [6]. However, further research is necessary to elucidate more thoroughly the

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pathophysiological pathways that underlie the association between adipokines and atherosclerosis, and their potential role as new therapeutic approaches and biomarkers.

Recently, the study of the secretome has emerged as a new strategy for analysing the formation of atherosclerotic plaques in humans [10]. The secretome is the sub-set of proteins released by a cell or tissue under certain conditions and shows a narrower dynamic range of proteins than serum or plasma, which means less complexity. Furthermore, studies on tissue secretome more closely resemble the in vivo situation than cell culture workflows.

The aim of this study was to analyse the presence of several adipo/cytokines with different profiles, pro- and anti-inflammatory: adiponectin, visfatin, lipocalin-2, resistin, IL-6 and TNFR2, and compare their differential expression in the secretome of an unstable carotid atherosclerotic plaque with the secretome in a non-atherosclerotic mammary artery. Moreover, in order to study whether the differences observed in adipo/cytokine levels were only a local effect or if they were also reflected in serum, we measured circulating levels in the group of patients suffering from atherosclerosis and in a serum group of healthy subjects.

Methods

Subjects/Samples

The study was approved by the institutional review board "Comitè d'Ètica d'Investigació Clínica, Hospital Universitari de Sant Joan de Reus" (10-04-29/4proj3). All participants gave written informed consent for participation in medical research.

Human unstable carotid atherosclerotic plaques were obtained from patients (men, $n = 18$) who underwent carotid endarterectomy at the Angiology and Vascular Surgery Unit of the Hospital Universitari Joan XXIII (Tarragona, Spain). Patients with cerebrovascular ischemia and internal carotid artery stenosis >75 % were included, diagnosed by colour Doppler assisted duplex investigation and arteriography. The diagnosis of unstable carotid atherosclerotic plaques was made by an experienced pathologist following the American Heart Association (AHA) guidelines [11].

Mammary arteries were used as non-atherosclerotic control arteries. Segments of mammary arteries (men, $n = 13$) were obtained during coronary revascularisation surgery at the Cardiovascular Surgery Department of the Hospital Germans Trias i Pujol (Badalona, Spain). Patients who had an acute illness, acute or chronic inflammatory or infective diseases, or malignant neoplastic disease were excluded.

We also recruited serum cohort of healthy men ($n = 16$), whose medical history included no cardiovascular event. Subjects who had an acute illness, acute or chronic inflammatory or infective diseases, or malignant neoplastic disease were excluded.

All subjects recruited were male. Blood samples were obtained from each individual immediately before surgery and after overnight fasting. Serum was obtained by standard protocols and preserved at −80 °C until use.

Clinical and biochemical assessments

A complete anthropometric, biochemical, and physical examination was carried out on each patient. Body height and weight were measured with the patient standing in light clothes and shoeless. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2). Laboratory studies included glucose, insulin, glycated haemoglobin (HbA1c), total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides, all of which were analysed using a conventional automated analyser. Insulin resistance (IR) was estimated using the homeostatic model assessment of IR (HOMA2-IR) [12].

Arterial tissue culture – obtaining the secretome

Tissue samples were transported from the surgery to the laboratory in phosphate buffered saline (PBS) at room temperature. Immediately upon arrival, the tissue was transferred to a Petri dish and washed with PBS. For mammary arteries, we removed the adventitia before incubation of the intima-media. All samples were then cut into similar-sized pieces about 3–5 mm in length and transferred to a 12-well tissue culture plate containing 2 ml/well of protein-free Roswell Park Memorial Institute medium (RPMI) (RPMI-1640, Gibco, Invitrogen, N.Y, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 50 mM HEPES. These procedures were all carried out under a laminar flow hood using sterile equipment. After 24 h of incubation at 37 °C and 5 % of CO_2, the media containing the secreted proteins, the so-called secretome, were collected, aliquoted and stored at −80 °C until used for analysis.

Additionally, a section of each atherosclerotic plaque was placed in phormol 10 % and further studied by an experienced pathologist from the Hospital Universitari Joan XXIII (Tarragona) following the AHA guidelines [11].

Measurements of adipo/cytokines levels

Defrosted secretome samples were centrifuged at 1200 rpm and 4 °C for 15 min. Then, they were analysed by enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions. Adiponectin (EMD Millipore, St. Charles, MI, USA), visfatin (AdipoGen, San Diego, CA, USA), lipocalin-2 (R&D Systems Inc, Minneapolis, USA), resistin (Biovendor, Modrice, Czech Republic), IL-6 (R&D Systems Inc, Minneapolis, USA) and TNFR2 (BioSource Europe, Nivelles, Belgium) were

determined in secretome samples. Only visfatin was determined in both secretome and serum samples. The adiponectin assay sensitivity was 0.2 ng/ml, and intra-assay and inter-assay coefficients of variation (CV) were 3.4 and 5.7, respectively. The visfatin assay sensitivity was 30 pg/ml, and intra-assay and inter-assay CV were 5.63 and 5.92, respectively. The lipocalin-2 assay sensitivity was 0.012 ng/ml, and intra-assay and inter-assay CV were 3.7 and 6.5, respectively. The resistin assay sensitivity was 0.012 ng/ml, and intra-assay and inter-assay CV were 5.9 and 7.6, respectively. The IL-6 assay sensitivity was 0.039 pg/mL, and intra-assay and inter-assay CV were 7.4 and 7.8, respectively. Finally, sTNF-RII assay sensitivity was 0.1 ng/ml, and intra-assay and inter-assay CV were 4.9 and 7.9, respectively. In order to normalize adipo/cytokine measurements, total protein concentration was assessed using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Statistical analysis

All the values reported are expressed as mean ± standard deviation (SD) and were analysed using the Windows SPSS/PC+ statistical package (version 22.0; SPSS, Chicago, IL, USA). Differences between groups were calculated using Student's t test or one-way ANOVA analysis. The strength of association between variables was calculated using Pearson's method for parametric variables and the Spearman Rho correlation test for non-parametric contrasts. *P* values <0.05 were considered to be statistically significant.

Results

Characteristics of the population studied

The general characteristics and biochemical measurements of the population studied are shown in Table 1. Subjects were classified according to the samples obtained: serum group of healthy subjects ($n = 16$), non-atherosclerotic mammary artery samples from patients undergoing coronary artery bypass ($n = 13$) and unstable carotid atherosclerotic plaque samples from patients undergoing endarterectomy ($n = 18$). The three groups studied had similar BMIs and they were all men. Anthropometrical and biochemical parameters showed no significant differences between non-atherosclerotic mammary artery and unstable carotid atherosclerotic plaque groups. As expected, carotid atherosclerotic plaque and mammary artery patients showed significant lower lipid profile because these subjects were taking lipid-lowering drugs. Table 1 also shows that the levels of glucose and HbA1c were significantly higher in the carotid atherosclerotic plaque and mammary artery group than in serum group of healthy subjects.

Table 1 Anthropometric measurements and metabolic analysis of the population studied

	Serum group of healthy subjects ($n = 16$) Mean ± SD	Coronary patients with non-atherosclerotic mammary artery ($n = 13$) Mean ± SD	Unstable carotid atherosclerotic plaque group ($n = 18$) Mean ± SD
Age (years)	52.47 ± 13.25	65.08 ± 10.48	69.17 ± 7.44[b]
BMI (kg/m^2)	32.19 ± 11.76	29.39 ± 3.36	27.74 ± 3.13
Glucose (mg/dl)	91.31 ± 14.24	129.19 ± 55.44[a]	123.56 ± 45.37[b]
HbA1c (%)	4.97 ± 0.39	6.81 ± 1.39[a]	6.29 ± 1.07[b]
Insulin (mUI/L)	12.76 ± 16.19	11.76 ± 7.15	7.21 ± 4.93
HOMA2-IR	1.62 ± 1.95	1.59 ± 0.97	1.01 ± 0.67
Triglycerides (mg/dL)	115.02 ± 71.38	110.33 ± 27.84	103.00 ± 40.61
Cholesterol (mg/dl)	192.33 ± 37.81	128.34 ± 23.92[a]	118.81 ± 34.54[b]
HDL-C (mg/dL)	49.13 ± 10.35	23.71 ± 4.64[a]	28.50 ± 6.98[b]
LDL-C (mg/dL)	120.17 ± 39.06	78.56 ± 19.48[a]	69.78 ± 26.21[b]

Subjects were classified according to the samples obtained: serum group of healthy subjects ($n = 16$), non-atherosclerotic mammary artery samples from patients undergoing coronary artery bypass ($n = 13$) and unstable carotid atherosclerotic plaque samples from patients undergoing endarterectomy ($n = 18$). *BMI* body mass index, *HbA1c* glycosylated haemoglobin, *HOMA2-IR* homeostatic model assessment 2- insulin resistance, *HDL-C* high density lipoprotein, *LDL-C* low density lipoprotein. Data are expressed as mean ± SD. $p < 0.05$ are considered statistically significant. [a]refer to the statistically significant differences between coronary patients with non-atherosclerotic mammary artery and serum group of healthy subjects. [b]refer to the statistically significant differences between unstable carotid plaque and serum group of healthy subjects. HOMA-2 is calculated using the HOMA Calculator version 2.2.2 (http://www.dtu.ox.ac.uk)

Adipo/cytokine levels in the secretome

To study the local role of adipo/cytokines in atherosclerosis, we evaluated the presence of adiponectin, visfatin, lipocalin-2, resistin, IL-6 and TNFR2 in secretomes of the unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery tissue cultures (Table 2). Of all the molecules analysed, visfatin was the only adipo/cytokine that was differently expressed in secretome

Table 2 Adipo/cytokine levels in secretome samples

	Unstable carotid atherosclerotic plaque group ($n = 18$) Mean ± SD	Coronary patients with non-atherosclerotic mammary artery ($n = 13$) Mean ± SD
Visfatin (ng/μg total protein)	0.100 ± 0.017	0.046 ± 0.012[a]
Adiponectin (μg/μg total protein)	0.311 ± 0.039	0.369 ± 0.096
IL-6 (pg/μg total protein)	0.048 ± 0.012	0.039 ± 0.008
Lipocalin-2 (ng/μg total protein)	0.009 ± 0.002	0.008 ± 0.001
Resistin (ng/μg total protein)	0.001 ± 0.001	0.001 ± 0.001
TNFR2 (ng/μg total protein)	0.007 ± 0.002	0.005 ± 0.001

IL-6 interleukin 6, *TNFR2* tumor necrosis factor receptor 2. Data are expressed as mean ± SD. $p < 0.05$ are considered statistically significant. [a]refer to the statistically significant differences between unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery group

samples. Specifically, visfatin levels were significantly higher in the unstable carotid atherosclerotic plaque than in non-atherosclerotic mammary artery secretomes (Table 2, $p = 0.021$). Conversely, the levels of adiponectin and IL-6 showed no significant differences between the two secretome groups analysed. Finally, the levels of lipocalin-2, resistin and TNFR2 were almost undetectable in the secretome samples. No significant correlations between adipo/cytokines were found.

Circulating Visfatin and adipocytokines levels in serum

As only differences in situ visfatin levels were observed and in order to study whether these differences were only a local effect or if they were also reflected in serum, we measured visfatin circulating levels in the group of patients suffering from atherosclerosis and in a serum group of healthy subjects ($n = 16$). Fig. 1 shows that there were no differences between unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery group. However, visfatin serum concentration was higher in both unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery groups than in the serum cohort of healthy subjects ($p = 0.037$ and $p = 0.001$; respectively). This difference remained significant after adjusting for age, BMI and glucose metabolism.

Then, we analysed the circulating levels of two adipo/cytokines with different profile, pro- and anti-inflammatory (IL-6 and adiponectin, respectively). We found that adiponectin circulating levels were significantly higher in the serum group of healthy subjects (29.20 ± 8.42) than unstable carotid atherosclerotic plaque group (11.23 ± 1.69, $p = 0.025$) and non-atherosclerotic mammary artery patients (9.26 ± 2.35, $p = 0.031$). However, we observed no differences in the circulating levels of IL-6 between groups. No significant correlations between these adipo/cytokines and visfatin were found.

Discussion

To date, the knowledge of the local action of the adipo/cytokines expressed in secretomes of atherosclerotic plaques is under development. In fact, most secretome studies have been carried out using proteomic techniques [10, 13]. The aim of this study was to analyse the presence of several adipo/cytokines with different profiles, pro- and anti-inflammatory in the secretome of an unstable carotid atherosclerotic plaque with the secretome in a non-atherosclerotic mammary artery. The main finding was that visfatin levels were significantly higher in the unstable carotid atherosclerotic plaque than in non-atherosclerotic mammary artery secretomes, suggesting a possible link between visfatin and unstable carotid atherosclerotic plaque.

Visfatin is a ubiquitous adipokine that is produced in adipose tissue, bone marrow, skeletal muscle, and liver with a physiological role not completely understood [14–16]. In the context of metabolic diseases, most studies have focused on increased circulating levels and adipose tissue expression of visfatin [17, 18]. Also, it was initially proposed as a clinical marker of atherosclerosis, endothelial dysfunction and vascular damage [19]. Also,

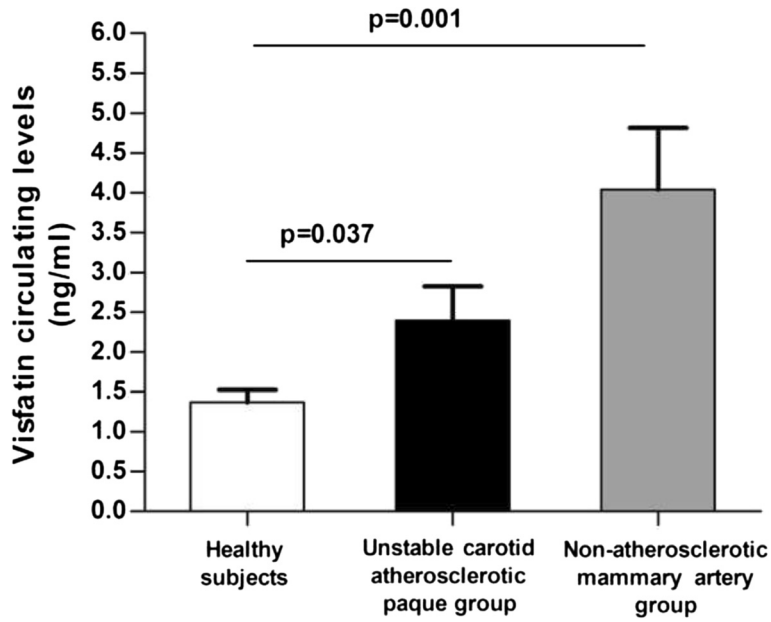

Fig. 1 Visfatin serum levels in different groups: unstable carotid atherosclerotic plaque group ($n = 18$), coronary patients with non-atherosclerotic mammary artery ($n = 13$) and serum cohort of healthy subjects ($n = 16$). $p < 0.05$ were considered statistically significant

visfatin is an active player promoting vascular inflammation, atherosclerosis development and progression, and plaque destabilization [19–21]. As far as the local effect of visfatin on atherosclerotic lesions is concerned, other authors studying the atheroma plaque directly have reported similar results to ours [22–24]. One of the studies that has most similarities with ours has reported that visfatin should be regarded as an inflammatory mediator, localized to foam cell macrophages within unstable atherosclerotic lesions, which potentially plays a role in plaque destabilization [22]. Moreover, Zhou et al. have reported that visfatin induces cholesterol accumulation in macrophages and accelerates the process of atherosclerosis [23]. Apart from the pro-inflammatory effect of visfatin on atherosclerosis, other possible direct mechanisms have been reported: promotion of smooth muscle cell proliferation, alteration of the expression and the activity of matrix metalloproteinases, greater atherosclerotic plaque vulnerability and impairment of endothelial vasodilatory responses [25–28]. The mechanism underlying elevated levels of visfatin in secretomes from unstable atherosclerotic plaques are not known nowadays. However, Dahl et al. have reported enhanced visfatin expression in symptomatic atherosclerotic plaques and also that visfatin had a combined ability of increasing TNF-α as well as to respond with increased expression on TNF-α stimulation. Therefore, this bidirectional interaction between TNF-α and visfatin could represent a pathogenic loop on unstable atherosclerotic lesions [22]. Another study has further demonstrated that the regulation of visfatin in macrophages is related to pro-atherogenic stimuli, including hypoxia, TNF-α and ox-LDL [29].

Although some authors have identified that visfatin is a potential inflammatory mediator in plaque destabilization [22], in our study we only included patients with cerebrovascular ischemia and unstable carotid atherosclerotic plaque. Therefore, we could not compare visfatin levels between stable and unstable carotid plaque secretomes. Although the biological mechanisms involving visfatin in the pathogenesis of atherosclerosis are not well-established, visfatin seems to be an active factor in the development and progression of atherosclerosis through its effects on cytokine and chemokine secretion, macrophage survival, leukocyte recruitment by endothelial cells, vascular smooth muscle inflammation and plaque destabilization [19, 23].

Regarding circulating levels, we found higher visfatin serum concentrations in patients suffering carotid atherosclerosis and coronary patients with non-atherosclerosis mammary artery who underwent coronary revascularisation surgery. In our study, mammary arteries have been used as control arteries, since previous studies have shown its lower incidence of atherosclerosis [30, 31]. However, it is important to remark that although mammary artery patients have non-diseased arterial secretome, they have atherosclerotic coronary disease. Likewise, in recent years, several studies have established positive associations between enhanced circulating visfatin levels and atherogenic inflammatory diseases, which suggest a possible role of visfatin in the atherosclerosis pathogenesis [19, 32]. Specifically, visfatin was associated with infarct-related artery occlusion, and also an association with coronary artery disease was found [33, 34]. On the other hand, some authors claim that high visfatin levels, instead of depicting changes in the atherosclerotic process are more likely to reflect changes in systemic inflammation in patients with cardiovascular disease [19]. Although our local and systemic results reinforce the first hypothesis, additional human studies are needed if these data are to be clarified.

Regarding the other adipo/cytokines, levels of lipocalin-2, resistin and TNFR2 were almost undetectable in the secretome samples. In addition, the levels of adiponectin and IL-6 showed no differences between the two secretome groups analysed. Several studies have described a protective role of adiponectin in cardiovascular diseases [35]. Although we did not find differences between secretome groups, we found higher serum levels of adiponectin in control individuals than in both unstable carotid atherosclerosis and non-atherosclerotic mammary artery patients. Further studies are needed to assess whether adiponectin can have a direct effect in situ by inhibiting the formation of an atherosclerotic plaque. Although IL-6 has been regarded as a pro-inflammatory cytokine that is classically associated with endothelial dysfunction and atherosclerosis [36], we found no differences in secretome or circulating levels between the group of patients suffering from atherosclerosis and the mammary artery. The reason for these discrepancies could be the dissimilarities of the studied populations.

Our results require the following observations. First, we have used mammary arteries as control arteries, since previous studies have shown a lower incidence of atherosclerosis. However, non-atherosclerotic carotid arteries would be the best choice but, unfortunately, are not available. Second, this study was cross-sectional, so it allowed us to detect correlations but not to formulate predictions. Future prospective studies are necessary to elucidate more thoroughly the association between some molecules such as visfatin and atherosclerosis, and also their potential role as new therapeutic approaches and biomarkers of unstable vs. stable plaques. As our study was conducted only including unstable plaques, we could only suggest doing further in order to confirm this hypothesis.

Conclusions

Of all the adipo/cytokines analysed in secretomes, visfatin was the only adipo/cytokine that was higher in unstable carotid artery plaque than in non-atherosclerotic

mammary artery secretomes. Regarding visfatin serum levels, there were no differences between unstable carotid atherosclerotic plaque and non-atherosclerotic mammary artery groups. However, these visfatin circulating levels were increased in comparison to serum cohort of healthy subjects. Prospective studies are needed to confirm whether visfatin could play a role as prognostic factor in the stability of atherosclerotic plaque.

Abbreviations

BMI, body mass index; HbA1c, glycosylated hemoglobin; HDL-C, high density lipoprotein; HOMA2-IR, homeostatic model assessment method insulin resistance; IL-6, interleukin 6; LDL-C, low density lipoprotein; PBS, phosphate buffered saline; RPMI, protein-free Roswell Park Memorial Institute medium; TNFR2, tumor necrosis factor receptor 2

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Authors' contributions

TA and GA were responsible for the study design and finalized the report; EGJ and AB contributed to data interpretation and drafted the manuscript; MC, AA and CA performed the general biochemical determinations; SM participated in technical analysis; VMP, EH, XR, MLC participated in patients selection; CR participated in the overall design. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Universitary Hospital Sant Joan de Reus (10-04-29/4proj3). All participants gave written informed consent for participation in medical research.

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