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En el día de hoy 17/07/17, reunido el tribunal de evaluación nombrado por la Comisión de Estudios Oficiales de Posgrado y Doctorado de la Universidad y constituido por los miembros que suscriben la presente Acta, el aspirante defendió su Tesis Doctoral, elaborada bajo la dirección de JOSÉ SABÁN RUIZ.

Sobre el siguiente tema: *C3 CONVERTASE AS A NOVEL BIOMARKER OF CARDIOVASCULAR PATHOLOGY, INSULIN RESISTANCE AND ENDOTHELIAL DYSFUNCTION*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[8] de (**no apto, aprobado, notable** y **sobresaliente**): SOBRESALIENTE

Alcalá de Henares, 17 de Julio de 2017

EL PRESIDENTE

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Con fecha 24 de julio de 2017 la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado, a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

- [x] Conceder la Mención de "Cum Laude"
- [ ] No conceder la Mención de "Cum Laude"

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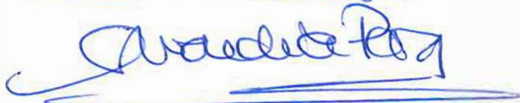

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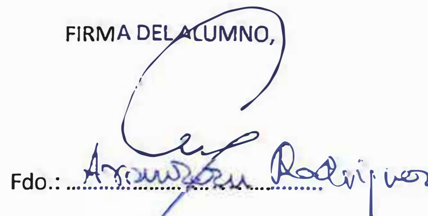

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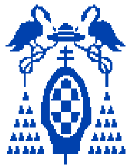
[8] La calificación podrá ser "no apto" "aprobado" "notable" y "sobresaliente". El tribunal podrá otorgar la mención de "cum laude" si la calificación global es de sobresaliente y se emite en tal sentido el voto secreto positivo por unanimidad.



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Comprobado que el expediente académico de D./Dª ____ reúne los requisitos exigidos para la presentación de la Tesis, de acuerdo a la normativa vigente, y habiendo presentado la misma en formato: ☐ soporte electrónico ☐ impreso en papel, para el depósito de la misma, en el Servicio de Estudios Oficiales de Posgrado, con el nº de páginas: __________ se procede, con fecha de hoy a registrar el depósito de la tesis.

Alcalá de Henares a _____ de ___________________ de 20_____

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PhD Program: Medicina (RD 778/1998)

C3 CONVERTASE AS A NOVEL BIOMARKER OF CARDIOVASCULAR PATHOLOGY, INSULIN RESISTANCE AND ENDOTHELIAL DYSFUNCTION

Doctoral Thesis presented by

ARÁNZAZU RODRÍGUEZ GUERRERO

Director:

PROF DR JOSÉ SABÁN RUIZ

Alcalá de Henares, 2017

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Abstract

Introduction: Atherothrombotic disease is the leading aetiology of mortality in the Western countries. Its main cause is not hypercholesterolemia but the Metabolic Syndrome (MetS). In the last three decades MetS evolved from a quintet (hyperglycaemia, hypertension, hypertriglyceridemia, low HDL and waist) to become an octet named Cardiometabolic Syndrome (CMS), where the triad, consisting of inflammation/metaflammation, oxidative stress (OS) and endothelial dysfunction (ED) is essential to understand its impact at the vascular level. Insulin resistance (IR) is located at the heart of both syndromes. From the inflammatory point of view, the most used marker has been the CRP with the inconvenience of being highly unspecific. Finding new markers has become the researchers' goal for the last years. The present work confirms that the C3 convertase in plasma can be useful as a marker and possibly as a pathogenic factor, which if confirmed, would postulate C3 as a target of future preventive strategies.
Objectives: Assess the behaviour of C3 convertase, key enzyme that controls the complement cascade in relation to MetS, CMS including ED, IR and cardiovascular (CV) risk in a population referred to cardiometabolic secondary care.
Methodology: An observational retrospective cross-sectional study was performed on a random cohort of adult individuals from Madrid. Specific cardiometabolic factors were measured as part of the clinical routine of the Cardiometabolic Unit. CV risk was calculated by REGICOR formula. IR was estimated by HOMA-IR formula in a non-exogenous insulin replacement group. Subjects were stratified by C3 complement quartiles to assess the distribution of the cardiometabolic markers. Multivariable regression analysis was applied to identify predictors of C3 variability.
Results: A total of n=374 subjects were selected (53.60±14.80 yr. old, 44.9% females), where 65% were hypertensive, 42% hyperglycaemic and 35% MetS. C3 complement levels were associated with: **1.** MetS: MetS diagnosis, each single MetS criterion, proportional number of MetS criteria and new MetS criteria (p<0.001). **2.** CMS: inflammation (CPR, fibrinogen, p<0.001) and metaflammation (Adiponectin, IL-6, p≤0.05), ED (TPA, PAI-1, VCAM, p≤0.050) and OS showed tendency (TBARS, p=0.084). **3.** IR: HOMA (p<0.001). **4.** CV risk: factors and CV risk (p<0.001). All of these correlations were independent of age and gender. Most of the previous variables were also correlated with C3 quartiles. Hypertriglyceridemia had the highest impact on C3 differing from clinical guidelines according to multivariate analysis (p<0.001).
Conclusions: In relation with the MetS quintet, C3 resulted a strong predictor of each criteria, C3 was proportional to number of MetS criteria and described a possible arbitrary threshold between two and three criteria suggesting earlier CV prevention through equal risk. In relation to the CMS octet, C3 also predicted strongly inflammation/metaflamation, ED and showed tendency with OS. In this order, hypertriglyceridemia, CRP, HTA, waist, HOMA and hyperglycaemia resulted predictors of the 34% of C3 complement variability by multivariate analysis. The association with HOMA may influence the results of this study but can not explain all the correlations. This study suggested that simultaneous evaluation of CRP and C3 would increase the intrinsic reliability of CRP as required in precise Cardiometabolic Medicine.

UNESCO International Nomenclature: 3207.04 Cardio-vascular pathology, 3207.02 Atherosclerosis, 3201.01 Clinical Pathology.

Keywords: C3 complement, C3 convertase, atherotrhombosis, MetS, IR, CV risk, CRP, ED, inflammation, atherosclerosis, fibrinogen, OS, REGICOR, HOMA, multi-regression analysis.

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UNESCO International Nomenclature / Áreas de Clasificación de la UNESCO

	Áreas Clasificación de la UNESCO	UNESCO International Nomenclature
3207.04	Patología Cardiovascular	Cardio-vascular pathology
3207.02	Aterosclerosis	Atherosclerosis
3201.01	Patología Clínica	Clinical Pathology

List of acronyms

A1c	Glycosilated haemoglobin	IR	Insulin Resistance
AAI	Ankle-Brachial pressure Index	IST	Insulin-suppression Test
AHA	American Heart Association	ITT	Insulin tolerance test
AHEAD	Action for Health in Diabetes	IVGTT	Intravenous Glucose tolerance test
AIDS	Acquired Immune Deficiency Syndrome	LADA	Latent Autoinmune Diabetes of Adults
AIT	Arterial Intima Thickness	LDLc	Low Density Lipoprotein
AMI	Acute Myocardial Infarction	Lpa	Lipoprotein-a
ATP-III	Adult Treatment Panel III	LPS	Lipopolysaccharides (Endotoxin)
BMI	Body Mass Index	MAP	Mean Arterial Pressure
BP	Blood Pressure	MBL	Mannose-binding Lectin
C3	C3 Complement	MCP-1	Monocyte chemotactic protein-1
CDC	Centers for Disease Control and Prevention,	MDA	Malondialdehyde
CHD	Coronary Heart Disease	MetS	Metabolic Syndrome
CMS	Cardio-metabolic Syndrome	MI	Metabolic Inflexibility
CPCs	Circulating Progenitor Cells	NADP	Nicotinamide Adenine Dinucleotide Phosphate
CRP	C-Reactive Protein		
CV	Cardiovascular	NCEP	National Cholesterol Education Program
CVD	Cardiovascular Disease	NEIR	Non-exogenous insulin replacement
DASH	Dietary Approach to Stop Hypertension	NHANES	National Health and Nutrition Examination Survey
DBP	Diastolic Blood Pressures		
DM	Diabetes Mellitus	NHLBI	National Heart Lung and Blood Institute
DNA	Deoxyribonucleic Acid	OGTT	Oral Glucose Tolerance Test
ECMU	Endothelium and Cardiometabolic Medicine Unit	NO	Nitric Oxide
		OMRON	Auto inflate Blood Pressure (Mod705 CP®)
ECPs	Endothelial Progenitor Cells	OS	Oxidative Stress
ECS	European Cardiovascular Society	PAI-1	Plasminogen Activator Inhibitor
ED	Endothelial Dysfunction	PAT	Peripheral Arterial Tone
EDTA	Ethylenediaminetetraacetic acid	PMH	Personal Medical History
ELISA	Enzyme-Linked Immunosorbent Assay	PH	Measure of the acidity or alkalinity
eNOS	Endothelial Nitric-oxide Synthase	QUICKI	Quantitative insulin sensitivity check index
ER	Endoplasmic Reticulum	REGICOR	Population Register of Cardiac Disease
ERS	Endoplasmic Reticulum Stress	RHI	Reactive Hyperemia Index
ESC	European Society of Cardiology	RNA	Ribonucleic Acid
FDA	Food & Drug Administration	ROS	Reactive Oxygen Species
FMD	Flow Mediated Dilatation	SAH	S-Adenosyl-L-homocisteine
FSIVGTT	Frequently Sampled IV Glucose Tolerance Test	SBP	Systolic Blood Pressures
		SD	Standard Desviation
GCP	Good Clinical Practice	SMC	Smooth Muscle Cell
GFR	Glomerular Filtration Rate	SPSS	Computer Statistical Program
HAA	Hitachi Automatic Analyser	T1DM	Diabetes Mellitus type 1
HBP	High Blood Pressure (hypertension)	T2DM	Diabetes Mellitus type 2
Hcy	Homocysteine	TAC	Total Antioxidant Capacity
HDLc	High Density Lipoprotein	TAP	Total Antioxidant Capacity of plasma
HIV	Human Immunodeficiency Virus	TBA	Thiobarbituric Acid
HOMA	Homeostatic Model Assessment	TBARS	Thiobarbituric Acid Reactive Substances
HR	Heart Rate	TGs	Triglycerides
HRP	Horseradish Peroxidase	TMB	3,3',5,5'-Tetramethylbenzidine
ICAM	Intercellular Adhesion Molecule	TNF-α	Tumor Necrosis Factor alpha
ICH	International Committee on Harmonization	tPA	Tissue Plasminogen Activator (PLAT)
IGF-1	Insulin-like Growth factor 1	uPA	Urokinase
IGT	Impaired glucose tolerance	UPR	Unfolded Protein Response
IL-6 IL-10	Interleukin-6 Interleukin-10	VCAM-1	Vascular Cell Adhesion Protein 1
IMMULITE	Chemistry Analyzer developed by DPC	WHO	World Health Organization
IDF	International Diabetes Federation	VLDL	Very-low-density lipoprotein
IPPF	International Professional Practices Framework		

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1. INTRODUCTION

Introduction

Cardiovascular diseases (CVDs) are the primary aetiology of morbidity and mortality in the world. An estimated 17.3 million people died from CVDs in 2008, representing 30% of world mortality, being the main cause of death. By 2030, more than 23 million people will die annually from CVDs. According to WHO data collected in 2013, 2.8 million deaths are caused every year by obesity and being overweight, being the fifth leading risk for global deaths [1]. Moreover, overweight and obesity account for 44% of the diabetes burden, 23% of the coronary heart disease and between 7% and 41% of certain types of cancer.

Even though over 80% of CVD deaths take place in low and middle-income countries, we have to keep in mind that the CVDs retain ultimate responsibility for 41% of the total mortality in western countries [2]. CVDs entail more than 2 million deaths per year in the European Union. One of every eight European men and one of every 17 European women will die before 65 years of age due to cardiovascular reasons. They are also the main cause of invalidity and decreased quality of life.

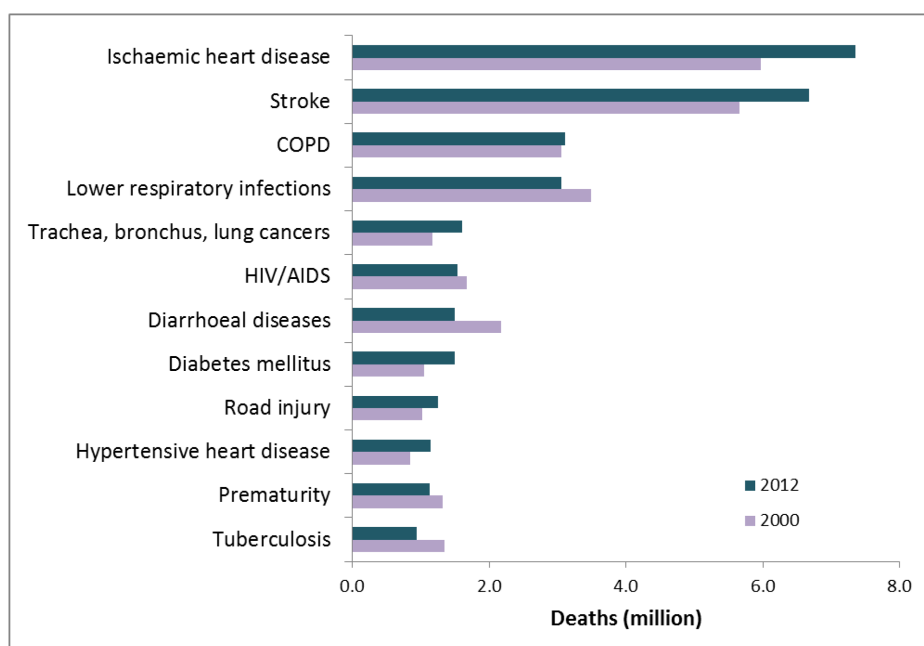

Fig. 1.1 Modified from comparison of leading causes of deaths, Global, 2000 and 2012, health statistics and information systems, World Health Organization.

It is known worldwide that most CVDs can be prevented by acting on the classical cardiovascular risk factors. These risk factors are mainly divided into three principal

groups such as behavioural, metabolic and other risk factors. Human behaviour is related to tobacco use, physical inactivity, alcohol abuse and unhealthy diet (rich in fat, sugar, calories and salt). The metabolic group agglomerates overweight and raised blood pressure, hyperglycaemia and hyperlipidaemia. Finally, the third heterogenic group conglomerates genetic disposition, psychological factors, age, gender and other risk factors such as hyperuricemia, hyperhomocysteinemia, hyperferritinemia, raised CRP, many others which we will study [3].

Medical professionals cannot forget that the governmental institutions, health organizations and also our professional duty is to promote, prevent and control the already known risk factors. Nevertheless, our duty as healthcare professionals and researchers has to advance a step further, pointing out the primary prevention and unknown risk factors such as C3 convertase.

The underlying pathologies of CVDs are mainly atherosclerosis or atherothrombotic disease, and at great distance rheumatic heart diseases, congenital heart diseases, disorders of the heart muscle, electrical conduction system of the heart and heart valve diseases [3, 4].

The leading pathogenic role is played by **atherosclerosis**, which develops over the years and is usually advanced by the time symptoms occur, generally in middle age. Acute coronary and cerebrovascular events frequently occur suddenly, and are often fatal before medical care can be given. Modification of risk factors has been shown to reduce mortality and morbidity in people with diagnosed or undiagnosed cardiovascular disease[5].

Atherosclerosis has moved from being considered a deposit and degenerative disease to be understood as a chronic inflammatory disease, which will develop an acute clinical event as a result of atheromatous plaque's disruption [4, 6]. Nowadays, the atheroma formation with superimposed thrombosis represents the underlying pathophysiological process in the development of cerebrovascular diseases, deep venous and pulmonary thrombosis and acute coronary syndromes (ACS), namely unstable angina, acute myocardial infarction and sudden death, arising due to partial or complete occlusion of the artery [7]. Atheroma formation can begin in infancy and progresses over decades until eventual weakening of the cap results in a plaque which is vulnerable to rupture and thrombosis [8]. On the other hand, there is mounting evidence to support a central role for inflammatory processes in the pathogenesis of CVDs, and similar inflammatory processes are also related to the pathogenesis of type two diabetes mellitus (T2DM), supporting the common soil hypothesis.

The inflammation does not start alone; it belongs to a triad with oxidative stress and endothelial dysfunction instead. The outcome of the chronic damage may trigger atheroma rupture and thromboembolic occlusion [9]. Therefore, the latest researching pathways are directed towards the inflammatory, oxidative stress and endothelial dysfunction biomarkers as the first step in the atheroma formation chain. The endothelium has emerged as the key regulator of vascular homeostasis. In that, it has not merely a barrier function but also acts as an active signal transducer for circulating influences that modify the vessel wall phenotype [10]. Alteration in endothelial function precedes the development of morphological atherosclerotic changes and can also contribute to lesion development and later clinical complications.

The biomarkers will play a crucial role in the diagnosis monitoring and treatment of the early as well as late stages of the CVDs development. These markers would represent the key factors to predict the cardiometabolic risk inherited to a person from his childhood, allowing us to develop a real primary prevention [11]. Elevation in markers of inflammation predicts outcomes of patients with acute coronary syndromes, independently of myocardial damage. their diagnostic role in basal conditions have not been sufficiently studied. Low-grade chronic inflammation, as indicated by levels of the inflammatory marker C-reactive protein (CRP), prospectively defines risk of atherosclerotic complications, but CRP is not an specific marker, condition that reduces its value in the clinical practice.

Therefore, the aim of this thesis will be the correlation between C3 convertase as key component of the intricate inflammatory cascade complement pathways, endothelial damage, reticulum and metabolic stress and cardiovascular risk. Nowadays, the secondary prevention with our current treatments such as statins or diuretics reduces the probabilities of CVDs when the arterial damage was already initiated. Then the question should be what if we could tackle this risk in early steps.

2. KNOWLEDGE REVIEW

2. Knowledge review

2.1. Atherosclerosis as low-grade inflammatory process

Atherosclerosis has moved from being considered a deposit degenerative disease to be understood as a chronic inflammatory disease which complications lead to acute clinical episodes when the atherosclerotic plaques are disrupted or become occlusive [12].

Firstly, we should differentiate between two key concepts, arteriosclerosis and atherosclerosis. Both processes jointly participate in the cardiovascular disease development.

Arteriosclerosis corresponds to a diffuse process characterised by a progressive loss of elasticity and increment of the arterial wall stiffness thereof. Therefore, arteriosclerosis is a generalized age-related process, which is presented as a diffuse thickening of the endothelial and medial layers of the arteries.

Atherosclerosis is a focal process of atheroma deposit localised in certain territories, such as proximal and distal thirds of the coronary arteries and carotid arteries bifurcation.

The formation of atherosclerotic plaque or atheromatous disease was first defined by Marchand in the early nineteenth century as an arterial lesion characterised by sclerotic fibrous tissue enveloping a central soft atheroma. This term remains as the cornerstone of the CVD but has evolved to a multifactorial background. The atheroma's basic components are lipid deposit and cellular and collagen progressive proliferation.

The atherogenic process starts at early age. The turnover of lipoproteins in and out of the subendothelium is a normal physiological process. But the generation of lipids' deposits is conditioned by the inflow of these lipoproteins and their resistance to oxidative modifications involve a change in their biological behaviour. The modified low-density lipoproteins (LDLs), especially oxidized, are cytotoxic and harmful to the endothelium, chemotactic for monocytes and also inhibit macrophages' migration. Oxidized LDLc induce the expression of tumour necrosis factor α (TNF-α) or interleukin 1 (IL-1), which favour the endothelial expression of adhesion endothelial molecules [13].

Monocytes phagocytise these modified LDLc becoming foam cells. If the circulating lipoprotein level exceeds the phagocytic capacity of monocytes, macrophages exert a chemotactic function on monocytes and smooth muscle cells of the arterial wall which become macrophages.

The lysis of these lipid-laden cells releases cholesterol crystals and catalytic enzymes contained in cell debris to the cellular interspace, triggering a local inflammatory process. This early precursor stage of the atheromatous plaque stage can be observed from the childhood as fatty streaks. The atheroma may be macroscopically visible at the end of puberty.

Due to the perpetuation of the pathogenetic mechanism outlined above a defensive endothelial wall mechanism through connective tissue reaction stabilises the plaque and generates a fibroathenoma. Rokitansy in 1852 postulated his inlayed theory where the initial mechanism was the fibrotic and thickening intima reaction which was secondarily loaded with lipid content. However, for Virchow (1856), the lipid infiltration determined the plaque formation. Both theories considered the endothelium as a passive agent of an undergoing fibrotic process. It was needed a century to reach the integration of both theories and consider atherogenesis as an inflammatory response to a endothelial aggression by Ross [14], where mechanical, chemical, biological and immunological stimuli develop endothelial dysfunction and damage. Endothelial injury triggers a response of monocyte infiltration, alters the endothelial antithrombotic ability, fibrinolytic properties and even vasomotor response.

These facts facilitate the platelet aggregation. Fuster [15] classified the endothelial damage into three stages:

- Stage I: functional but not morphological damage due to local changes in the blood flow (flexure areas, hypertension, arterial branches) or inside the wall itself (ischemia vasa vasorum). When these lesions remain the release of proteolytic enzymes, free radicals, etc. the endothelial damage leads to stage two.
- Stage II: endothelial denudation with intimal damage keeping intact the internal elastic lamina. In this phase platelet aggregation and fibrin deposit, secondary to activation of the coagulation cascade, may lead to thrombosis [5].
- Stage III: intima and media layers are involved Inflammation is considered the key regulatory process and predisposes to thrombogenesis. There is an early

involvement of both monocytes and macrophages during atherogenesis. These inflammatory monocytes express high levels of beta transforming growth factor (TGF-β), tumour necrosis factor (TNF), interleukin (IL-1) and angiogenic mediators, such as vascular endothelial growth factor (VEGF). Mast cells produce various mediators such as histamine, leukotrienes, chymase, trytasa, interleukin-6 (IL-6), interferon gamma (IFN-γ) and are involved in adaptive immunity. Successive evidence attribute them a key regulatory role in inflammation, immunity, atherosclerosis and its complications[7].

Multiple factors contribute to the pathogenesis of atherosclerosis, including endothelial dysfunction, dyslipidemia, inflammatory, and immunologic factors, plaque rupture and smoking. In particular, endothelial dysfunction is induced by oxidized low density lipoprotein (LDLc), can be considered as a final common pathway [19] and is felt to be caused principally by loss of endothelium-derived nitric oxide [18]. This starting point of endothelial damage generated by inflammation processes triggers the atheroma formation process.

2.2. Atheroma formation from healthy endothelium and progression to CVD

Fatty streaks represent the first step of the atheroma formation. The histologists describe it as focal thickening of the intima with accumulation of lipid-laden macrophages (foam cells), which constitute the hallmark of the early atheroma, and extracellular matrix[16]. Hematopoietic stem cells migrate, and proliferate populating the intima [17]. Lipids accumulate both intracellular land extracellular deposits producing the fatty streak binding and trapping low density lipoprotein and T lymphocytes. [10]. The smooth muscle cells accumulated within the deep layer of the fatty streak are susceptible to apoptosis, which is associated with further macrophage accumulation. Their vesicles can calcify into chronic atherosclerotic plaques [11]. A dense cap of collagen will cover a lipid core and micro-vessels will supply oxygen and nutrients forming the atheroma's own microvasculature network (vasa vasorum). The atheroma will extend from the adventitia through the media and into the thickened

intima [13]. These thin vessels are prone to disruption, haemorrhage and progression of the coronary atherosclerosis [14,15].

We described below a comprehensive modified classification based on morphologic descriptions of the coronary atheroma formation and progression and symptoms (fig.2.). This modified clasification combines the American Heart Association (AHA) consensus drawn by Fuster [12] and modified by Virmani [18] with Stary original classification.

Comprehensive atheroma formation classification:

a. Early lesions:

Nonatherosclerotic intimal lesions: Absent Thrombosis. Reversible lesions. Asymptomatic.

- Type I: Intimal thickening, fatty dot: Normal accumulation of smooth muscle cells (SMCs) in the intima in the absence of lipid or macrophage foam cells. This lesion is only visible under microscopy.

- Type II: Intimal xanthoma or fatty streak: Superficial (luminal) accumulation of foam cells without a necrotic core or fibrous cap; based on animal and human data, such lesions usually regress. Yellowish lesion which is macroscopically visible. Foaming cells are seen arranged in rows and accompanied by SMC and T lymphocytes. Extracellular lipid deposits are only visible with electronic microscope. 99% of children between 2 and 15 years old have these lesions at aortic level.

- Type III: preatheroma. Pathologic intimal thickening: SMC-rich plaque with proteoglycan matrix and focal accumulation of extracellular lipid. The deposit of extracellular lipids or "core" is visible with a conventional microscope. This deposit interrupts the ordered arrangement of the foam cells and displace the SMC of the intima. There is no decrease in vessel lumen because collagen formation, thrombosis and bruising do not occur in the plaque.

b. Advanced lesions

b.1. Progressive atherosclerotic lesions: Absent thrombosis or thrombus mostly mural and infrequently occlusive. From this phase the lesions are potentially symptomatic but normally silent.

- Type IV. Atheroma: is developed from the third decade of life. The blood lumen may start diminishing. There is a lipid nucleus constituted by an initially delimited accumulation of extracellular lipids at the intima level, which evolves to disorganized nucleus by eccentric thickening. SMC are displaced by this lipid accumulation. A layer of peptidoglycans (PG) separates this nucleus and the intimal endothelial cells filtered by macrophages, lymphocytes and SMC. At the same time, some capillaries emerge surrounding the lipid nucleus and some foam cells and SMC start apoptosis . This atheroma can evolve to a thin fibrous cap atheroma: a thin, fibrous cap (< 65 μm) infiltrated by macrophages and lymphocytes with rare or absence of SMCs and a relatively large underlying necrotic core; intraplaque haemorrhage/fibrin may be present.

- Type V: Fibrous cap atheroma: When the layer of PG is substituted by fibrous tissue such as collagen it results in type V lesion. In the fibrous atheroma we find a well-formed fibrous layer that covers the lipid nucleus, composed basically by collagen. This collagen is synthesized by the SMC in response to the disorganization of the intima and replaces the matrix of proteoglycans. It occurs from the fourth decade onwards. This lesion is susceptible to necrosis fissure and thrombosis and also presents a stenosis of the arterial lumen to a greater or lesser degree:

  - Early necrosis: focal macrophage infiltration into areas of lipid pools with an overlying fibrous cap.

  - Late necrosis: loss of matrix and extensive cellular debris with an overlying fibrous cap.

  The lipid nucleus will continue generating capillaries, favouring micro-haemorrhages. At the tunica media level of the artery wall, an increase in

macrophages, lymphocytes and lipid deposit weakens the arterial wall and facilitates the appearance of aneurysms.

b.2. Lesions with acute thrombi: Symptomatic.

- Type VI: complicated atheroma. The type IV and V lesions are susceptible to fissures and thrombosis through its weaker areas resulting in type VI lesion with luminal thrombosis or no communication of thrombus with necrotic core.
    - Plaque rupture: Fibroatheroma with fibrous cap disruption. The luminal thrombus communicates with the underlying necrotic core producing occlusive or nonocclusive thrombus.
    - Plaque erosion: Plaque composition, as above plus no communication of the thrombus with the necrotic core. It can occur on a plaque substrate of pathologic intimal thickening or fibroatheroma. Usually nonocclusive thrombus.
    - Although thrombosis in general will be favoured by disruption of plaques, in other cases they will occur without previous ruptures, favoured by predisposing personal factors. Some identified fibrinolysis inhibitor factors are diabetes, smoking status, increased fibrinogen or high levels of Lp (a).
- Type VII: Calcified nodule atheroma: Eruptive (shedding) of calcified nodules with an underlying fibrocalcific plaque with minimal or absence of necrosis. Usually resulting in nonocclusive thrombus.

b.3. Lesions with healed thrombi: absent thrombosis.

- Type VIII: Fibrotic plaque (without calcification): Collagen-rich plaque with significant luminal stenosis. These lesions may contain large areas of calcification with few inflammatory cells and minimal or absence of necrosis and represent healed erosions or ruptures
- Type VIII calcified: Fibrocalcified plaque (+/- necrotic core)

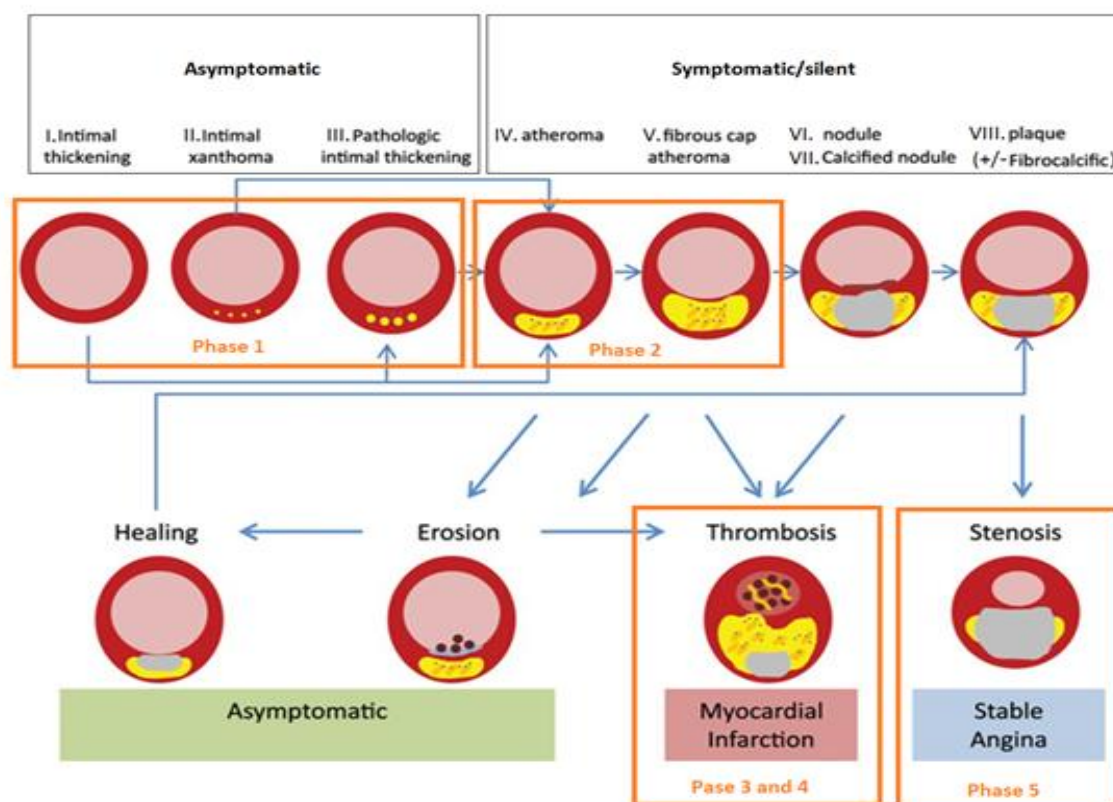

Fig. 2.1: Clinical phases of coronary atherosclerosis modified from Fuster: Phase 1 (types I to III of Stary): consists of a small plate, which progresses very slowly. It is common in subjects younger than 30 years of age and is usually asymptomatic. Phase2 (types IV and V of Stary): characterized by atheroma rich in extracellular lipids, and capable of producing an asymptomatic stenosis. Phases 3 and 4 (type VI of Stary) make unpredictable the course of atherosclerosis and they occur with rupture of the plate that is accompanied by a mural thrombus. Phase III symptoms comprise angina unstable angina, infarction or sudden death. Phase 5 (type VII and VIII Stary) vessel occlusion occurs without rupture of the plaque. This type of lesion produces a third of the coronary occlusions and is morphologically accompanied by a greater myocytes proliferation.

Atheroma formation associates coronary artery remodelling with abnormal arterial physiology and development of clinical symptoms [16]. Positive remodelling is linked to unstable plaques in patients presenting with unstable angina; meanwhile, negative remodelling is associated with obstructive plaques in patients with stable angina [17].

2.3. Endothelium's role in vascular pathology

2.3.1. Endothelial complexity

Healthy endothelium has been described as an unicellular layer of endothelial cells (ECs), which coats the blood vessels (arterial and venous), lymph vessels, heart chambers, corpora cavernosa and eye anterior chamber. It comprises an area over a 1000 square metres (6 tennis courts), it weighs about 4 kg, being regarded as the greatest virtual organ of our body, which regulates the flow of 7200 litres of blood a day and the cells are oriented in the direction of the blood flow [19]. The endothelium is a unique structure. Twenty years ago was seen as a passive coating which facilitated the passage of cells and molecules into the surrounding tissues. In the past two decades the EC has been studied in the umbilical cord, synovial villi, foreskin, placenta, breast and abdominal adipose tissue. This layers maintains vascular tone and organs blood perfusion, maintains vascular patency, regulates hemostasis balancing thrombosis and clotting, is a barrier for toxics, controls inflammation and regulates angiogenesis.

As an organ of enormous complexity, the endothelium is involved in embryogenesis, histogenesis, organogenesis, wound healing, angiogenesis, tumorigenesis and metastasis. The endothelial tissue also plays a vital role in host defence and is involved in the organization of thirteen barriers: I alveolar-capillary, placenta, liver, glomerular, blood-brain, blood-nerve, blood-cerebrospinal fluid, blood-ocular (blood-retinal and blood-aqueous), hematic-testicular, hematic-splenic, hematic-thymic and hematopoietic. The endothelium is the inner skin of the human body. It is considered a paraneurona belonging to the diffuse neuroendocrine system. The EC plays an important role in the regulation of capillary's permeability, lipoprotein's metabolism and tissue's aging. Actively involved in immunological reactions (systemic lupus erythematous, scleroderma, Raynaud's phenomenon, psoriasis, preeclampsia, Kawasaki disease, asthma event), inflammatory (rheumatoid arthritis), tumour growth and metastatic process. Furthermore EC synthesises neuropeptides, neurotransmitters, cytokines, growth factors, adhesion molecules and membrane receptors. It also expresses autocrine, paracrine and endocrine functions[20].

One of the most striking features of the endothelial lining is its ability to keep the blood soluble even in prolonged contact with the vessel wall, as well as their participation in

clot formation. The molecular mechanism of the hemocompatibility of normal endothelium is given by the expression of thrombomodulin, and plasminogen activators of type heparan sulfate glycosaminoglycans may interact with antithrombin III, all produced by the endothelium. But at the same time is able to synthesize endothelium stabilizing molecules of the blood clot and cause thrombosis to synthesize plasminogen activator inhibitor (PAI - 1), tissue factor, interleukin-1 and alpha tumour necrosis factor. All these cytokines are synthesized by the endothelium. As well as being a multifunctional organ, the endothelium is also defined as compartmentalized and specialized organ depending on the anatomical region where is located [21].

The endothelial lining has vasoconstrictor and vasodilator, procoagulant and anticoagulant, pro-and anti-inflammatory functions. It also promotes and inhibits cell growth and promotes and stops the process of angiogenesis. In addition, the endothelium actively participates in the inflammatory response and immune phenomenon. The endothelium, with circadian behaviour, early morning is different, increases the synthesis of PAI - 1 and decreases fibrinolytic activity, which is consistent with increased sympathetic activity and increased platelet aggregation. Therefore, it is suspected that this is the reason why cardiovascular and stroke are more common early in the morning.

The endothelial cells are rich in lipids (cholesterol, phospholipids, sphingolipids) proteins (caveolin, actin PCK) and enzymes (nitric oxide synthases). Zawadzki and Furchogtt suggested that ECs secreted substance with vasorelaxant properties and an intense investigation that led to the identification of the endothelium-derived relaxing factor started, nowadays known as nitric oxide (NO). NO is considered the epitome of healthy endothelium, from the metabolic conversion of L-arginine to L-citrulline. NO has vasodilatory effectsand is a physiologic inhibitor of smooth muscle growth and promotes apoptosis with an unclear role in relation to angiogenesis[22]. Other vasoactive substances released by the endothelial prostacyclin, bradykinin, angiotensin II and endothelin. The factors released by endothelial cells will be crucial in the regulation of vascular tone.

A proper balance between the vasoconstrictor factors released by the endothelium (angiotensin II and endothelin), and vasodilators (prostacyclin, bradykinin and NO) allow the maintenance of normal vascular tone, which under physiological conditions is slightly vasodilator. NO is a paracrine mediator, different than angiotensin and

antidiuretics [23]. NO is a molecular gas with an extremely short half-life, unstable out of the system and difficult to measure.

The normal endothelium does not generally support binding of white blood cells. However, with an atherogenic diet, early patches of endothelial cells start to express on their surface selective adhesion molecules which will bind leukocytes. The VCAM-1 (vascular cell adhesion protein 1) precisely attracts monocytes and T lymphocytes found in the early human nascent atheroma [24] The usual loci for atheroma formation are located at branch points in arteries, where the endothelial cells experiment the not laminar flow's aggression. This aggression reduces the production of nitric oxide (NO), natural anti-inflammatory molecule with vasodilator properties and increases the production of intercellular adhesion molecules (ICAM-1) and proteoglycans. These mechanisms promotes multiplied by 4 the promotion of early atheromatous lesions attracting inflammatory cells which will perpetuate the inflammatory response.

Inflammatory processes are involved in the promotion, evolution and also contribute to precipitate the acute thrombotic complications of the atheromatous plaque. The macrophages produce proteolytic enzymes which will degrade the collagen support and procoagulant tissue factors which will trigger thrombosis response [25] as already described, inflammation contributes across the spectrum of cardiovascular disease, including the earliest steps in atherogenesis.

To date, elevated levels of several inflammatory mediators among apparently healthy men and women have proven to have predictive value for future vascular events. In particular, prospective epidemiological studies have found increased vascular risk in association with increased basal levels of cytokines such as IL-6 and TNF-α [12], cell adhesion molecules such as soluble ICAM-1, P selectin, and E selectin and downstream acute-phase reactants such as CRP, fibrinogen, and serum amyloid [13].

Several traditional cardiovascular risk factors track with these inflammatory biomarkers, in particular, central obesity and body mass index due to adipocytes can produce inflammatory cytokines, and a common underlying disorder of innate immunity may well link obesity, accelerated atherosclerosis, and insulin resistance. In support of this hypothesis, very recent observations show that elevated levels of both IL-6 and CRP associate not only with the subsequent development of atherosclerosis, but also with the development of type II diabetes, even among individuals with no current evidence of insulin resistance [26]. During the last decades the CRP (C reactive proteína) appeared like the most promising inflammatory biomarker [27].

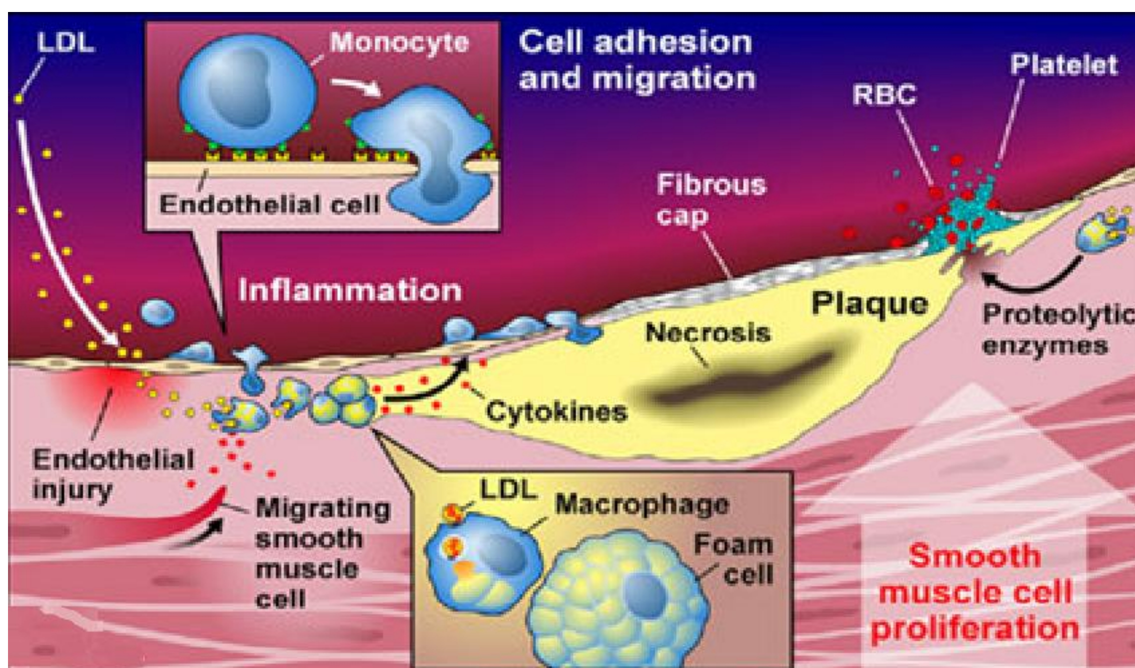

Fig. 2.2: Atherosclerosis is a chronic low-grade inflammatory process started in the endothelium. The atherosclerosis formation is triggered and chromicised maintaining endothelial damage by constant toxics such as tobacco, HBP, hyperlipidaemia or hyperglycaemia among others and subsequently initiating the inflammation cascade. Modified from "Ethnicity, Metabolism and Vascular Function: From Biology to Culture" Caballero E. 2008.

But these already classic inflammatory biomarkers are not the sole new molecules which can predict and measure the cardiovascular events and their evolution. There are multiple soluble immune effector molecules found in arterial lesions, such as antibodies and complement proteins. Complement activation, either by the antibody-dependent classical pathway or the alternative or lectin pathways, generates proinflammatory mediators. A good example of them, are C3a and C5a, which activate endothelium and enhance leukocyte recruitment to inflammatory sites. The complement cascade is involved in the stimulation and regulation of antibody responses. In particular, the CD19/ CD21 receptor complex on B cells links the complement system to the activation of B cells [13].

Patients with atherosclerotic disease produce antibodies specific to atheroma antigens. These antigens groups include oxidized low-density lipoproteins, and heat shock protein. The complement system is involved in both the inductive and effector phases of humoral immune responses. Due to this dual mechanism by which these antibodies

influence atherogenesis, the process complexity is not clearly explained. However, several studies demonstrate that complement is involved in modulating atherogenesis [28]. Furthermore, antibody-independent activation of complement in arterial lesions may contribute to the inflammatory process of atherosclerosis and several studies provide evidence that complement activation is involved in atherogenesis. Besides this, components of the complement system are frequently found in human atheromas [29]. C3 and C4 deposition in arterial lesions has also been demonstrated [30]. Additionally, RNA analysis studies indicate that complement genes are expressed locally within the plaques [20].

In relation to this fact, the **complement system**, found by J Bordet in the 19th century, represents one of the most important figures of the inflammation cascade. The complement cascade acts as a protein functional system. These proteins are synthesized by hepatocytes, tissue macrophages, blood monocytes, and epithelial cells of the genitourinal tract and gastrointestinal tract. In the bloodstream, they develop an amplified self-regulated enzymatic response during the inflammatory process. This process includes several functions such as opsonization (enhancing phagocytosis of antigens), chemotaxis (attracting macrophages and neutrophils), cell lysis (rupturing membranes of foreign cells), and agglutination (clustering and binding of pathogens together). All of these are basic steps in the formation and rupture of the atheroma plaque.

2.3.2. Endothelial dysfunction (ED)

ED represents the inability of the small arterioles to vasodilate when necessary, leading to microvascular dysfunction and possible myocardial ischemia. The NO-mediation is compromised due to reduced production and favoured consumption. Beyond this main action, ED also promotes leukocyte and platelets activation and adhesion, increases the permeability of the arterial wall, favouring the protein oxidation, cell proliferation and atherosclerosis[31].

Besides NO, other markers of ED are circulating progenitor cells (CPCs), atherosclerosis, hyperlipidemia, DM, smoking habit, ischemia and aging among others. EPCs are regenerative cells that are rarely found in blood in healthy individuals. EPCs are reported to participate in neovascularization after stroke, remodelling ischemic cardiac tissue and correlated to microvascular peripheral endothelial function [32, 33]. On top of EPCs and NO, atherosclerosis is also involved in impaired vasodilation,

augmented vasoconstriction through serotonin upregulation and microvascular dysfunction. DM, as well as tobacco, contributes to ED increasing oxidative stress. Additionally, hyperlipidemia reduces flow response to acetylcholine and hypertension reduced relaxation mediated by the endothelium [34, 35].

2.3.3 Evaluation of the endothelial function

The endothelium has emerged as the key regulator of vascular homeostasis, damaged endothelial function precedes the development of morphological atherosclerotic changes. Endothelial function can be evaluated directly or indirectly. All the direct testing techniques involve the microvascular response to endothelial-dependent stimuli such as reactive hyperhemia or vasoactive substances. Unfortunately endothelial function testing, using direct or indirect methods, is not routinely used in everyday clinical practice.

Direct testing is infrequent in the clinical studies due to its invasive nature involving biochemical and cellular integrity via coronary angiography. The coronary microvascular response to acetylcholine or adenosine is tested by blood flow measurement via intracoronary Doppler[36]. A healthy endothelium results in vasodilation and ED in vasoconstriction as observe in angina[37]. Besides angiography, an impedance plethysmography test measures forearm blood flow but also requires the direct intravascular administration of vascular agonists.

Due to the invasive character of the direct ED measurements, the assessment of the endothelium function in this study was indirect via cytokines as endothelial dysfunction markers, such as vascular adhesion molecule-1 (VCAM-1) and plasminogen activator inhibitor-1 (PAI-1).

Most cardiovascular risk factors activate molecular machinery in the endothelium that results in expression of chemokines, cytokines, and adhesion molecules, like VCAM-1 and PAI-1.

Vascular cell adhesion molecule 1 (VCAM-1):

VCAM-1 mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelial wall after endothelial cells are activated by pro-inflammatory cytokines such as TNF-α and IL-6, leading to vascular inflammation and atherosclerosis [38]. Mediators of endothelial dysfunction, such as

hypercholesterolemia, smoking, or oxidative stress, enhance endothelial cell activation, measured by VCAM-1 levels, leading to increased vasoconstriction, smooth muscle proliferation, platelet aggregation, leukocyte adhesion and LDLc oxidation[38].

VCAM-1 has been associated independently of traditional cardiovascular risk factors and inflammatory markers with common carotid artery intima–media thickness and endothelial dysfunction[39]. An alternative method to assess endothelial function, more accurate than the measurement of instable NO gas, involved the measurement of biomarkers of endothelial activation and dysfunction (VCAM-1). Elevated circulating levels of adhesion molecules have been associated with cardiovascular risk factors and predict atherosclerosis and cardiovascular events [40].

Plasminogen activator inhibitor-1 (PAI-1):

PAI-1 is the principal inhibitor of tissue-type and urokinase-type plasminogen activators, which convert plasminogen to plasmin. PAI-1 stimulates cell migration by binding to the low-density lipoprotein receptor-related protein. Then, fibrinolysis is regulated by PAI-1 which prevents the escape of this potentially destructive protease system. Increased PAI-1 levels predispose patients to the formation of atherosclerotic plaques prone to rupture with a high lipid-to-vascular smooth muscle cells ratio as a result of decreased cell migration.

Overexpression of PAI-1 has been linked to endothelial dysfunction, metabolic syndrome and cardiovascular disease [41]. PAI-1 has been also implicated in adipose tissue development, control of insulin signalling in adipocyte, atherothrombosis, diabetogenesis and insulin resistance [42]. The PAI-1 production has been positively associated to oxidative stress, TNF-α, cortisol and IL-6 levels, disturbances of the renin-angiotensin and insulin glycolipid control systems[43, 44].

2.4. Inflammatory cascade: complement system interaction with pro-inflammatory factors

The fundamental role of the inflammatory cascade in the pathogenesis of CVDs has been globally recognised. Inflammation contributes to the development and progression of atherosclerotic lesion formation, plaque rupture and thrombosis [45]. The most consistently studied and demonstrated factor in relation to prediction of CVDs

development has been the CRP [46, 47]. CRP levels can be induced by a wide variety of stimuli, including acute and chronic infection and are elevated in various inflammatory diseases such as rheumatologic pathologies as demonstrated in several studies and meta-analysis[48]. The lack of specificity related to CRP encourages the authors to expand the research to other inflammatory markers and provide us with supporting evidence of the functional role for the complement activation in the pathogenesis of CVDs through pleiotropic effects on endothelial and hematopoietic cell function and haemostasis[49].

Inflammatory response is part of a complex biological mechanism activated by harmful stimuli involving immune cells, blood vessels and molecular mediators. Chronic inflammation leads to progressive simultaneous destruction and healing processes of the affected tissue. A chronic inflammatory disorder may lead to a variety of diseases, such as atherosclerosis, allergic reactions as asthma, immune system disorders and rheumatologic diseases among others.

Inflammation is currently viewed as a complex pathophysiologic process that engages hundreds of mediators and different cell types and tissues and can be initiated by any stimulus causing cell injury. This complex cascade is triggered by a variety of stimuli:

- Inflammatory mediators (bacterial/viral/fungal sources, endotoxins, cytokines, histamine, oxidized products, complement fragments, etc.)
- Depletion of anti-inflammatory mediators (nitric oxide, IL-10, glucocorticoids, albumin, etc.)
- Fluid stress, transients of gas pressure or temperature, etc.

The intricacy of this inflammatory cascade involves multiple steps as follow:

- Early Cell Responses: ion exchange, depolymerisation, degranulation, release of inflammatory mediators, strengthening endothelial permeability and upregulation of adhesion molecules.
- Tissue Degradation: neutrophil entrapment into vessels, transvascular migration, platelet aggregation and thrombosis, red cell aggregation, protease release, oxygen free radical formation, apoptosis and organ dysfunction.
- Initial Repair: downregulation of anti-inflammatory genes, upregulation of pro-inflammatory genes (cytokines), monocyte and T-Lymphocyte infiltration.
- Repair: release of growth factors, connective tissue growth, revascularization.

The complement system is actively involved in the inflammation cascade in a multifaceted way regulating actively various steps of the inflammatory response, including changes in vascular flow and calibre, the increase in vascular permeability, extravasation of leukocytes, and chemotaxis. Complement components are activated in plasma. Complement may regulate other inflammatory mediators or be associated with a direct action on target cells and can independently participate in the regulation of inflammation, in either the presence or absence of an infection [50].

Three biochemical pathways activate the complement system: the classical complement pathway, the alternative complement pathway, and the lectin pathway. These three activation pathways converge at the formation of the C3 convertase which cleave C3, the central effector protein of the complement cascade, to C3a and C3b.

The classical complement pathway typically requires for activation antigen-antibody complexes (immune complexes) generating a specific immune response, whereas the alternative and mannose-binding lectin pathways can be activated by C3 hydrolysis or antigens without the presence of antibodies (non-specific immune response). The activation of the alternative pathway via generation of hydroxilated C3 surges and a surveillance mechanism enabling rapid responses to invading pathogens or modified self-cells. Lectin and alternative pathways also constitute the amplification loop for complement activation [51].

The classical pathway is activated by the interaction antibody-antigen to cells surface mediated by the C1 binding and subsequent cleavage of C4 and C2. On the surface of the active cell a serine protease, namely C3 convertase, will synthetize an anaphylotoxin, C3a, which will promote opsonisation. This response will also beamplified by C3 convertase generating further C3b [52]. **The MBL pathway** is similar to the classical pathway, albeit antibodies are substituted by lectin proteins[53].

Conversely, **the alternative pathway** owns the ability to initiate the complement cascade without requiring contact with proteins. The thioester bond of C3 produces a significant quantity of self-activated C3 which binds B factor. C3 convertase will also cleavage C3 amplifying the response. The C3b proteins produced by the classical pathway will generate a positive feedback loop to form C3 convertase [54].

The three pathways of activation generate homologous variants of the protease C3-convertase. In all of them, C3-convertase cleaves and activates component C3, creating C3a and C3b, and causing a cascade of further cleavage and activation events[55].

C3 complement plays a central role in the activation of the complement system. Its processing by C3 convertase is the central reaction in both classical and alternative complement pathways. **C3 convertase** belongs to the family of serine proteases as a part of complement system which eventuate in opsonisation of particles, release of inflammatory peptides, C5 convertase formation and cell lysis. C3 convertase is mainly synthetized in the liver, in smaller proportion in the adipose tissue and it is present circulating in plasma to participate in the complement cascade.

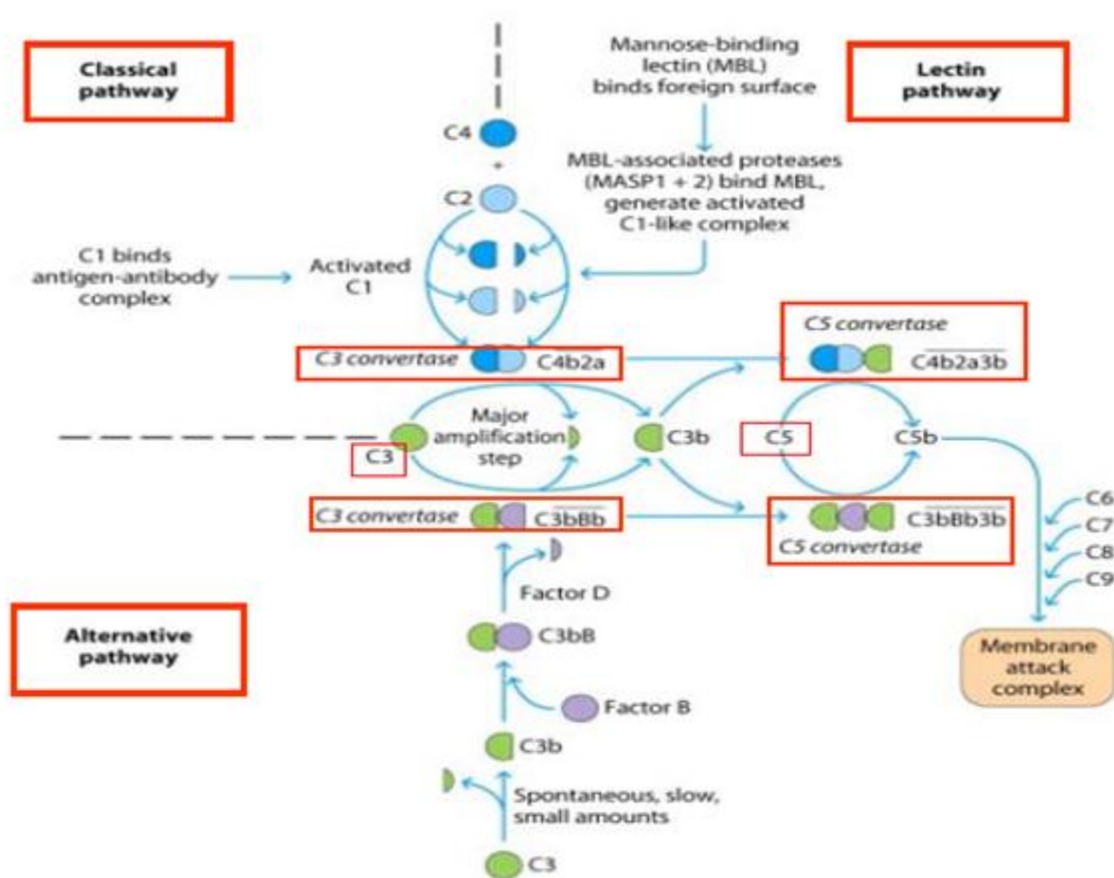

Fig.2.3. Overview of the complement activation pathways, modified from Kuby et al.'s algorithm, Inmunology 2003.The three pathways of complement activation: classical, lectin (mannose-binding lectin (MBL)) and alternative converge at formation of the C3 convertase products. C3b acts as an opsonintargeting C3b surfaces for phagocytosis. C3a and C5a act as anaphylotoxins, promote chemotaxis and the degranulation of mast cells.

The smaller fragment called C3a is released and stimulates inflammation through the chemo-attractant activity. C3b fragment, becomes covalently attached to the cell surface or to the antibody molecules through the thioester domain at the site of complement activation. Derived from proteolytic degradation of complement C3, C3a anaphylatoxin is a mediator of local inflammatory process. It induces the contraction of smooth muscle, increases vascular permeability and causes histamine release from mast cells and basophilic leukocytes. It also interacts with adipogenic proteins that stimulate triglyceride (TG) synthesis and glucose transport in adipocytes, regulating fat storage and playing a role in postprandial TG clearance.

After cleavage and binding to cell surface, the C3b fragment is ready to bind a plasma protein called Factor B. The Factor B (a zymogen) is cleaved by a plasma serine protease Factor D releasing a small fragment called Ba and generating a larger fragment called Bb that remains attached to C3b. Thus, the alternative pathway C3 convertase is formed and is able to cleave C3 now [56]. On the other hand, during the classical or lectin pathways, the C3 convertase contains different proteins of complement system – C4b and C2a. The cleavage of C4 and C2 is mediated by serine proteases - C1 complex in classical pathway and Mannose-binding lectin-associated serine proteases in lectin pathway. C4 is homologous to C3, and C4b contains an internal thioester bond, similar to that in C3b, that forms covalent amide or ester linkages with the antigen-antibody complex or with the adjacent surface of a cell to which is antibody bound. C2 is cleaved by C1s to a smaller fragment called C2b and larger fragment called C2a that binds to C4b. The fragments C4a and C2b are released [57].

C3 convertase exists in two forms (C3bBb and C4bC2a) but both of them cleave C3, central molecule of complement system (hence the name "C3-convertase"). C3b binds to the surface of pathogens, leading to greater internalization by phagocytic cells by opsonization. C3a is the precursor of an important cytokine (adipokine) named ASP and is usually rapidly cleaved by carboxypeptidase B. C3a and C5a have anaphylatoxin activity, directly triggering degranulation of mast cells as well as increasing vascular permeability and smooth muscle contraction.

This accelerated spiral has its own regulatory systems, thus C3-convertase can be inhibited by Decay accelerating factor (DAF), which is bound to erythrocyte plasma membranes via a glycophosphatidilinositol anchor and blocks the membrane attack complex [58]. C3 convertases are unstable, their average half-life lasts 10 to 20 minutes. C3 convertases are deactivated spontaneously or their dissociation is

facilitated by the regulators of complement activation proteins, such as decay acceleration factor (DAF), complement receptor 1 (CR1), C4b-binding protein and Factor H. C3 convertase assembling is suppressed by the proteolytic cleavage of C3b (and C4b), mediated by Factor I in the presence of membrane cofactor protein (MCP, CD46), C4b-binding protein, CR1 or a plasma-glycoprotein Factor H.

These negative control processes are essential for the protection of self-tissue [59]. On the contrary, C3 convertase cleaves C3 producing C3b, which can form an additional C3 convertase. This positive-feedback effect is a unique feature of the alternative pathway of complement and results in the deposition of large numbers of C3b molecules on the surface of activating particles [60] Properdin (Factor P) is the only known positive regulator of complement activation that stabilizes the alternative pathway convertases (C3bBb).

The complement deficiency would result in increased susceptibility to infection and to complement-tissue damaged. Deficiency of C3 and C4 have been linked to glomerulonephritis sue to an increased deposit of immune complexes in the glomerula [61]. Dysregulations in the alternative complement cascade have demonstrated an accelerated development of atherosclerosis and thrombosis and point to the potential role for complement C3 in CVDs [62, 63]. As an example, some studies have demonstrated that elevated levels of C3 at the time of an acute ischaemic event have been associated with worse outcomes and predict restenosis following endarterectomy[64].

Already defined atherosclerosis as a chronic low-grade inflammatory disease comprises the action of T-Lymphocytes, mast cells and macrophages at the early lesions. A healthy endothelium maintains vascular tone, flow and patency, inhibits cell adhesion and suppresses activation of the coagulation cascade via the secretion of multiple molecules such as nitric oxide (NO), endothelin I and prostacyclin [20].

The atherosclerosis developing process will start via endothelial cell activation towards to an inflammatory, vasoconstrictive and thrombotic phenotype. This atherogenic endothelial cell phenotype increases the expression of cellular adhesion molecules, namely vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin. These adhesion molecules promote binding of inflammatory cells to the activated endothelium and support their migration into the arterial intima magnifying the inflammatory response within the arterial wall [65]. C5a and C5b interact with endothelial cells regulating these cellular adhesion molecules,

therefore, contribute to endothelial activation and leukocyte infiltration into the wall[49, 66].

The activation of the alternative complement cascade also shows pathophysiological relevance in its role as rise giver to fibrin clots of denser structure. C3 has been identified as a clot component which bounds to fibrin with high affinity supporting a functional relationship between elevated C3 and prolonged fibrinolysis[67].

Cross-talks among complement, coagulation cascades and atherosclerosis therefore occurs at multiple levels to coordinate haemostatic and immune response. The C3 seems to play a central role in the complement system and its regulation is exerted by a complex mechanism composed by its own intrinsic decay, stabilization by properdin, disassembly by glycoprotein B1H, inactivation by C3b inactivator and protection by activators from the alternative pathway. Consequently, C3 convertase plays the junction point role of the three complements pathways and its study as an inflammatory biomarker and acute phase reactant seems the logic approach in the cardiovascular pathogenesis environment [68].

There is rising interest in identifying new inflammatory risk factors for cardiovascular disease, to improve our understanding of the cardiovascular events development biology and to account for the cases of heart disease that cannot be explained by known risk factors.

Investigation of the newly adopted risk factors frequently involves the study of circulating biomarkers. In recent years, a spirited debate has arisen regarding the validity and usefulness of these new measures. A careful assessment of the evidence suggests that most newer biomarkers are not ready for routine clinical use in the primary prevention setting. The traditional risk factors perform quite well with regard to the prediction of future cardiovascular risk. Inadequate recognition and control of the 'classic' risk factors continues to account for a large number of avoidable cardiovascular events. At the same time, new insights into disease mechanisms should lead to the development of novel preventive therapies, regardless of how well biomarkers themselves perform in risk stratification. Furthermore, new developed technologies allow the profiling of large panels of genes, transcripts, proteins, or small molecules and facilitate the discovery of newer biomarkers capable of providing both mechanistic insight and true prognostic utility, and here C3 convertase plays a main role again.

Our research team has not been the first one which paid attention to this protein. In 1987 Muscari et al noticed the humoral immunity changes in patients with coronary ischemia [69] and later when continuing their studies he described for the first time the possible predictive value related to coronary disease [28]. Five years later the same team linked the C3 protein levels to insulinemia in patients with personal history of atheroeslerosis [29]. Another Italian team paid attention to the relation of the C3 with the hypertension [70]. Lately, other authors correlate C3 serum levels with the central obesity and smoking habit [71]. Several researches during the last 3 decades are intensifying the fence attack towards the final target and seeding the possible relations of the C3 convertase levels with the diverse cardiometabolic risk factors. Most of the classical cardiovascular risk factors are included in the metabolic syndrome (MetS) entity. Subsequently, most studies reveal a tendency pointing out the C3 convertase as the main character of the complement cascade and the future metabolic syndrome biomarker.

2.4.1. C-reactive protein as first inflammatory marker linked to metabolic syndrome

Circulating levels of several inflammatory biomarkers have been studied to assess their value in predicting CVD. The best characterized and standardized biomarker of inflammation is C-reactive protein (CRP). The largest study to date that examined the association between inflammation and the MetS was the NHANES III study [72].

CRP was described as an inflammatory protein produced by hepatocyte and activated monocyte under the influence of cytokines such as interleukin (IL) -6 and tumour necrosis factor-alpha [73], being acute phase reactant in stress situations. Clinical evidence since the 1990s has demonstrated the relationship between inflammatory process, atherogenesis, plaque rupture and cardiovascular event. The elevation of serum concentrations of acute phase reactants, such as CRP [74] and IL-6 suggests that chronic inflammation of the coronary artery wall can play an important role in plaque rupture. This leads to a growing interest in the study of inflammatory biomarkers as markers of underlying atherosclerosis in apparently healthy individuals and the risk of recurrent events in patients with atherosclerotic vascular disease [75, 76]. Many studies have linked the metabolic syndrome to CRP since [77-79].

Despite the lack of specificity of CRP to narrow inflammation aetiology, a significant association has been demonstrated between elevated serum CRP levels and the prevalence of underlying atherosclerosis. CRP has also been correlated to risk of recurrence of cardiovascular events among patients with established disease, incidence of early CVDs events [80, 81] and drugs used in the treatment of cardiovascular diseases reduce serum levels of CRP. Therefore, it is possible that inflammation contributes reducing the beneficial effects of these drugs.

As a direct pathogenic role, CRP has been found in atherosclerotic lesions, as well as linked to LDLc facilitating the action of macrophages, inducing the expression of adhesion molecules and the production of interleukin-6 and monocyte chemotactic protein-1 (MCP-1) in endothelial cells and recruitment of monocytes and lymphocytes [82]. As possible consequence the risk of ischemic heart disease was found significantly higher in individuals with higher serum CRP levels.

The Centres for Disease Control and Prevention and the American Heart Association (CDC / AHA) (Pearson et al., 2004) define low, medium and high cardiovascular risk values as <1, 1 to 3, and> 3 mg / L of serum CRP respectively, these values correspond to the approximate tertiles in the general population. They also suggest that a value greater than 15 mg / L should initiate a search for a source of infection or inflammation. For patients with coronary heart disease> 3 mg / L would predict stable coronary disease and passing the threshold >10mg / L would be more predictive of acute coronary syndrome [83]. Among apparently healthy men, the plasma concentration of CRP predicted the long-term risk of a first myocardial infarction, ischemic stroke or peripheral vascular disease [84].

Some studies associate CRP with the risk of future acute events in patients with stable angina and angiography [85] or the degree of calcification of the coronary arteries in the CT-scan. In patients with established coronary disease, a strong correlation between initial CRP and future acute coronary events has been demonstrated [86]. Several studies point to CRP as a predictor of the development of heart failure, new-onset diabetes, a marker of rapid progression of coronary disease in revascularized patients correlation of serum levels of CRP with induction of ischemia in Stress tests [74, 87].

Previous studies have shown that MetS factors are individually associated with decreased endothelium-dependent vasodilatation, such as obesity, low HDL cholesterol, IGT, hypertriglyceridemia, and hypertension. Also, insulin resistance is associated with endothelial dysfunction. Subsequently several authors established the

link between CRP and endothelial dysfunction. To demonstrate this theory, in-vivo studies [88] have shown that CRP impairs endothelial vasoreactivity and decreases eNOS activity. Knowing that patients with MetS are in a procoagulant state as evidenced by increased circulating plasminogen activator inhibitor-1 (PAI-1, some authors have shown that CRP induces PAI-1 and decreases tissue plasminogen activator (tPA) in endothelial cells [89, 90]leading to endothelial dysfunction.

2.5. Evolution of the metabolic syndrome to cardio-metabolic syndrome

The **metabolic syndrome** (MetS) was defined for the first time by Reaven in 1988 as "syndrome X" or "insulin resistance syndrome" [91]. Since then, several studies and definitions have been conducted and copious pieces of literature have been produced as shown in the figure 2.4.

The MetS concept has evolved to cardio-metabolic syndrome (CMS) by the addition of inflammation and reticular stress to the equation [92]. In fact, MetS and CMS are complementary pathophysiological concepts. If we include biomarkers of inflammation, oxidative stress, endothelial dysfunction and pro-thrombotic state, we refer to CMS, otherwise MetS.

Metabolic syndrome represents a cluster of interrelated risk factors that promote the development of atherosclerotic vascular disease and are commonly associated with insulin resistance and type 2 diabetes. These include hyperglycaemia, dyslipidaemia, hypertension and abdominal obesity [93]. Dyslipidaemia involves elevated triglycerides (TGs) and low-density lipoprotein cholesterol (LDLc) and low high-density lipoprotein cholesterol (HDLc).

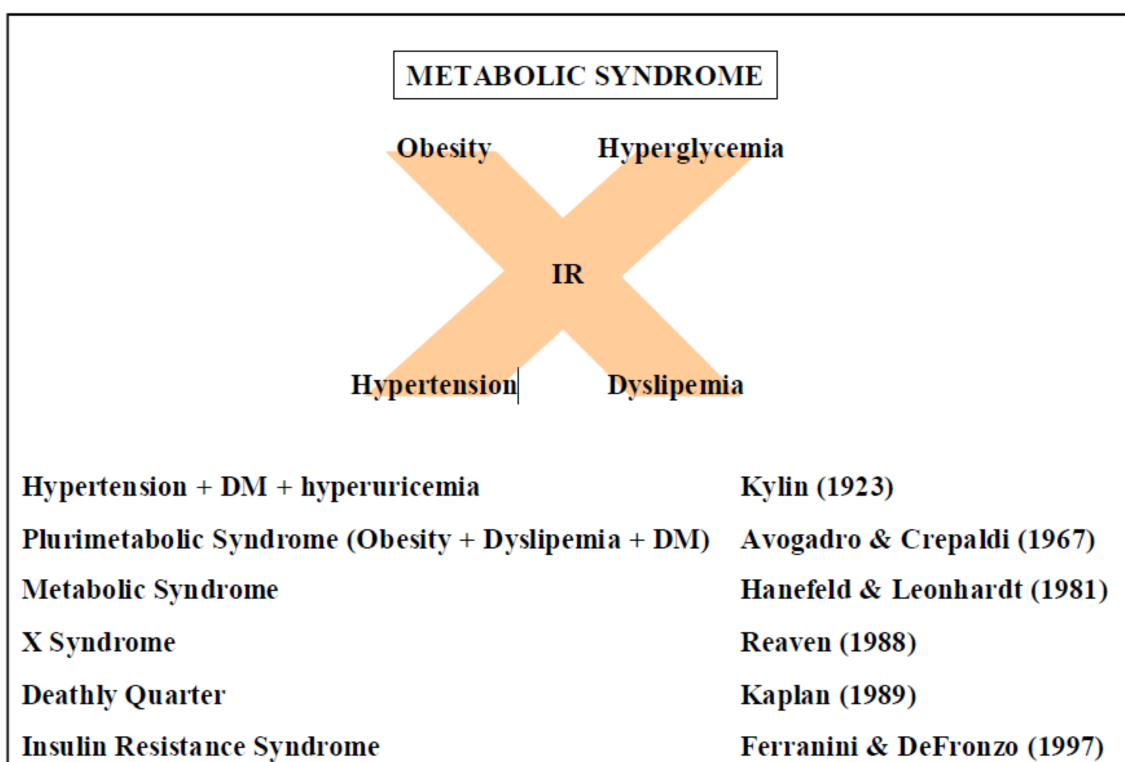

Fig. 2.4: History evolution of the Metabolic Syndrome (MetS) concept. Modified from Anti-Obesity Drug Discovery and Development 2014 [94].

Cardio-metabolic syndrome (CMS) definition evolves one step further, adding the promotion of pro-inflammatory and pro-thrombotic states. The mayor mechanisms of the underlying forces of CMS are abdominal obesity, insulin resistance, obesity-induced inflammation, reticulum and metabolic stress. It is also important to analyse the role of low-grade inflammation or meta-inflammation in fat tissue, as well as two new metabolic phenomena, metabolic Inflexibility and endoplasmic reticulum stress, and their interaction with the vascular triad: endothelial dysfunction, oxidative stress and vascular inflammation.

Despite the MetS worldwide prevalence, there is still a lack of a uniformly accepted definition and great controversy with regard to the pathogenesis of MetS [35].

In our research team, we agreed with the most widely accepted definition, which was proposed by the National Cholesterol Education Program-Adult Treatment Panel-III (NCEP-ATPIII).

Their criteria require three or more of the following parameters: waist circumference (WC) >102 cm in men and >88 cm in women, HDLc <40 mg/dl (<1.04 mmol/l) in men

and <50 mg/dl (< 1.29 mmol/l) in women, TG ≥150 mg/dl (≥1.7 mmol/l), blood pressure (BP) ≥130/85 mmHg and fasting glucose ≥100 mg/dl (≥6.1 mmol/l)[95].

The clinical criteria for the metabolic syndrome diagnosis according to the ATP-III can be summarized into three or more of the following parameters:

1. Variable waist circumference according to the different specifications related to populations, genders and location.
2. Raised hypertriglyceridemic levels (or patient under specific treatment) > 150 mg/dl.
3. Reduced plasmatic C-HDLc levels (or patient under specific treatment) < 40 mg/dl (males) <50 mg/dl (females)
4. Hypertension (or patient under specific treatment):
    i. Systolic blood pressure ≥130 mmHg
    ii. Diastolic blood pressure ≥ 85 mmHg
5. Raised plasmatic fasting glucose ≥100 mg/dl

Besides this definition, there are two other commonly used. The first one proposed by the International Diabetes Federation (IDF) [95], and a second one by the National Heart Lung and Blood Institute (NHLBI) and the American Heart Association (AHA). According to the IDF definition, MetS is diagnosed if an individual has abdominal obesity, that is waist size ≥94 cm in European men and ≥80 cm in European women and ≥2 of the remaining four criteria of the NCEP-ATP III definition. Cut-off points for hypertension, TG and HDLc levels are the same but glycaemia is considered abnormal at lower levels (FG≥100 mg/dl).

The definition proposed by the AHA/NHLBI in 2005 retained most of the NCEP-ATPIII criteria but adopted the same waist thresholds for some ethnic groups (e.g., South Asians) but their cut-off points for fasting glucose levels [≥100 mg/dL (5.6 mmol/L)] are lower following the IDF definition [96].

Recently, the 2009 Joint Interim Societies (JIS) proposed a MetS definition to unify the aforementioned three definitions. This definition uses the same thresholds for TG, BP, and HDLc considers WC based on ethnicity (≥94 cm (males) or ≥80 cm (females) for a Mediterranean population, but this is not a mandatory criteria though) and finally glycaemia (FG≥100 mg/dl).

Three or more of these criteria are required for diagnosis. All these criteria are presented in table 2.1.

Clinical measure		WHO (1998)[14]	EGIR (1999)	NCEP-ATP III[14] (2001)	AACE (2003)	1DF (2005)[15]	AHA
Insulin resistance		IGT, IFG, Type 2 DM or Insulin resistance *	Plasma insulin ≥75th percentile *	None †	IGT or IFG §	None	None
Body weight	M	WHR>0.90	WC ≥94 cm	WC ≥102 cm	BMI ≥ 25 kg/m^2	Increased WC (population specific)	WC ≥102 cm
	F	WHR >0.85 and/or BMI >30 kg/m^2	WC ≥80 cm	WC ≥ 88 cm			WC ≥ 88 cm
Lipid		TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)
	M	and/or HDL-C <35 mg/dl (0.9 mmol/l)	And/or HDL-C <39 mg/dl (1.0 mmol/l)	HDL-C <40 mg/dl (1.03 mmol/l)	And HDL-C <40 mg/dl (1.03 mmol/l)	HDL-C <40 mg/dl (1.03 mmol/l)	HDL-C <40 mg/dl (1.03 mmol/l)
	F	HDL-C <39 mg/dl (1.0 mmol/l)	HDL-C <39 mg/dl (1.0 mmol/l)	HDL-C <50 mg/dl (1.3 mmol/l)	HDL-C <50 mg/dl (1.29 mmol/l)	HDL-C <50 mg/dl (1.3 mmol/l)	HDL-C <50 mg/dl (1.3 mmol/l)
Blood pressure (mmHg)		≥ 140/90	≥ 140/90	≥ 130/85	≥ 130/85	≥ 130/85	≥ 130/85
Glucose		IGT, IFG or Type 2 DM	IGT or IFG (but not diabetes)	>110 mg/dl [6.1 mmol/l] (including DM)	IGT or IFG (but not DM)	≥ 100 mg/dl (5.6 mmol/l) [includes DM]	≥ 100 mg/dl (5.6 mmol/l)
Others		Micro-albuminuria			Other features of insulin resistance		

Table2.1: listed MetS diagnosed criteria differentiated by each health organization standards, gender and personal characteristics. Modified from Indian Journal of Endocrinology and Metabolism, 2012. Abbreviations: as shown in list of acronyms.

Summing up, obesity predisposes the individual to increased risk of developing diabetes mellitus (DM). It reflects our contemporary world’s sedentary lifestyle, over-nutrition, and resultant excess adiposity due to overweight. It seems to affect about one-fourth to one-fifth of the Mediterranean population, and its prevalence increases with age due to a reduction in energy requirements and consumption [97]. The “Look AHEAD Study” has shown that a body weight reduction of 7% improves glycemic control and cardiovascular risk factors in subjects with type 2 diabetes[98].

The physiopathological effects of adipose tissue are related to the specific site where fat is stored [99]. There is a clear functional distinction between visceral or intraperitoneal fat, extraperitoneal (peripancreatic and perirenal) and intrapelvic (gonadal/epididymal and urogenital) adipose tissues, all of them presenting a higher

metabolic activity than subcutaneous peripheral adipose tissue [100]. Moreover, abdominal obesity with low metabolic activity, android obesity, is strongly associated with MetS, cardiometabolic risk and cardiovascular disease, therefore the importance of the waist circumference measurements [101]. The term *adiposopathy* or *sick fat*, described by Dr Saban-Ruiz, refers to an excess of adipose tissue which results in pathogenic enlargement of fat cells and functional abnormalities, including endocrine and immune disorders.

Obesity is already considered as a low-grade chronic inflammation disease or meta-inflammation that comprises fat and vascular stroma [101]. This meta-inflammation involves adipocytes and the stroma (vascular endothelial cells) is infiltrated by macrophages, and leukocytes. As a result, the macrophages secrete pro-inflammatory cytokines and reactive oxygen species (ROS). The pro-inflammatory cytokines levels, such as IL-6, TNF-α, resistin and leptin are raised in obese patients along with reduced adiponectin. ROS activates stress pathways and disrupt metabolic processes as the insulin signalling cascade, energy homeostasis, lipid metabolism increasing the production of triglycerides and leading to MetS.

Two concepts have been recently added to complete the MetS physiological picture: Metabolic Inflexibility (MI) and the Endoplasmic Reticulum Stress (ERS).

The metabolic inflexibility is considered the ability to switch from fat to carbohydrate oxidation. Consequently, a healthy organism counts with a great adaptability to the fat from diet, so it is able to suitably metabolize this fat, while maintaining body weight. This process is mediated by genetic and hormonal factors [102, 103] .

On the contrary, metabolically inflexible subjects present decreased adaptability to fat ingestion, which appears to usually be impaired in insulin-resistant subjects. In MI subjects, a fat accumulation occurs, fatty acids are stored in muscles and the liver and thus weight gain occurs as food intake increases. In addition, consumption of local glucose at muscular tissue is increased and consumption of postprandial glucose is decreased. MI individuals can lead to IR by interfering with the insulin-signalling cascade [104].

MI is closely related to IR, and both processes are intimately linked Endoplasmic Reticulum Stress. ER is a cellular organelle that integrates the protein, lipid, and glucose metabolism. The secretion of inflammatory mediators increases as fat accumulation grows. The inflammatory mediators promote lipogenesis and impair

mitochondrial respiratory chain function and increases the generation of ROS, leading to mitochondrial calcium overload, and oxidative stress From chronic inflammation of adipose tissue and metabolic inflexibility, [105].

Endoplasmic reticulum stress has been considered as the first step which leads to oxidative stress, vascular inflammation, endothelial dysfunction, T2DM and MetS.

Because of the increased importance of obesity**, waist circumference** has been recommended as a screening tool to evaluate the risk of developing metabolic syndrome, diabetes and cardiovascular disease since 1995, though exact cut off points for maximum waist circumference have been debated [106]. The risk of developing diabetes (controlled for age, sex, race, and smoking) is 4.12 (2.72–6.24) times higher if waist is larger than International Diabetes Federation (IDF) recommendations [107].

The risk of developing cardiovascular disease is greater for persons with intra-abdominal obesity who have high levels of triglycerides. A recent study conducted in the United Kingdom proposes that screening for increased waist circumference and hypertriglyceridemia (the hypertriglyceridemic-waist phenotype) is an inexpensive approach for identifying patients with excess intra-abdominal adiposity and associated metabolic abnormalities[108]. This theory has been tested with confirmative results across the world, from China [42] to Brazil [109] and Puerto Rico [110].

Several studies suggest that measuring triglycerides and waist circumference is particularly important in patients with normal traditional risk scores. Patients who have hypertension, diabetes or raised cholesterol will be identified with traditional methods. However, patients with hypertriglyceridemic waists and normal values on traditional risk scores had double or triple the risk of developing heart disease [111].

There are several cut-off points of maximum waist circumference in relation to the different health organizations, ethnicity and gender. The European Society of Cardiology (ESC) establish the cut-off point in 102 cm for men and 88 cm for women, while the International diabetes Federation (IDF) specify a cut-off point specifically for Mediterranean population lower than that, with 94 cm for men and 80 cm for women. Consensually, the WHO considers the IDF's cut off point as high risk and the ESC's cut off point as very high risk to develop cardiovascular diseases.

Population	Organization	Men waist circumference	Women waist circ.
Europeans	IDF	≥94 cm	≥80 cm
Europeans	ECVs	≥102 cm	≥88 cm
Caucasians	OMS	≥94 cm high risk ≥102 cm very high risk	≥ 80 cm ≥ 88cm
Asians	IDF	≥ 90 cm	≥ 80 cm
Asians (excluiding Japanese)	OMS	≥ 90 cm	≥88 cm
Japanese	Obesity Jap. Soc.	≥ 85 cm	≥80 cm
Chinese	Coop. Task Force	≥85 cm	≥80 cm
Mediterranean	IDF	≥94 cm	≥80 cm
Subsaharians	IDF	≥94 cm	≥80 cm
South Americans	IDF	≥90 cm	≥80 cm
USA	AHA/ATP III	≥102 cm	≥88 cm
Canadians	Health Canada	≥102 cm	≥88 cm

Table 2.2: cut off points for waist circumference according to ethnicity, gender and health organization.

Beyond CVD and DM, the metabolic syndrome is also associated with higher urinary albumin excretion, lower glomerular filtration rate (GFR) and a greater prevalence of chronic kidney disease [46]. Other co-morbidities include non-alcoholic fatty liver disease [47-49], sleep-disordered breathing [112], and hypogonadism in males [113] .

Furthermore, MetS has been associated with increased incidence of some types of cancer, such as pancreatic cancer, with DM being the key component for this correlation [52, 53], and breast cancer [54] One of the proposed mechanisms for this association may be related to increased insulin and insulin-like growth factor-I (IGF-I) activities observed in MetS. Elevated serum insulin concentrations observed in MetS

and IR states increase the level and bioavailability of IGF-I, which in turn plays a key role in the development and progression of several diseases.

In nondiabetic, normotensive overweight individuals, serum triglyceride concentration, the ratio of triglyceride to high density lipoprotein HDLc concentrations, and fasting insulin concentration are useful markers. This makers identify those who may be insulin resistant, as measured by an insulin suppression test.

Optimal cut-points were identified as 130 mg/dL for triglycerides, 3.0 (1.8 SI units) for triglyceride-to HDLc ratio and 15.7 µU/mL for insulin, respectively [55]. The co-segregation of overall obesity (increased BMI), abdominal obesity (raised waist circumference), raised blood pressure, increased fasting glucose levels, raised triglyceride levels, and low HDLc concentration suggest the existence of metabolic syndrome, which is closely related with the development of insulin resistance.

2.6. The metabolic triad

The association between T2DM and inflammation was noted in early 20th century when sodium salicylate therapy showed lower levels of glycosuria and further evidence emerged when aspirin treatment improved blood glucose control in T2DM [114]. Oxidative stress and increased ROS are associated with chronic hyperglycaemia and both play a role in endothelial dysfunction (ED) development. Chronic hyperglycaemia, as seen in diabetes, leads to ED and subsequently reduces the bioavailability of NO and increases the platelet activation, SMC and expression of adhesion molecules participating from the first step in the development of atherothrombosis.

Thus, hyperglycaemia is likely to induce and perpetuate inflammation through increased mitochondrial reactive oxygen species (ROS) formation [115]. Furthermore, studies have shown that improving glycaemic control of T2DM can reduce plasma inflammatory proteins such as C3 complement [116]. T2DM may be implicated in the glycation of C3 and fibrinogen enhancing the incorporation of C3 in fibrin clots and promoting a prothrombotic estate [67]. The discovery in the 1990s that adipose cells were capable of secreting the pro-inflammatory cytokine, TNF-α, which subsequently able induced insulin resistance changed our approach [117].

2.6.1.Insulin resistance as component of the metabolic triad

The concept of a vasculo-metabolic theory interrelates two pathogenic bidirectional ways in the process of generating and maintaining low-grade fat inflammation and activation of adipose tissue stroma [94]. This theory of a meta-process combines the vascular triad, which includes endothelial dysfunction, oxidative stress and vascular inflammation, and the metabolic triad, which is composed by insulin resistance (IR), metabolic inflexibility (MI) and endoplasmic reticulum (ER) stress.

Metabolic flexibility has been defined as the capacity for the organism to adapt fuel oxidation to fuel availability. In metabolic patients the inability to modify fuel oxidation in response to changes in nutrient availability has been implicated in the accumulation of intramyocellular lipid and insulin resistance. Insulin-resistant patient become metabolically inflexible (MI) and their ability to switch from fat to carbohydrate oxidation is usually impaired during a hyperinsulinemic clamp [104].

ER stress plays a role in the pathogenesis of diabetes, obesity, cardiovascular diseases [118] and myocardial damage by contributing to pancreatic beta-cell loss and insulin resistance. Components of the unfolded protein response (UPR) play a dual role in beta-cells, acting as beneficial regulators under physiological conditions or as triggers of beta-cell dysfunction and apoptosis under situations of chronic stress such as chronic high glucose and fatty acid exposure.

High fat feeding and obesity induce ER stress in liver, which suppresses insulin signalling via kinase activation and contributes to cytokine-induced beta-cell death. The B-cell mediators, namely cytokines IL-1beta and interferon-gamma, induce severe ER stress and NO-mediated depletion of ER calcium and amplify the proapoptotic pathways [119]. Moreover, the endoplasmic reticulum stress induces autophagy response, a catabolic and degradation process for long-lived proteins and unnecessary or damaged organelles [120]. In healthy conditions starvation and cellular nutrient limitation triggers this stress, but also pathological conditions given in cardiometabolic subjects produce the autophagic response as prof. Y. Ohsumi, 2017 Medicine Nobel Prize, and other authors noted [121].

As a confirmation of this vasculo-metabolic theory hyperinsulinemia also comes along with raised levels of PAI-1 (Plasminogen-Activator Inhibitor-1), main inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the activators of plasminogen and hence fibrinolysis, and it appears to be also associated with endothelial dysfunction, left ventricular hypertrophy and coronary disease.

2.6.2.Insulin resistance as single cardio-metabolic factor

The **insulin resistance** (IR) is intimately associated with abdominal obesity and a variety of abnormalities that impact upon the cardiovascular system, such as T2DM, hypertension, an atherogenic lipid profile that includes hypertriglyceridemia and low serum HDL-cholesterol concentrations, and coronary disease. Adipose cells are metabolically active and lead to the hyperglycaemic hyperinsulinaemic state which characterised T2DM. Fatty hepatocytes and adipocytes increase the recruitment of inflammatory cells. These active inflammatory macrophages raise the cytokine production, which in conjunction with insulin resistance through impairment of adipocyte differentiation generate a positive feedback for hepatic and skeletal muscle lipid accumulation.

Insulin resistance has been defined as a subnormal biological response to normal insulin concentrations. In clinical practice, insulin resistance refers to a state in which a given concentration of insulin is associated with a subnormal glucose response [122]. This abnormal response is to both, endogenous and exogenous insulin. Insulin resistance, instead of being a rare complication of the diabetes' treatment is now recognized as a component of several disorders, including the following:

- Major causes of insulin resistance: Inherited states of target cell resistance, leprechaunism (insulin-receptor mutations), Rabson-Mendenhall syndrome (insulin-receptor mutations), type A syndrome of insulin resistance (insulin-receptor mutations in some) and lipodystrophies.
- Secondary insulin resistance: obesity (adipocytokines may contribute), excess counter regulatory hormones (glucocorticoids, catecholamines, growth hormone, placental lactogen), type 2 diabetes mellitus (secondary to obesity and other factors), inactivity, stress, infection (counter regulatory hormones), pregnancy (placental lactogen), immune mediated (anti-insulin antibodies), starvation, uraemia, cirrhosis and ketoacidosis.
- Multifactorial aetiology of insulin resistance: HBP, polycystic ovary syndrome, MetS, T2DM.

Insulin resistance contributes to the pathophysiology of diabetes and is a hallmark of obesity, metabolic syndrome, and many cardiovascular diseases. Therefore,

quantifying insulin sensitivity/resistance in patients was of great importance for epidemiological and clinical studies, and eventual use in clinical practice [123].

In a research setting, the hyperinsulinemic euglycemic insulin clamp technique has been considered to be the gold standard, and intravenous glucose tolerance test (IVGTT) and/or the insulin tolerance test (ITT)/insulin suppression test are the tests most frequently used [124]. It is an invasive test and takes about two hours. The insulin is perfused through a peripheral vein and in order to balance the glycaemia a glucose 20% is also infused.

Low-dose insulin infusions are more useful for assessing the response of the liver, whereas high-dose insulin infusions are useful for assessing peripheral insulin action. Levels between 4.0 and 7.5 mg/min are not definitive and suggest "impaired glucose tolerance," an early sign of insulin resistance [123]. The main limitations of the glucose clamp approach are that it is an invasive technique, time consuming, expensive, and requires an experienced operator to manage the technical difficulties.

The modified insulin suppression test is another measure of insulin resistance is developed by Gerald Reaven at Stanford University. The test suffers from less operator-dependent error than the clamp. Nonetheless, it is also requires a complicated methodology with infusion of octreotide, somatostatin, insulin and glucose. Subjects with steady-state plasma glucose level greater than 150 mg/dl are considered to be insulin-resistant [125].

However, both techniques are impractical for routine clinical use, the methods to directly measure insulin resistance are invasive, complex, and costly. Given the complicated nature of the "clamp" technique (and the potential dangers of hypoglycemia in some patients) to directly measure the insulin sensitivity, alternatives have been sought to simplify the measurement of insulin resistance.

Several authors developed simple surrogated indexes to calculate the relation in between insulin resistance and sensitivity. The first was the **Homeostatic Model Assessment** (HOMA), and a more recent method is the Quantitative insulin sensitivity check index (QUICKI). Both employ fasting insulin and glucose levels to calculate insulin resistance, and both correlate reasonably with the results of clamping studies. Simple surrogate indexes of insulin sensitivity/resistance are inexpensive quantitative tools that can be easily applied in almost every setting, including epidemiological studies, large clinical trials, clinical research investigations, and clinical practice.

To develop these indexes we have to assume that our patients accomplish a strictly fasting conduct along the previous night to reflect a primary hepatic insulin sensitivity versus resistance during a basal state of plasma glucose and Insulin levels. Both methods are widely used in our department of endothelial pathology, HOMA has been chosen for this study because of practicality.

The **homeostasis model assessment** (HOMA) was developed in 1985 by Matthews and cols.[126]. At that time the steady-state basal plasma glucose and insulin concentrations were determined by their interaction in a feedback loop. A computer-solved model was used to predict the homeostatic concentrations which arose from varying degrees beta-cell deficiency and insulin resistance.

Matthews and cols. compared the patient's fasting values with the model's predictions and allowed a quantitative assessment of the contributions of insulin resistance and deficient beta-cell function to the fasting hyperglycaemia. The accuracy and precision of the estimation was determined by comparison with independent measures of insulin resistance and beta-cell function using hyperglycaemic and euglycaemic clamps and an intravenous glucose tolerance test. HOMA-IR had a reasonable linear correlation with glucose clamp and minimal model estimates of insulin sensitivity/resistance in several studies of distinct populations [127]. Both, the original HOMA and the updated HOMA2 assume a feedback loop between the liver and β-cell [128].

The approximating equation for insulin resistance, in the early model, used a fasting plasma sample, and was derived by use of the insulin-glucose product, divided by a constant: (assuming normal-weight, normal subjects < 35 years, having 100% β-cell function an insulin resistance of 1). In the table below referred to the primary HOMA model, IR is insulin resistance and % β is the β-cell function. Insulin is given in mU/L. Glucose and insulin are both analyzed during fasting basal conditions. The HOMA model follows the formulas presented below:

$\text{HOMA-IR} = \dfrac{\text{Glucose} \times \text{Insulin}}{22.5}$	$\text{HOMA-IR} = \dfrac{\text{Glucose} \times \text{Insulin}}{405}$
$\text{HOMA-}\beta = \dfrac{20 \times \text{Insulin}}{\text{Glucose} - 3.5}\%$	$\text{HOMA-}\beta = \dfrac{360 \times \text{Insulin}}{\text{Glucose} - 63}\%$
Fasting Glucose in Molar Units mmol/L	**Fasting Glucose in mass units mg/dL**

In clinical practise, most studies using HOMA employ an approximation described by a simple equation to determine a surrogate index of insulin resistance. The denominator of 22.5 is a normalizing factor. This factor is determined by the normal individual results: the product of normal fasting plasma insulin of 5 µU/ml and normal fasting plasma glucose of 4.5 mmol/l typical of a “normal” healthy individual = 22.5. Therefore, for an individual with “normal” insulin sensitivity, HOMA-IR = 1. Even so, the coefficient of variation for HOMA-IR varies considerably depending upon the number of fasting samples obtained and the type of insulin assay used [63]. Hence, Log (HOMA-IR) is useful for evaluation of insulin resistance in individuals with glucose intolerance, mild to moderate diabetes, and other insulin-resistant conditions. However, in subjects with severely impaired or absent β-cell function, HOMA-IR may not give appropriate results. The updated HOMA2 adjusted the assessment of HOMA%S and HOMA%B in subjects with glucose levels ≤25 mM, accounts for renal glucose losses, assumes reduced suppression of HGP and increased insulin secretion in response to glucose levels >10 mM, and allows for the use of total or specific insulin assays [128].

The formula was based on the principle that assumes glucose concentrations are regulated by insulin-dependent hepatic glucose production, whereas insulin levels depend on the pancreatic β-cell response to glucose concentrations. Thus, deficient β-cell function reflects a diminished response of β-cell to glucose-stimulated insulin secretion. Likewise, insulin resistance is reflected by diminished suppressive effect of insulin on hepatic glucose production. Decreases in [beta]-cell function were modeled by changing the [beta]-cell response to plasma glucose concentrations. Insulin sensitivity was modeled by proportionately decreasing the effect of plasma insulin concentrations at both the liver and the periphery [129]. In either situation, the glucose turnover in the model remains constant. In the HOMA model, no distinction has been made between hepatic insulin sensitivity and peripheral insulin sensitivity. The model predicts fasting basal levels of plasma glucose and insulin for any given combination of pancreatic β-cell function and insulin sensitivity.

On the other hand, the **quantitative insulin sensitivity check index** (QUICKI) is derived using the inverse of the sum of the logarithms of the fasting insulin and fasting glucose:

$$1 / (\log(\text{fasting insulin } \mu U/mL) + \log(\text{fasting glucose mg/dL}))$$

This index correlates well with glucose clamp studies (r = 0.78), and is useful for measuring insulin sensitivity (IS), which is the inverse of insulin resistance (IR). It has the advantage of that it can be obtained from a fasting blood sample, and is the preferred method for certain types of clinical research [130]. During development of QUICKI, sensitivity analysis of data from the first 20 min of an FSIVGTT revealed that physiological fasting steady-state values of plasma insulin and glucose contain critical information about insulin sensitivity as determined by the reference standard glucose clamp [131] . Since fasting insulin levels have a non-normal skewed distribution, log transformation improves its linear correlation with insulin sensitivity.

To accommodate some clinical circumstances such as where fasting glucose is inappropriately high and insulin is inappropriately low, addition of log (fasting glucose) to log (fasting insulin) provides a reasonable correction such that the linear correlation with insulin sensitivity, is maintained in both diabetic and non-diabetic subjects. Log (HOMA) is roughly comparable to QUICKI in this regard. Nevertheless, both indexes are mathematically related, QUICKI is proportional to 1/log (HOMA-IR).

For selected patients, who have been brought under the oral glucose tolerance test, we can perform another test with dynamic properties. Obviously, dynamic testing requires more effort and cost than simple fasting blood sampling. Dynamic indexes that depend on dynamic testing take into account both fasting steady-state, dynamic postglucose load plasma glucose and insulin levels. Glucose disposal after an oral glucose load or a meal is mediated by a complex dynamic process that includes absorption, glucose effectiveness, neurohormonal actions, incretin actions, insulin secretion, and metabolic actions of insulin that primarily determine the balance between peripheral glucose utilization and hepatic glucose production. Logically, the oral glucose intake is more physiological than intravenous glucose infusion, taking into account the possible variability in the glucose absorption. For this specific patients spectrum in our unit we develop the Matsuda index.

The oral glucose tolerance test is a simple test widely used in clinical practice to diagnose glucose intolerance and T2DM. After an overnight fast, blood samples for determinations of glycaemia and insulin concentrations are taken at 0, 30, 60, and 120 min following a standard oral glucose load (75 g). The OGTT mimics the glucose and insulin dynamics of physiological conditions. more closely than conditions of the OGTT

is less invasive than the glucose clamp, IST, or FSIVGTT. Besides this, OGTT provides useful information about glucose tolerance but not insulin sensitivity/resistance per se.

Because of this Matsuda and cols. proposed an insulin sensitivity index (ISI Matsuda) where the insulin secretion activity could be obtained at the same time as the insulin action was measured, where fasting glucose and insulin data are taken from time 0 of the OGTT, and the mean data represents the average values obtained during the whole process:

$$ISI_{(Matsuda)} = 10{,}000/\sqrt{[(G_{fasting} \times I_{fasting}) \times (G_{OGTTmean} \times I_{OGTTmean})]}$$

Nevertheless, HOMA or log(HOMA) are used widely in large epidemiological studies, prospective clinical trials, and clinical research studies. A big portion of them relate the insulin resistance measured by HOMA model to metabolic and hemodynamic alterations and higher cardio metabolic risk [67-70]. A cross-sectional and longitudinal research established relationships between C-reactive protein (CRP), a marker of low-grade inflammation, and insulin resistance and whether the association was independent of obesity and oxidative stress [132]. Inflammation, measured by plasmatic CRP levels, showed a significant positive association with insulin resistance. During the last decade several authors have developed successful research which correlates C3 convertase, inflammation, metabolic syndrome, diabetes, hyperlipidemia, polycystic ovary syndrome, and new cardiovascular risk factors to HOMA and insulin resistance [72-77]. All of them seed a fruitful growing field to develop future studies which will connect a logical chain from C3 convertase, arterial chronic inflammation, metabolic risk factors, insulin resistance and cardiovascular events.

Besides this, the criteria for metabolic syndrome are just a part of the cardiovascular risk factors. We have to remember that the classic risk factors also include smoking and a sedentary lifestyle. Into the bargain, during the last 3 decades the authors have included new interesting risk elements such as polycystic ovary syndrome, chronic liver disease, hyperferritinemia among others.

2.7. New components of metabolic syndrome

2.7.1. Hyperuricemia

Kyllin observed the role of hyperuricemia as a component of the metabolic syndrome. This observation was forgotten in the 20th century, but strong evidences during the last two decades reintroduced the hyperuricemia in the complex cocktail of cardiometabolic factors. Hence, large epidemiologic studies have shown that hyperuricemia is associated with an increased incidence of cardiovascular events, specifically coronary heart disease. Lately, hyperuricemia has been also correlated to metabolic syndrome [133], also in Mediterranean population [134]. It has been also demonstrated an increased mortality rate [135], mainly in patients with heart failure, due to a decrease in the tissue perfusion. It is unclear if hyperuricemia has a causal effect, but it may develop HBP and oxidative stress, or is simply a marker for HBP, dyslipidaemia, and diabetes [136].

Lehninger described uric acid as the end product of purine degradation, nucleic acids and nucleoproteins [133, 137]. Hyperuricemia is a frequent metabolic disorder in general population [138]. The observed prevalence in studies of Tecumseh and Framinghan was about 5%. Uric acid overload is usually caused by an unhealthy lifestyle and the progression from asymptomatic hyperuricemia to urate deposition and advanced gout with organ damage varies among individuals.

Body Mass Index (BMI) has been identified as the best predictor for levels of uric acid, showing a strong positive correlation [139]. Uric acid overload has been considered as a component of the called "insulin resistance syndrome " or "Syndrome X" or Metabolic Syndrome (MetS). Hyperuricemia is also associated with the consumption of drugs most frequently the use of diuretics and antihypertensives. Therefore, before considering hyperuricemia as a component of the syndrome, some authors recommend to rule it out as "secondary hyperuricemia". Hyperuricemia has been linked to cardiovascular pathology such as myocardial infarction, stroke, hypertension and heart failure [140, 141].

Uric acid can change its chemical activity of antioxidant to prooxidant when entering the atherosclerotic plaque [142]. Within the plaque, the uric acid contributes to oxidize

lipoproteins in patients with metabolic syndrome and diabetes mellitus type 2. The association between uric acid and MetS promotes sinergically the renin angiotensin aldosterone system activation. Angiotensin II is a potent inducer of NADPH oxidase, which increases NADPH, this in turn increases the reactive oxygen species (ROS) in the arterial intima-media layers [143]. Meanwhile, hyperinsulinemia increases the renal reabsorption of urate.

The association between MetS and increased uric acid levels was highlighted by Professor Reaven (Hayden, 2004), discoverer of the syndrome-X, then called metabolic syndrome. Lately uric acid levels have been correlated to Mets, insulin resistance and C3 complement in Japanese population [144]. In an experimental model of MS developed in rats an increase of uric acid in the plasma, concomitant with increased triglycerides, hyperinsulinemia and hypertension was observed [145]; as well as, increased reactivity of the left ventricular myocardium together with endothelial dysfunction expressed as attenuation vasodilator response to acetylcholine in aortic rings according to Rosa, et al. in 2005.

The mechanisms that can raise uric acid in hypertensive are as follows: first, reduced blood flow, second, microvascular local ischaemia, third, increased lactate production by the aforementioned ischemia, which blocks the secretion of urate the proximal tubule, increased degradation of RNA and DNA which increases the uric acid synthesis by xantine-oxidase protein [146]. All this increases ROS production neutralizing endothelial nitric oxide and produce vascular endothelial dysfunction. Treatments to reduce uric acid concentrations, allopurinol and oxypurinol (inhibitors of xanthine oxidase), reverse the reduced synthesis of endothelial nitric oxide in patients with heart failure and T2DM. Despite controlling blood pressure with medication, hyperuricemia is significantly associated with increased incidence of cardiovascular events. The DASH diet (Dietary Approach to Stop Hypertension) not only reduces blood pressure but also the the plasma concentration of uric acid and its co-morbidities [147].

2.7.2. Hyperferritinemia

Hyperferritinemia is associated with inflammatory processes such as metabolic syndrome [148], chronic rheumatological disorders, chronic anemia or hemochromatosis, iron overload after transfusions, chronic non-alcoholic liver disease,

hemochromatosis and other hereditary diseases. Therefore, it is undisputed its relationship with the inflammatory generation of atherosclerotic plaques and consequently with markers of the inflammatory cascade and acute phase reactants.

Iron overload in vital organs, even in mild cases, increases the risk of atherosclerosis [149], dyslipidaemia [150], heart failure [151], metabolic syndrome[152], myocardial infarction [150], hypothyroidism [153], hypogonadism [153], osteoarthritis, osteoporosis, liver disease (cirrhosis), numerous symptoms and in some cases premature death. Mismanagement resulting in iron overload can accelerate neurodegenerative diseases such as Alzheimer's, early onset Parkinson's, Huntington's, epilepsy and multiple sclerosis [154].

The liver is the major reservoir of iron. Excess stored iron in the liver causes hyperinsulinemia via both decreased insulin extraction and impaired insulin signalling. On top of this hyperinsulinemia encourages and increase of iron deposition as a positive feedback [155]. Iron deposits within hemosiderin in different cells, including b-cells, induce apoptosis and impaired response to insulin in the liver, muscle, and adipose tissue [156]. Subsequently, iron overload leads to diabetes by progressively reducing their β-cell function. Disruption of iron homeostasis, either in excess or in defect, results in impaired adipocyte differentiation and may affect insulin action by modulating the degree of adiposity. Iron-enriched diets reduce the adipocyte size and its insulin sensitivity [157].

The relationship between hyperferritinemia and various cardiovascular risk factors has been shown in multiple studies. A long list of metabolic risk factors has been correlated to iron overload such as diabetes mellitus [116], central obesity [158], metabolic syndrome [152], hypertriglyceridemia and dyslipidaemia [159], hepatic steatosis [160], insulin resistance [161], damage vascular [162], lipid metabolism disorders [163] among others. Increased transferrin saturation due to iron overload showed a dose-dependent association with an increased total mortality [164]. Excess iron store contributes to the pathogenesis of MetS, T2DM, entothelial dysfunction and oxidative damage [165].

2.7.3. New cardiovascular biomarkers

Multiple cardiometabolic markers have been involved during the last two decades in the complex process of atheroma formation. Some of them were used in the daily clinical activity of the Endothelium and Cardiometabolic Medicine Unit (ECMU). These available data represented an excellent opportunity to compare the C3 complement activity with the new cardiometabolic markers participation in the cardiovascular pathology development.

2.7.3.1. Inflammatory biomarkers: Adiponectin TNFα IL6 IL10

Inflammation, already explained earlier on in this chapter, was widely known as to play a key role in the development and progression of cardiovascular diseases and obesity has been linked to many proinflammatory and cardiovascular conditions. It has also been observed that adipokines play an important role in systemic and local inflammation and adipose tissue have a more important role than previously thought in the pathogenesis of cardiometabolic syndromes. Adipokines (such as leptin, TNF-α, PAI type 1, IL-1β, IL-6, and IL-8) are proinflammatory and increased in cardiometabolic pathologies. As Inflammatory markers and inmunomodulatory factors TNF-α and adiponectin intersect with the inflammation associated with both cardiovascular and metabolic pathologies.

Tumour necrosis factor-α (TNF-α):

TNF-α as part of the inflammatory cascade, plays a key role in the formation of atherosclerotic lesions [166]. Among many inflammatory markers TNF-α emerged as a pleiotropic cytokine that influences intermediary metabolism. TNF-α has proinflammatory properties, which play crucial roles in the innate and adaptive immunity, cell proliferation, and apoptotic processes. Tumour necrosis factor-α (TNF-α) has been correlated with chronic inflammatory conditions where a shift toward a proatherogenic lipid profile and impaired glucose tolerance occurs and worsen prognosis [167]. Adipose tissue secretes inflammatory cytokines, such as TNF-α, which in turn contribute to impaired glucose tolerance, insulin resistance, and T2DM. The TNF-α cytokine is produced by different kind of cells (macrophages, monocytes, T-

cells, smooth muscle cells, adipocytes, and fibroblasts). In animal models, administration of TNF-α led to severe impairment of glucose tolerance and insulin sensitivity [168]. TNF-α has emerged as an important contributor to the development of atherosclerotic lesions, by promoting the expression of adhesion molecules on endothelial cells, recruitment and activation of inflammatory cells and initiation of the inflammatory cascade inside the arterial wall. Has been demonstrated that TNF-α directly interferes with the metabolic pathways of TGs and cholesterol and the development of dyslipidaemia, insulin resistance, metabolic syndrome and cardiovascular diseases [169].

Secondly, TNF-α is able to induce proatherogenic changes in lipoproteins and influences the lipid metabolism. This action has been seen in patients with acute and chronic inflammatory disorders, such as sepsis and AIDS. Both pathologies, in which increased TNF-α concentrations occur, have been shown to have increased TG concentrations. Also, the administration of TNF-α and endotoxin (LPS) to mice and humans resulted in an acute TNF-α induced hypertriglyceridemia in several studies. The effects of TNF-α on plasma triglycerides concentration occur through effects on both adipose tissue and liver triglycerides metabolic pathways. Besides increasing the concentration of TG-rich VLDL particles, TNF-α may also alter their composition, making them proatherogenic[170]. TNF-α is also involved in the cholesterol metabolism although the mechanisms are nor clear, high concentrations of TNF-α have been correlated to a reduction of HDLc concentrations and may decrease hepatic cholesterol catabolism and excretion [171].

Finally, TNF-α, by decreasing insulin sensitivity, interferes in the glucose metabolis[172]. Obesity has been proved as a state of low-grade chronic inflammation with increased concentrations of C-reactive protein, IL-6, and other inflammatory markers in plasma. Consequently other authors investigated the interaction between inflammation, TNF-α and diabetes. In 1993, TNF-α was the first inflammatory marker demonstrated to be involved in the pathogenesis of insulin resistance [173]. It was demonstrated that adipocytes became insulin-resistant when exposed to TNF-α by inhibiting the insulin-stimulated tyrosine kinase activity of the insulin receptor [174]. The TNF-α also stimulates lipolysis in the adipose tissue, thus increasing the plasma concentration of the free fatty acids that eventually contributes to the development of the insulin-resistant phenotype. All these factors contribute to the fact that TNF-α was also correlated to endothelial dysfunction [175].

Adiponectin:

Adiponectin is a cytokine, “adipokine”, produced almost exclusively in adipose tissue. Its concentrations are higher in healthy individuals and decline in pathological and inflammatory conditions such as diabetes, hypertension, obesity and cardiovascular pathologies inversely to TNF-α levels [176, 177]. Low adiponectin levels are inversely related to high levels of inflammatory markers, such as C-reactive protein (CRP), in patients with obesity, type 2 diabetes, and cardiovascular diseases [178].

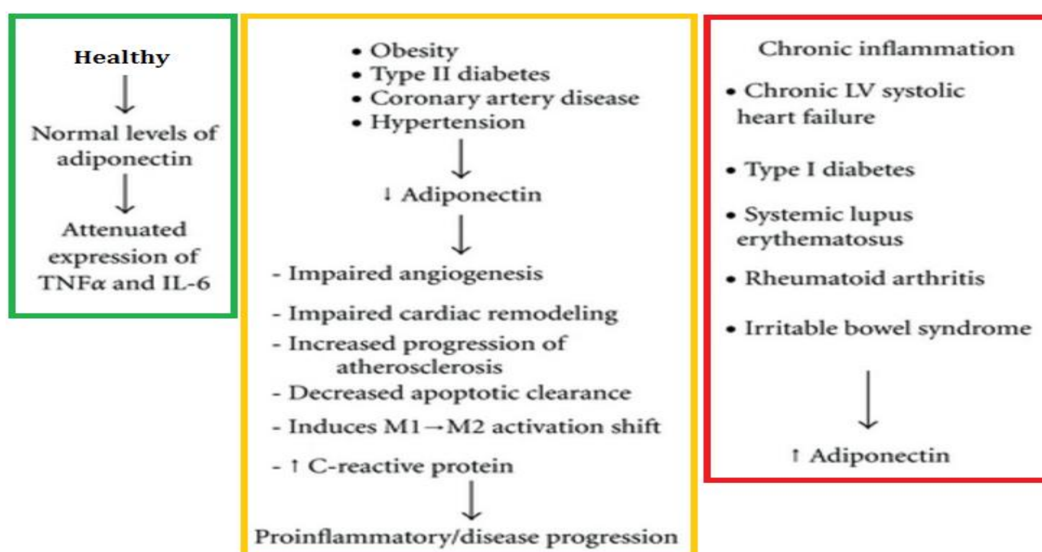

Fig. 2.5: In healthy individuals, adiponectin maintains anti-inflammatory properties. Cardiometabolic pathologies where adiponectin levels decrease result in pro-inflammatory signalling and exacerbation of disease.

Adiponectin inhibits the expression of TNF-α in adipocytes, and likewise, both TNF-α and IL-6 inhibit the production of adiponectin [173]. Negative regulation of adiponectin expression also results from hypoxia and oxidative stress . In cardiometabolic patients, adiponectin levels are decreased, and the ability of adiponectin to inhibit the inflammatory processes becomes limited. Hypoadiponectinemia also contributes to insulin resistance, impaired endothelium-dependent vasodilatation, impaired ischemia-induced neovascularization, hypertension progression, and diastolic heart failure [70, 174-176].

Thus, adiponectin mediates protective effects cardio-metabolic and vascular disease presumably by its anti-inflammatory actions and protects the heart against ischemia-reperfusion injury through its ability to suppress myocardial inflammation and apoptosis [177].

Interleukin-6 (IL-6):

IL-6 is a multifunctional signalling cytokine which modulates responses to cardiovascular diseases and an independent indicator of vascular homeostasis. IL-6 is produced by a wide variety of vascular cells, including macrophages, lymphocytes, fibroblasts, endothelial cells and smooth muscle cells, and its secretion is up-regulated in response to inflammation, angiotensin II, oxidative stress, endothelial damage and vascular injury. IL-6 upregulates AT1R gene expression, it leads to increased angiotensin-mediated vasoconstriction and ROS production, and thereby plays an important role in mediating endothelial dysfunction. Consistent with this idea, it was observed that IL-6 deficiency protects against angiotensin-induced endothelial dysfunction [179, 180].

As an inflammatory factor, IL-6 promotes macrophage differentiation, growth arrest and eventual apoptosis. IL-6 stimulation causes these cells to increase in size, develop irregularly shaped nuclei, larger vacuolar cytoplasm and become surface adherent inducing thrombosis. Through direct membrane receptor or trans-signaling modalities, IL-6 has diverse actions including modulating endothelial-dependent vasorelaxation, monocyte differentiation, platelet function, pro-coagulant state, myocardial hypertrophy, and effects on obesity and intermediary metabolism [181].

IL-6 levels are positively correlated to cardiovascular risk and myocardial re-infarction, obesity, diabetes, metabolic syndrome and inversely correlates with AHA functional classification, ejection fraction, and survival [182]. IL-6 has actions locally in the vessel wall in coronary atherosclerotic plaques interacting with angiotensin-stimulated vessels. Here, IL-6 is predominantly expressed by fibroblasts and activated macrophages in the adventitial and endothelial layers signalling pathway during very early phases of atherosclerosis [183].

Interleukin-10 (IL-10):

. IL-10 has been considered the prototype of anti-inflammatory interleukins [184]. Contrarily to the previously mentioned pro-inflammatory cytokines, IL-10 acts as an endothelial protector. Its effector functions include a shift of T-cell cytokine expression, down-regulation of the production of pro-inflammatory cytokines by macrophages and represent the autocrine negative feed-back and cross-talk inhibition of cytokine signalling. IL-10 inhibits the induction of the pro-inflammatory cytokines such as TNFα and IL-6. IL-10 also enhances B cell survival, proliferation, and antibody production.

Moreover, IL-10 reduces vascular injury by its paracrine effect mediated by modulation of immune function by reduction in inflammatory gene expression [185].

IL-10 is expressed in human atherosclerotic plaque reducing atherogenesis and improving the stability of plaques. Increased IL-10 serum levels are associated with improved systemic endothelial vasoreactivity in patients with coronary artery disease [186]. Besides this, IL-10 has been linked to myokines showing that exercise promotes an increase in circulating levels of IL-10 and fosters an environment of anti-inflammatory cytokines. Therefore, IL-10 levels have been inversely associated to metabolic syndrome, vascular diseases, obesity, diabetes, fatty liver and insulin resistance [187].

Lipoprotein(a) (Lp(a)):

As cardiovascular risk marker, Lp(a) was a lipoprotein subclass discovered by Berg in 1963. Genetic and epidemiologic studies have identified Lp(a) as a risk factor for cardiovascular diseases. Lp(a) consists of an LDL-like particle and the specific apolipoprotein(a), which is covalently bound to the apoB of the LDL-like particle. The mechanism and sites of Lp(a) catabolism, but the kidney has been identified as playing a role in Lp(a) clearance from plasma and Apo(a) is expressed by hepatocytes [188]. Lp(a) as atherogenic carrier attracts inflammatory cells to vessel walls, reduces plasmin generation and leads to inflammation and smooth muscle cell proliferation. Its association with proteins of the acute phase response has been demonstrated [189].

Among its main functions, Lp(a) contributes to the process of atherogenesis. The macorphages low density lipoprotein receptor mediates de Lp(a) catabolism via endocytosis and lysosomic degradation leading to macrophagic accumulation of lipids. Lp(a) promotes endothelial dysfunction via selective impairment of vasodilation, binding extracellular matrix components and oxidizing LDLc [190]. Lp(a) also leads to thrombogenesis due its structural similarity to plasminogen stimulates PAI-1 secrection [191]. Moreover, Lp(a)'s competitive inhibition with fibrinogen reduces fibrinolysis. Lp(a) also binds to macrophages via a high-affinity receptor that leads to foam cell formation and the deposition of cholesterol in atherosclerotic plaques.

High Lp(a) has become a new modest independent predictor of atherosclerosis, especially myocardial infarction, and a coagulant risk of plaque thrombosis indicator after adjustment for traditional cardiovascular risk factors [192]. Lp(a) is also associated with unstable angina and complex coronary lesions, suggesting a possible role in

plaque rupture and coronary thrombosis. In patients with an acute coronary syndrome, Lp(a) concentrations predict increased risk of cardiac death [193]. The European Atherosclerosis Society recommended to measure Lp(a) levels in patients with moderate or high cardiovascular risk.

2.7.3.2. Oxidative stress markers: TAP, homocysteine.

Total antioxidant capacity of plasma (TAP):

Oxidant factors, such as superoxide anion (O2-) and hydrogen peroxide, are produced in the body as a consequence of normal aerobic metabolism. These oxidant molecules interact with reactive oxygen species (ROS). Under normal physiologic conditions, the production of oxygen free radicals and peroxides is balanced by a system of antioxidants preventing oxidative damage [194]. The plasma capacity to scavenge ROS is measured by the TAP.

At the cellular level, enzymatic antioxidants, such as superoxide dismutase and catalase, convert ROS to oxygen and water. Several non-enzymatic antioxidants, such as vitamins E and C, also tackle free radicals. In chronic inflammation, atherosclerosis, the free radicals are presented in excess leading to the pathologic condition of oxidative stress [195]. Measurement of the combined enzymatic plus non-enzymatic antioxidant capacity of plasma provides an indication of the overall capability to counteract reactive oxygen species (ROS), resist endothelial oxidative damage and combat oxidative stress.

TAC has been inversely related to the presence of obesity diabetes, metabolic syndrome insulin resistance, intima-media thickness and cardiovascular diseases [196-198]. Antioxidants inhibit lipid peroxidation and therefore play a protective role in the development of cardiovascular disease by preventing the formation of early atherosclerotic lesions. Plasma concentrations of individual antioxidants could be measured separately in laboratory, but these measurements are time-consuming and costly. Therefore, since antioxidant effects of antioxidant components of plasma are additive, several methods have been developed to determine the total antioxidant plasma status [199].

Homocysteine (Hcy):

Hyperhomocysteinemia has been described as high levels of Hcy in blood. Etiologically, hyperhomocysteinemia is the consequence of a genetic disorder, certain drugs or related to nutritional deficiency in vitamins B6, B9 and B12. Hcy concentrations can be reduced by vitamins supplements.

Hyperhomocysteinemia has been associated with cardiovascular pathology. Hcy has atherogenic and prothrombotic properties, promotes intimal thickening, elastic lamina disruption, smooth muscle hypertrophy, marked platelet accumulation, and the formation of platelet-enriched occlusive thrombi [200]. Supplements with folic acid lower Hcy concentrations and reduce endothelial dysfunction [201] and oxidative stress [202, 203]. Hyperhomocysteinemia screening has been recommended in Canada for people at moderate CVD risk.

Hcy promotes vascular injury by recruiting leukocytes, up-regulating pro-inflammatory interleukines expression and secretion[204], interacting with LDLc and macrophages activation which release the lipids into atherosclerotic plaques and increasing smooth muscle cell proliferation and enhances collagen production [205]. Hcy is also a pro-thrombotic factor inhibiting plasminogen activation, heparin sulphate and endothelium-mediated platelet-aggregation inhibition [206]. Evidence shows that hyperhomocysteinemia is a risk factor for venous thromboembolic disease (pulmonary embolism and deep vein thrombosis) [207, 208].

Thiobarbituric acid reactive substances (TBARS)

TBARS are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS assay using thiobarbituric acid as a reagent. The TBARS assay has been assessed as an indicator of oxidative stress in a multiple cardiovascular disease models [209].

In vivo, plasma TBARS concentration can be normalized through supplementation with various antioxidants [201]. TBARS were found to be elevated in the serum of cigarette smokers, patients with documented coronary artery disease and carotid atherosclerotic plaque progression. Serum levels of TBARS could predict major cardiovascular events and the need for a major vascular procedure independently of traditional risk factors.

Animal and human studies therefore support a potential role of lipid oxidation in predicting the progression of CVD and response to therapies [210].

3. HYPOTHESIS AND OBJECTIVES

3. Hypothesis and objectives

3.1 Hypothesis

C3 complement levels are positively correlated to higher cardiovascular risk and endothelial dysfunction in patients referred to cardiometabolic secondary care

3.2 Objectives

Primary

1. Assess the parallelism between C3 complement levels and cardiovascular risk score amongst the Spanish population (REGICOR).

Secondary

1. Assess the role of C3 complement as a counterpart of the inflammatory cascade in correlation to other inflammatory markers.
2. Study the correlation of C3 levels with insulin resistance as measured by the HOMA model.
3. Evaluate the C3 levels' correspondence with metabolic syndrome criteria.
4. Examine the C3 convertase measurement parallelism with classical cardiovascular factors in cardiovascular risk prognosis.
5. Analyse the role of C3 complement as an oxidative stress marker and its interrelationship with other oxidative stress markers.
6. Appraise the correspondence between C3 complement measurements with endothelial damage biomarkers.

4. MATERIAL AND METHODS

4. Material and methods

4.1. Study design

An observational, cross-sectional, retrospective study was designed. For the initial database, we selected all participants who were referred to secondary cardiometabolic care at the ECMU during their first visit between December 2002 and September 2012 and had their C3 complement levels measured.

This research was designed to evaluate the aforementioned objectives. The study sample population was selected from an original cohort of n=776 according to the exclusion objectives (figure 5.2).

Study design and population diagram:

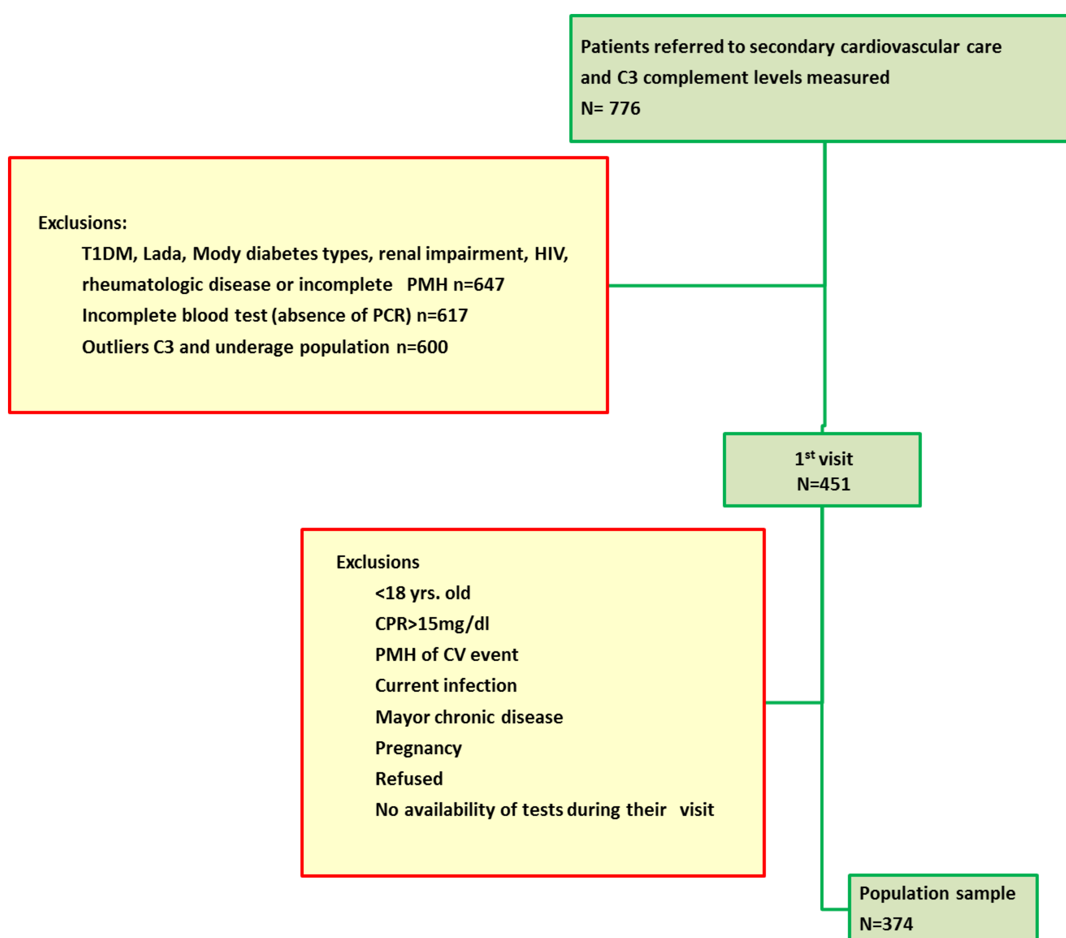

Figure 4.1: Study design inclusion and exclusion criteria's flow chart obtaining the baseline population sample.

4.2. Study population

Our cohort evaluated consisted of patients from the Endothelium and Cardiometabolic Medicine Unit (ECMU) at Ramon y Cajal University Hospital (Madrid, Spain).

The random sample included Spanish individuals from the province of Madrid referred to cardiometabolic secondary care from primary care, plus voluntary healthy first-degree family members of these cardiovascular pathology patients. The group of patients referred to secondary care comprises a heterogenic cluster of cardiometabolic pathologies and healthy individuals including the following: diabetes, dyslipidaemia, hypertension, obesity, MetS, first-degree family history of early coronary disease (parents or siblings), coronary, cerebral or peripheral established atherosclerosis, precocious menopause (<40 years), hyperferritinemia (exception: alcoholism in activity) and voluntary healthy family members.

All the individuals studied were of Spanish and South American origin, both Caucasian.

Exclusion criteria

i) Subjects under age (younger than 18 years old).
ii) Individuals having CRP values >15 mg/L, given that extreme CRP values are usually not associated with cardiometabolic disorders [211].
iii) Patients with a history of current infection in the month preceding the study, such as acute hepatitis.
iv) Chronic systemic inflammation or rheumatologic chronic diseases were not elicited, such as renal impairment with creatinine levels higher than 2 mg/dl, chronic rheumatologic disease, HIV, active cancer under treatment protocol, kidney, pulmonary or liver disease.
v) Type 1 diabetes and LADA, because of their different genetic characteristics, metabolic and cardiovascular morbidity behaviour.
vi) Participants with previous or current cardiovascular event such as history of myocardial infarction, angioplasty, coronary artery bypass or cerebrovascular ischemia/stroke, symptomatic congestive heart failure, atrial flutter or unstable angina, because the aim of this study is the primary prevention of cardiovascular events.

vii) Subjects with outlier C3 complement values, according to Reed's theory, establishing 1/3 ratio as a cut-off value, where observed values smaller than one-third of the range R, to avoid bias from outlaying observations [212].

viii) Additionally, any other condition that could interfere with study participation, such as pregnancy, alcohol or drug abuse and severe neurological or mental disorder.

After the inclusion and exclusion criteria were applied, the resulting sample contained 374 participants in primary cardiovascular prevention, free from cardiovascular events.

4.3. Ethics

The study conformed to the principles embodied in the Declaration of Helsinki (Appendix 10.2) and conducted in accordance to the research activity within the clinical routine of the ECMU. The researches at the unit follow the ethical guidelines of Good Clinical Practice (GCP) of the International Committee on Harmonization (ICH) and the law Biomedical Research (14/2007 of 3 July). Verbal informed consent was obtained from all study participants after having listened to an explanatory note about the ongoing scientific researching characteristics of the service before study enrolment.

Following the 15/1999 Spanish Personal Data Protection Law, the patents included in the study were informed of their right of access, rectification and deletion of data. In case of publication of the study results, the identity of the participants would not be disclosed.

Participants in this study did not receive any form of compensation for their participation. There are no declared conflicts of interest.

4.4. Measurements of variables

4.4.1. Demographic variables

In both studies, the participants' age was measured in years and gender was divided between males and females.

4.4.2. Anthropometric parameters

i) Weight was measured without shoes in light indoor clothes using a scale in kilograms (Kg).

ii) Waist circumference was measured with a tape (Roche LI95 63B 00; Roche Diagnostics, Mannheim, Germany), with the subject standing and wearing only underwear, at the level midway between the lower rib margin and the iliac crest (centimetres). Waist circumference high risk threshold for high risk population followed the Regicor and ATP-III recommendations >102 cm for male patients and >88 cm for female patients.

iii) Height was measured in centimetres (cm) using an upright scale which consisted of a scale platform, a height scale, a height rod and a balance scale validated by the European Community standards.

iv) Body mass index (BMI) was calculated as weight divided by height squared, in kilograms per square meter (kg/m^2). BMI measurements were divided into normal weight (18-25), overweight (25-30) and obese (>30), according to the WHO recommendations.

4.4.3. Haemodynamic parameters

i) Blood pressure (BP) was measured in the sitting position on the right arm, and the mean of 2 recordings at least 3 minutes apart was recorded with OMRON 705 CP device, after 5 min of rest in the seated position. Each measurement of BP included systolic and diastolic blood pressures (SBP and DBP), considering the threshold for high blood pressure measurements >135/85 mmHg according to the ATP-III recommendations.

ii) Mean Arterial Pressure (MAP) describes the average blood pressure of an individual during a single cardiac cycle. It is determined from the following formula using the diastolic (DBP) and systolic (SBP) blood pressures previously measured: MAP=DBP + 1/3 (SBP-DBP).

iii) Pulse pressure was calculated as the result from the difference between the systolic and diastolic pressure readings.

iv) Heart rate (HR) was recorded with OMRON 705 CP device, after 5 min of rest in the seated position on the right arm.

4.5. Personal Medical History (PMH)

PMH including age (years), gender, previous diseases and allergies, 1st-degree family history of CVD (parents or siblings before the age of 55 years for men and 65 years for women), as well as physical inactivity (< 90 min/week of walking), smoking status, treatments and surgeries were recorded at baseline examination by a doctor at the ECMU during the first visit. Self-reported cigarette smoking status was categorized into non-smokers, former smokers (more than three months smoking-free) and current smokers.

4.6. Laboratory measurements and formulas

Overnight fasting blood samples were processed within 90 min of blood collection. Then, fasting serum concentrations of total cholesterol, triglycerides, HDL cholesterol, fasting glucose, creatinine and uric acid were measured (Hitachi Automatic Analyser).

The table 4.1 lists the measured biochemical parameters, units and method of measure at the Ramon y Cajal hospital general laboratory.

LDL-cholesterol was estimated indirectly with the Friedewald equation. The LDLc was analysed directly by the laboratory when chylomicrons were present and when plasma triglyceride concentration exceeded 400 mg/dL to avoid the limitations of the Friedewald equation.

The homeostatic model assessment score was calculated following the given formula: HOMA score = Fasting insulin (µU/mL) x fasting plasma glucose (mmol/L)/22.5.

The cardiovascular laboratory is located at the service of **The Endothelium and Cardiometabolic Medicine Unit (ECMU)**. The ECMU, which is part of the Internal Medicine Service of the Ramón y Cajal Hospital, was conceived in 1998, the same year that the first researchers on endothelium pathology were awarded with the medicine Nobel Prize. The ECMU is a unit with clinical and research activity [213].

Currently the unit is composed of a multidisciplinary group of professionals, doctors, nurses, nutritionists, geneticists, biologists, biochemists and engineers.

General laboratory. Biochemistry parameters	**Units**	**Measurement method**
CRP	mg/L	Behring nephelometer
Albuminuria	mg/24h	Nephelometry
Aldosterone	ng/dL	RIA Inmunotech
Creatinine	mg/dl	HITACHI autoanalyser
Complete Blood Count (polimorphonuclear leukocytes and monocytes)	n x 103/µl	GEN-S Coulter
HDLc	mg/dl	HITACHI autoanalyser
Triglycerides	mg/dl	HITACHI autoanalyser
Fasting glucose	mg/dl	HITACHI autoanalyser
Ferritin	µg/dl	MSSA
Fibrinogen	mg/dl	Behring System
GFR, glomerular filtration rate	ml/min per 1.73 m^2	Cokrofl-Gault equation. HPLC
A1c	%	HPLC (High pressure liquid chromatography)
LDLc	mg/mL	Friedewald formula
Uric acid	mg/dl	HPLC

Table 4.1: Biochemistry parameters measured at the hospital general laboratory, units and methodology.

Vascular damage is assessed in daily clinical practice by: determining 22 cardiometabolic risk biomarkers measurements at ECMU laboratory (inflammation, endothelial dysfunction, oxidative stress, chemokines and adhesion biomarkers), general blood indicators measured at the hospital general laboratory, blood pressure and glycaemia ambulatory monitoring, five non-invasive hemodynamic examinations (SphygmoCor, EndoPat, DRT4, FMD-Celermajer, transcranial Doppler), indicators of atherosclerosis AAI (ankle arm index) and AIT (arterial intima thickness) and the evaluation of cardiorespiratory fitness.

At the ECMU laboratory, focussed in cardiovascular and metabolic risk, whole blood was collected into anticoagulant-treated tubes (e.g., EDTA-treated or citrate-treated). Blood samples were clotted and centrifuged prior to testing. The cells where removed

from plasma by centrifugation for 10 minutes at 1000 –2000 × g using a refrigerated centrifuge. The platelets were depleted by centrifugation for 15 minutes at 2000 × g.

The supernatant plasma was transferred into 0.5mL aliquots using a Pasteur pipette. The aliquots were stored at -80ºC for assessment of biomarkers. Biomarkers were measured using commercial cytokine enzyme-linked immunosorbent assays.

The complete list of biomarkers measured in daily practice in the ECMU laboratory and used in this research is displayed in more detail in the table 4.2.

ECMU laboratory Biochemical parameters	Units	Measurement method
Adiponectin	µg/ml	Elisa Thermofisher
Antioxidant capacity of plasma(TAC)	µM	Copper Reducing Equivalents. Colorimetry
Complement C3	mg/dl	Nephelometry
Complement C4	mg/dl	nephelometry
Homocysteine (Hcy)	µM/L	IMX, Abott
Interleukin-6 (IL-6)	pg/ml	Chemiluminescent /Immunometric Assay. Immulite.DCP Labs
Interleukin-10 (IL-10)	pg/ml	Chemiluminescent /Immunometric Assay. Immulite.DCP Labs
Insulin (in blood)	µU/mL	Inmunometric assay. Inmulite.DCP
Lipoprotein-a (Lpa)	mg/dL	NLatex Lp(a) Reagent
Plasminogen activator inhibitor-1 (PAI-1)	ng/ml	ELISA Meranini
Thiobarbituric acid reactive substances (TBARS)	µM/L	Cayman
Tumoral necrosis factor (TNFα)	pg/ml	Chemiluminescent /Immunometric Assay. Immulite. DCP Labs
Tissue Type Plasminogen Activator (tPA)	pg/ml	ELISA Eiboscience
VCAM	ng/ml	ELISA Menarini

Table 4.2: parameters measured at the ECMU laboratory on clinical routinely basis, units and used methods.

C3 complement levels in plasma**,** main variable of this research, were measured from the stored aliquots by human competitive ELISA kit. A C3 complement specific antibody was pre-coated onto well plates and blocked. Standard plasma samples were added to the wells which included C3 complement detection proteins. The plates were washed with buffer solution. Streptavidin-Peroxidase Conjugate was added and

unbound conjugates were washed away with a second wash buffer. Chromogen solution was added and catalysed by Streptavidin-Peroxidase enzymatic reaction producing visible blue colour that changes into yellow after adding acidic stop solution. The density of yellow coloration was inversely proportional to the amount of C3 complement captured in plate. The procedure is summarized in the figure 4.2.

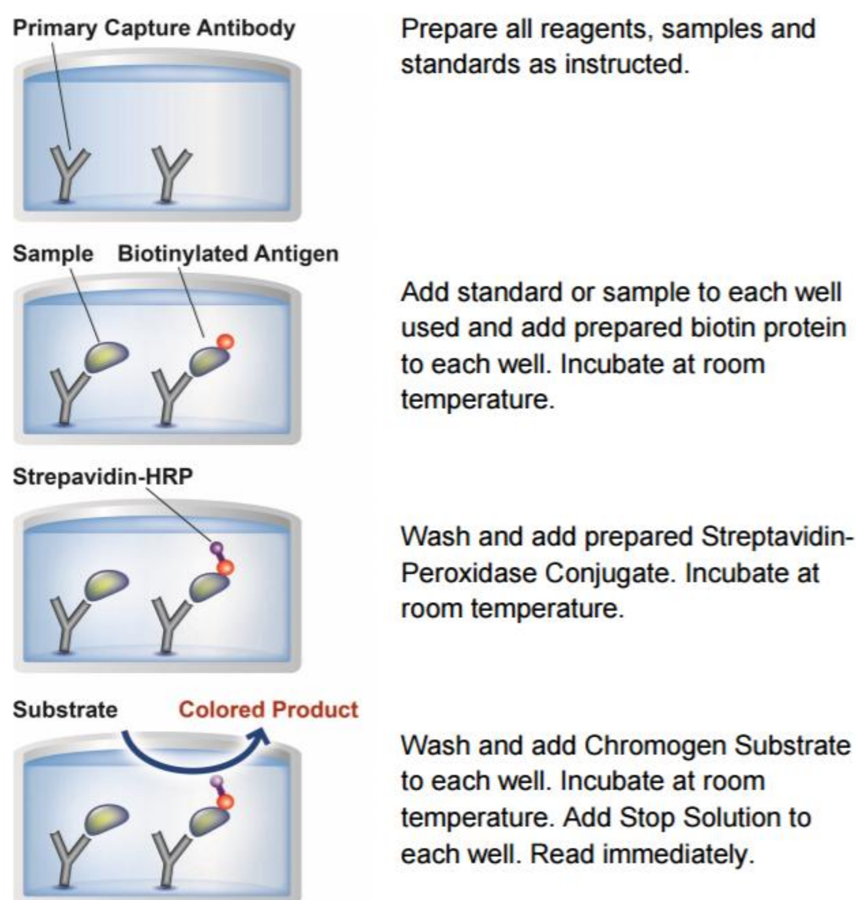

Figure 4.2: Summary of the nephelometry procedure to measure C3 complement concentrations by ELISA. Modified from Abcam ELISA kit manual.

4.6.1. Inflammatory biomarkers measurement methods

Inflammatory markers, such as PCR and fibrinogen, provide us with information about the degree of vascular inflammation. Beside the classical inflammatory markers, adipokines and cytokines (IL-6, adiponectin, IL-10hs and TNF-α) are released by the fat cells and / or vascular stroma in close contact with fat. These new inflammatory markers report the degree of inflammation of active adipose tissue (abdominal obesity).

Adiponectin and IL-10 are considered anti-inflammatory, anti-aggregant and anti-hyperglycaemic markers. However, TNF-α and IL-6 are considered pro-inflammatory, pro-aggregative and hyperglycaemic markers.

Adiponectin concentration in plasma aliquots is measured by enzyme-linked immunosorbent assay (Thermofisher® ELISA kit). The ELISA kit is based on solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay.

An antibody against the specific antigen is coated onto the wells of the microtiter strips. During the first incubation, samples are added to the coated wells to allow the antigen from the samples to bind to the immobilized (capture) antibody. After washing, an antigen-specific human antibody is added that binds to the immobilized antigen captured. After removal of excess detection antibody, a horseradish peroxidase enzyme is added. This binds to the detection antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a stabilized substrate solution is added, which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of antigen present in the original specimen. This process is illustrated in the figure 4.3.

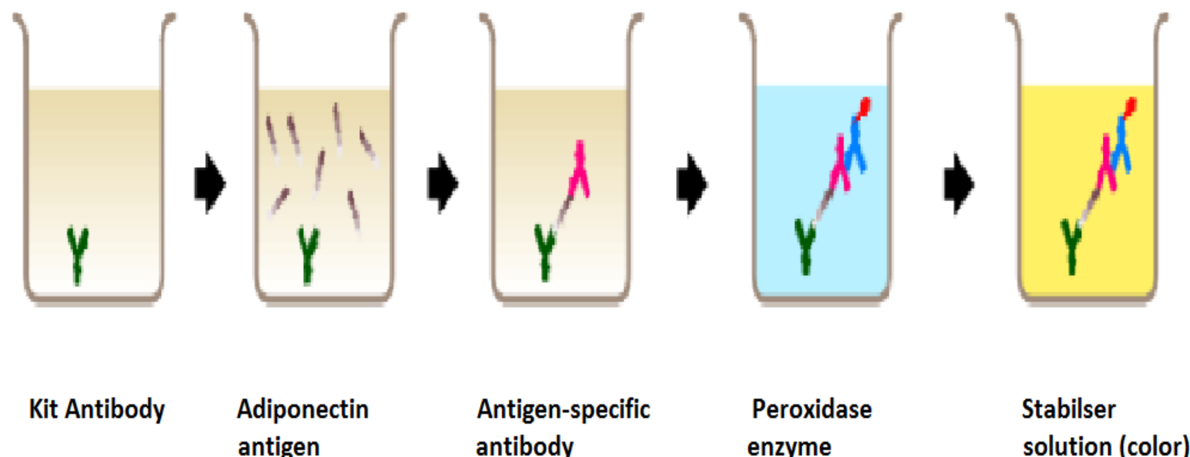

Figure 4.3: Pictogram of the adiponectin concentration measurement procedure by ELISA sandwich method.

Interleukin-6 and interleukin-10 concentrations (IL-6, IL-10) were measured by an immunometric method. DPC IMMULITE immunoassay system uses enzyme-amplified chemiluminescence chemistry for the detection of IL-6. INMULITE device comprises several subsystems to process the assay tube minimizing human errors. After loading

a carousel with alkaline phosphatase-labelled reagents, followed in each case by an assay tube, the assay tubes are transferred to the incubation carousel. The alkaline reagent initiates the reaction and the tubes are intermittently agitated during incubation. After incubation, the assay tubes are shuttled to the wash station. A chemiluminescent substrate for alkaline phosphatase is added and the tubes are transferred to the luminometer. The light output is measured with a photomultiplier tube in the photon-count. Counts are converted to analysed concentration by use of stored standard curves.

Tumoral necrosis factor (TNF-α) concentration in plasma was measured by chemiluminescent-immunometric assay (Immulite® DCP Labs ELISA Kit) following the procedure explained in the figure 4.4.

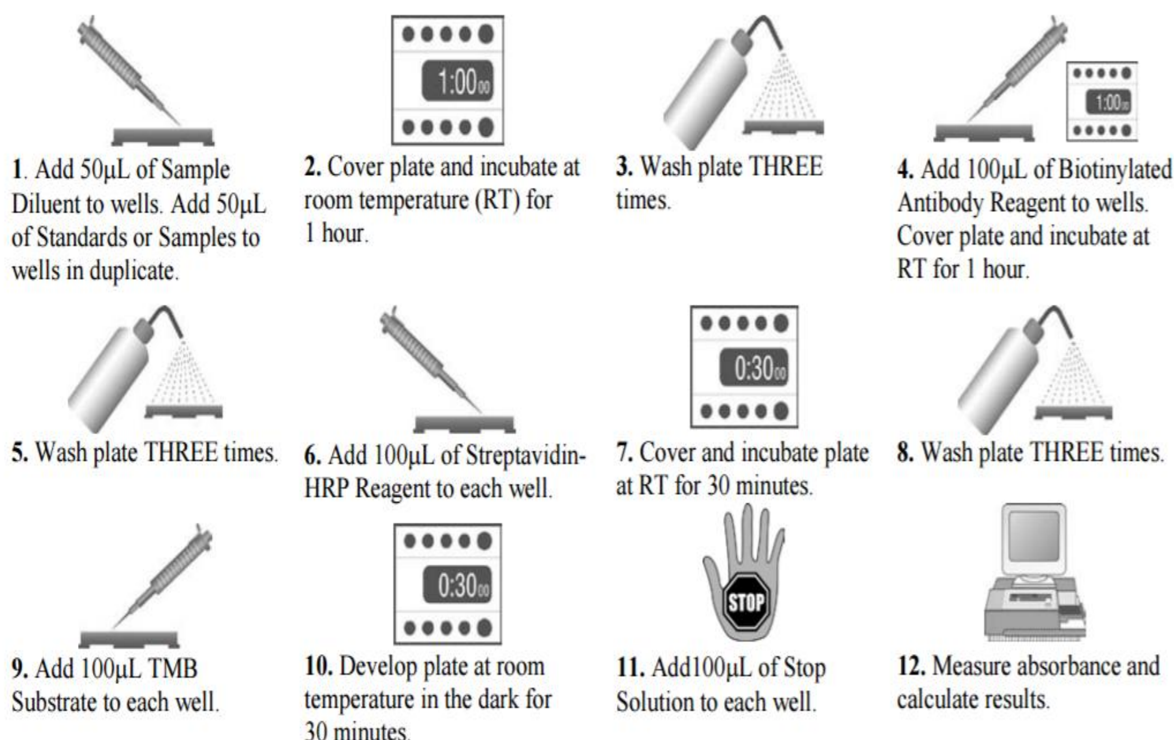

Figure 4.4: Summary of the laboratory technical procedure steps to measure TNF-α concentration, modified from DCP Labs manual.

4.6.2. Endothelial damage measurement methods

Endothelial peptides values, such as PAI-1 and TPA, are related to endothelial damage by any of the classical cardiovascular risk factors (hypertension, smoking, dyslipidaemia, hyperglycaemia, etc). Chemokines and adhesion molecules, such as VCAM-1, were also activated in the early stages of endothelial dysfunction and responsible for the infiltration of the monocyte into the wall. Meanwhile, nitric oxide measurement was considered a controversial technique for this research because its concentrations were not reliable due to its instability in frozen samples.

PAI-1 antigen (Imulyze) was assayed by ELISA using kits from Biopool-Menarini. PAI-1 is synthesized and secreted by many tissue and cell types. Free PAI-1 is relatively unstable in its active form and readily converts into a latent, inactive form; however, binding of vitronectin to PAI-1 stabilizes the active form. The active form of PAI-1 binds tightly with tPA in a 1:1 ratio. After the formation of an initial docking complex, the proteases cleave the reactive central loop of PAI-1 to form a stable, covalent complex, resulting in the inactivation of the targeted serine protease. The ELISA protocol is similar to the ELISA kits for other variables explained above.

Tissue Type Plasminogen Activator (TPA) in plasma was also measured by an ELISA kit. Specific antibodies were pre-coated onto the well plates and blocked. Streptavidin-peroxidase conjugate was added and unbound conjugates were washed away by buffer solution. TMB is catalysed by streptavidin peroxidase to produce a blue colour product that changes into yellow after adding acidic stop solution. The density of yellow coloration was directly proportional to the amount of TPA captured in plate.

Vascular cell adhesion molecule-1 (VCAM-1) human solid-phase sandwich ELISA Kit quantified natural and recombinant soluble VCAM-1 in human serum. VCAM-1 is a member of the immunoglobulin gene superfamily. ELISA technical procedures were similar the ELISA techniques explained above.

4.6.3. Oxidative stress measurement methods

The increase of oxidative stress (OS) is one of the essential characteristics of endothelial damage. OS along with endothelial dysfunction and vascular inflammation form a triad in the initial phases of a prothrombotic status. OS can be measured by OS markers, such as total antioxidant capacity of plasma (TAP), thiobarbituric acid reactive

substances (TBARS) and homocysteine. The oxidative degradation, called lipid peroxidation, of lipids by reactive oxygen species (ROS) results in the formation of highly reactive lipid peroxides. Decomposition of lipid peroxides results in the formation of TBARS.

The **TBARS** generated during oxidative stress were measured by a microplate-based TBARS Parameter Assay Kit which quantifies TBARS levels in plasma by setting up a reaction between MDA and two molecules of 2-thiobarbituric acid (TBA). In the presence of heat and acid, MDA reacts with TBA to produce a coloured end-product that can be measured. Intensity of the colour corresponds to the level of lipid peroxidation in the sample. This process is summarised in more detail in the figure 4.5.

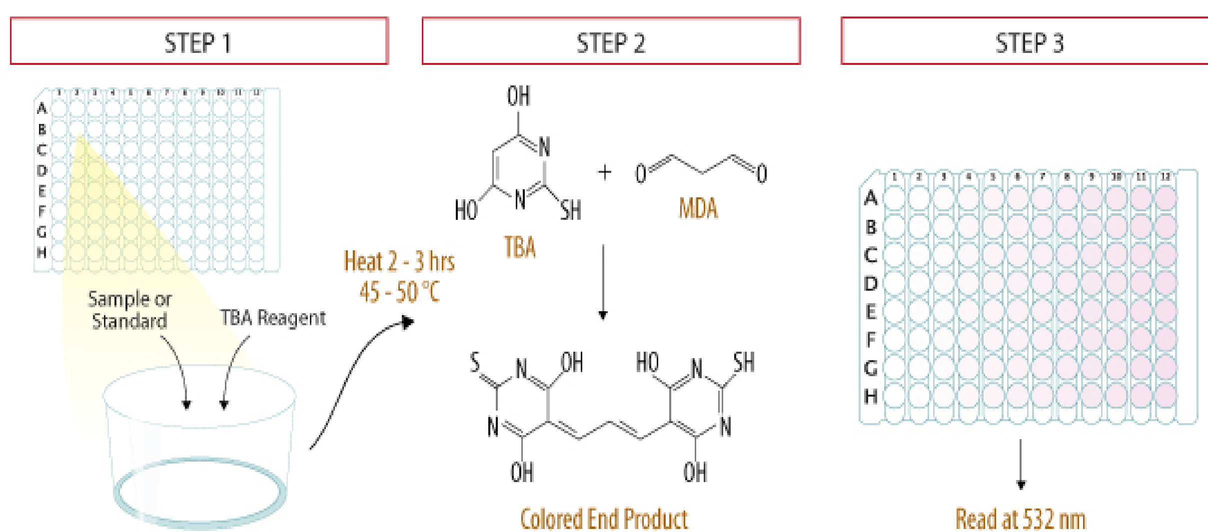

Figure 4.5: Summary of the TBARS measurement method by nephelometric assay. Step 1: Acid-treated samples and TBA reagent are added to the included well microplates. Step 2: The microplates are incubated at 45-50 °C for 2-3 hours to produce a coloured end product. Step 3: The microplates are read and the intensity of the colour corresponds to the level of lipid peroxidation in the sample (TBARS Parameter™ Kit for Measuring Oxidative Stress).

Total Antioxidant Capacity (TAP) was measured by assessing the total copper antioxidant capacity of the combination of both small molecule antioxidants and proteins in plasma. Plasma samples are compared to a known concentration of uric acid standard within a 96-well microtiter plate. Samples and standards are diluted with

a reaction reagent upon the addition of copper. Cu2+ ions are converted to Cu+ by both small molecules and proteins. The TAC Assay is based on the reduction of copper by antioxidants such as uric acid. The reduced copper further reacts with a coupling chromogenic reagent that produces a color and gives a broad absorbance peak proportional to the total antioxidant capacity. The net absorbance values of antioxidants are compared with a known uric acid standard curve. Antioxidant capacity is determined by comparison with the uric acid standards.

Homocysteine (Hcy) concentrations were measured by an automated latex-enhanced immunoassay for the quantitative determination of total L-homocysteine in human citrated plasma. Hcy levels in plasma were measured in three stages as explained in the figure below. The degree of agglutination is inversely proportional to the concentration of Hcy in the sample and is determined by measuring the decrease of transmitted light caused by the aggregates (figure 4.6)

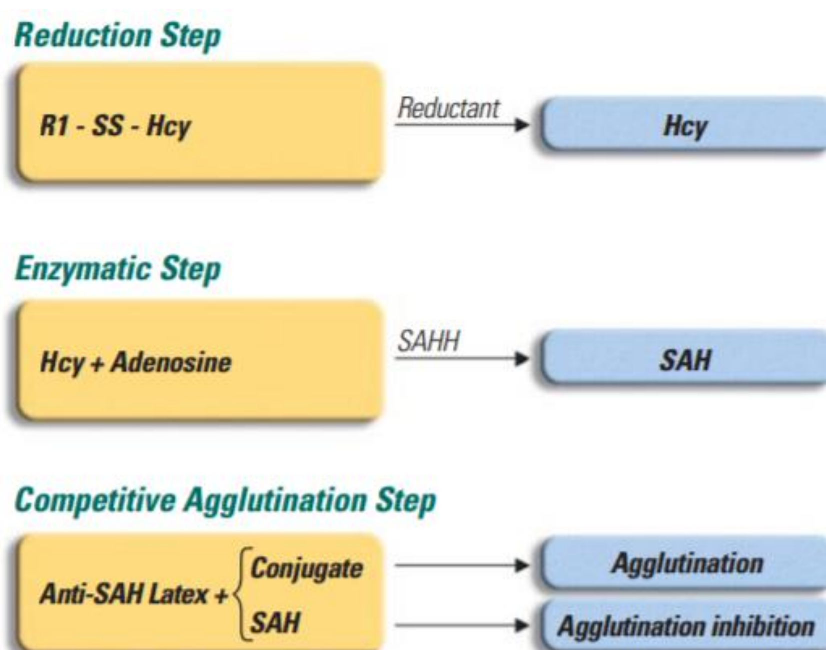

Figure 4.6: Stages of Homocystein measurement: 1. Reduction of mixed disulphides and protein-bound forms of Hcy present in the plasma samples to free Hcy. 2. Enzymatic conversion of free Hcy to S-adenosyl-L-homocysteine in the presence of adenosine. 3. SAH-antibodies conjugate allows competitive immunoprecipitation reaction between anti-SAH latex and free SAH/conjugate. Modified from HemosIL® Homocysteine manual.

4.7. Variables Definitions

4.7.1. Metabolic syndrome

Complete metabolic syndrome [214] diagnosis was met when at least three of five of the ATPIII criteria listed below were present.

Incomplete metabolic syndrome diagnosis for this study was met when one or two ATPIII SM criteria were present.

A healthy individual, metabolic syndrome-free, was defined when none of the ATPIII criteria were present.

i) Abdominal obesity, recognized by increased waist circumference, was the first criterion listed when waist circumference was greater than 88cm (women) or 102cm (men).
ii) Patient with raised triglycerides (≥150 mg/dL) and/or treated with fibrates or nicotinic acid.
iii) Individual with reduced HDL cholesterol (men <40 mg/dL, women <50 mg/dL) and/or under lipid lowering treatment.
iv) Elevated blood pressure (≥130/85 mm Hg) and/or under antihypertensive treatment.
v) Raised fasting plasma glucose (≥100 mg/dL) and/or under antidiabetic treatment.

4.7.2. Homeostasis model assessment of insulin resistance (HOMA-IR)

Homeostatic Model Assessment (HOMA) is a method for assessing β-cell function and insulin resistance (IR) from basal (fasting) glucose and insulin or C-peptide concentrations. Insulin resistance was calculated with standard methods [215] HOMA-IR using an approximation described by a simple equation to determine a surrogate index of insulin resistance:

$$\text{HOMA-IR} = \frac{\text{Glucose} \times \text{Insulin}}{22.5}$$

The denominator of 22.5 is a normalizing factor. This factor is determined by the normal individual results: the product of normal fasting plasma insulin of 5 μU/ml and normal fasting plasma glucose of 4.5 mmol/l typical of a "normal" healthy individual = 22.5 (assuming normal-weight, normal subjects < 35 years, having 100% β-cell function an insulin resistance of 1). Therefore, for an individual with "normal" insulin sensitivity, HOMA-IR = 1.

Glucose and insulin are both analyzed during fasting basal conditions. Log (HOMA-IR) is useful for evaluation of insulin resistance in individuals with glucose intolerance, mild to moderate diabetes, and other insulin-resistant conditions. However, in subjects with severely impaired or absent β-cell function, HOMA-IR may not give appropriate results. To avoid this eventuality we used the HOMA2 adjusted computer system. HOMA2 adjusted the assessment of previous HOMA in subjects with glucose levels ≤25 mM, accounts for renal glucose losses, assumes reduced suppression of HGP and increased insulin secretion in response to glucose levels >10 mM, and allows for the use of total or specific insulin assays.

Despite this improvement with HOMA2, for the secondary objectives studies, which imply insulin resistance calculation, patients under exogenous insulin treatment had to be excluded (n=54). The assumptions about hepatic extraction included in the model do not apply when a subject is being treated with exogenous insulin. The insulin-glucose HOMA model cannot be used to assess β-cell function in those taking exogenous insulin[216].

4.7.3. REGICOR

The REGICOR model calibrates and adjusts the Framingham CHD functions for Spanish population in cardiovascular primary prevention by substituting the prevalence of CHD risk factors and incidence found in Framingham with the same values for Spain. The REGICOR model replaces the incidence of the Framingham equation with the data observed in the population of Gerona through the REGICOR population-based registry of cardiac disease in 1995.

The data observed in REGICOR were validated for the entire Spanish population through the VERIFICA study. Studies with REGICOR have enabled the variables included in the Framingham equation to be refined by including antihypertensive and lipid-lowering therapies. The REGICOR model estimates the 10-year probability of developing a CHD event according to the risk factor profile and HDL levels ranging from 35 to 59 mg/dl. Tables with the general model of all the Framingham coronary events were calculated using the equation published in 1998 by Wilson et al. that follows the described calibration method. The following ratio was used:

H0(t)/FramAll / H0(t)/FramHard

Where **t** is the follow-up time, 10 years in our case; H0(t)/FramAll is the coronary event rate including Framingham angina and silent AMI, and H0(t)/FramHard is the rate of symptomatic AMI, fatal or non-fatal. The heart event rate in males of Girona in the REGICOR registry (3.5%) is multiplied by 1.400 to obtain an estimated total coronary event rate (4.9%). This allows calculation of 95.1% male population without events (100-4.9%). In females the hard event rate (1.1%) is multiplied by its quotient (1.910) to obtain the estimated total coronary event rate (2.2%). 97.8% female population resulted without events (100-2.2%).

In this study the cardiovascular risk in 10 years thresholds have been assigned as being <5% low cardiovascular risk, 5-10% moderate risk and >5% high cardiovascular risk, as established by the REGICOR model. Due to an insufficient number of high cardiovascular risk patients in some statistical analyses moderate and high risk patients are grouped as high cardiovascular risk patients.

Two examples of REGICOR function color-coded charts for hazard intensities in the risk factor combinations for diabetic and non-diabetic of overall CHD risk for the Spanish population according to the adapted equation are presented in the figure 4.7. These are separated for male/female and diabetic/non-diabetic population. The tables below are calculated for average HDL cholesterol (47.5 mg/dL) in 35 to 74 year old Spanish males.

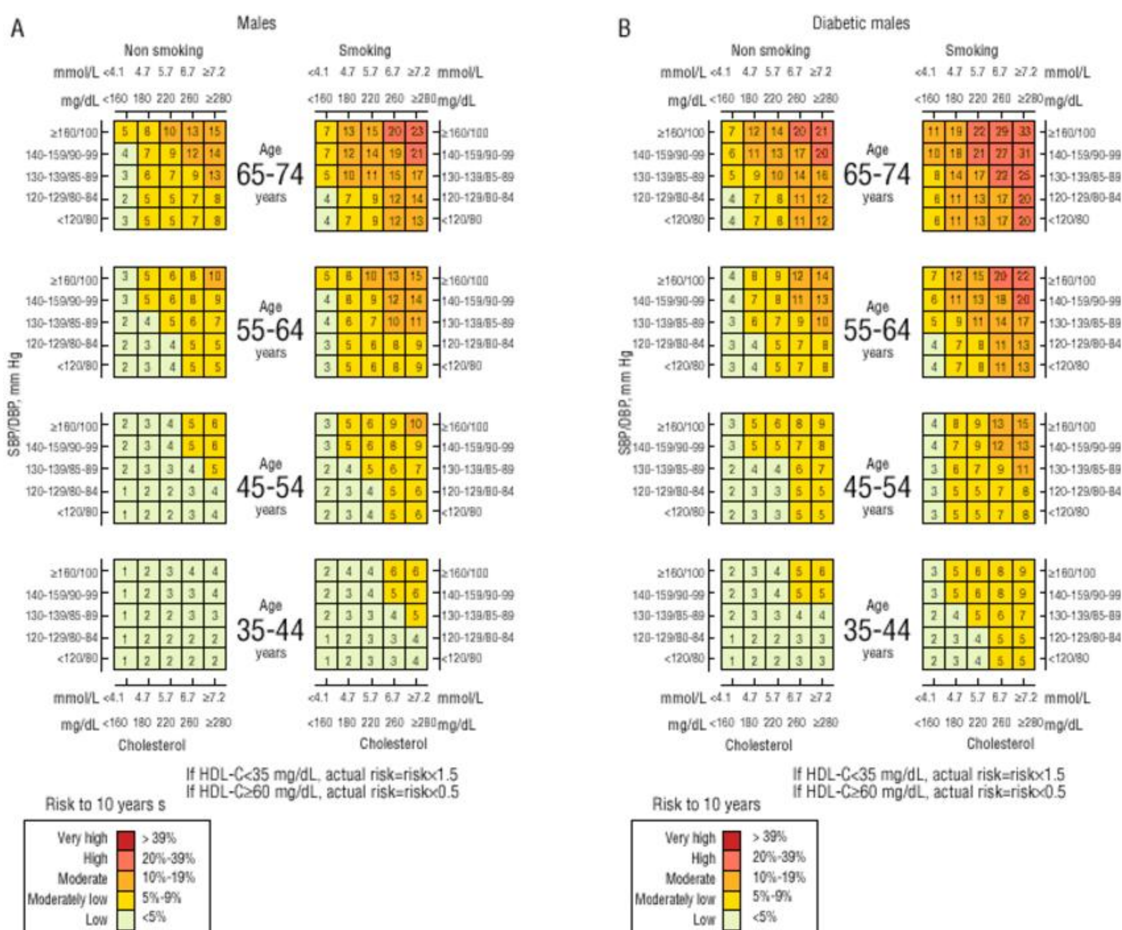

Figure 4.7: Example of REGICOR cardiovascular color-coded charts in non-diabetic and diabetic men divided into smokers and non-smokers, where low risk represents <5% possibilities of suffering a cardiac event in ten years, moderate risk is between 5% and <10% and high risk is ≥10%.

4.8. Data collection and coding methodology

Data collection process

Data were obtained by a detailed personal medical history of the previous years via a questionnaire developed by a permitted doctor following the privacy policies of the Ramon y Cajal Hospital. The physical examination of the cardiovascular system, electrocardiogram test if required and the non-invasive endothelial function were performed by a permitted physician offering a chaperone if required. Blood samples

were taken by a permitted nurse following the phlebotomy Ramon y Cajal hospital policies.

The results of the tests mentioned above were extracted from medical records codified into ECMU numbers to preserve patients' privacy. Data were collected manually from printed reports to an intranet electronic database electronically secured by a permitted laboratory biologist and a permitted doctor between August 2010 and August 2013. Three permitted professionals, a doctor, a laboratory technician and an IT engineer reviewed the data base three times to reduce personal bias.

4.9. Statistical data analyses

Statistical analyses were performed using SPSS 15 for Windows (SPSS, Inc., Chicago, IL, USA).

4.9.1. Descriptive analysis

Qualitative variables were described as percentages. Quantitative continuous variables were determined as mean ± standard deviation (SD). The confident intervals were 95%.

4.9.2. Hypothesis testing

Prior to hypothesis testing, variables were examined for normality by Kolmogorov-Smirnov test. Differences at baseline in quantitative variables were assessed by student t-test for independent samples for a normal distribution or Mann-Whitney test for non-normal distribution. Anova test was performed for comparisons of independent sample means of more than two groups. Differences in qualitative variables were assessed by chi-square test. The significance level predetermined p-value for differences between groups was agreed at 0.05.

The correlation between C3 levels and several continuous analytical variables was studied by linear Pearson coefficient agreeing statistical significance at $p<0.05$.

Serum complement C3 was used as a continuous variable and as quartiles for logistic regression analyses to ascertain whether a threshold effect existed. The quartiles were calculated from the median forming cut points. For correlations, a value of $P \leq 0.05$ on the 2-sided test was considered statistically significant.

4.9.3. Association of variables

A multivariable linear regression analysis corresponding to the outcome measure (dependent variable) was developed to identify predictors. Backward variable selection was used for modelling, with the standard significance level for contrasts $P \leq 0.05$. Independent variables associated with an outcome of $P \leq 0.20$ in univariate analysis were included in the corresponding multivariate model.

The collinearity of the maximum models was assessed with the criteria proposed by Belsley [217]. Normality and homocedasticity assumptions in each final model were assessed by analysing model residuals. Model validation was evaluated by bootstrap techniques and leave-one-out cross-validation.

The maximum predictive model was determined by C3 levels as the continuous dependant variable. The independent selected variables, were: age, gender, CRP levels, presence or absence or MS, low or high cardiovascular risk according to REGICOR model for Spanish population (<5% or >5% risk in 10 years), insulin resistance measured by HOMA, hyperhomocysteinemia, Lpa levels, smoking status, smoking status adjusted by age, presence or absence of MS ATPIII criteria for hypertriglyceridemia, high blood pressure, hyperglycaemia, waist, low HDL and hypercholesterolemia. The selection of the independent variables was made in relation to the previous univariate analysis results and their interest in the study objectives.

Smoking status was included twice in the regression model, as independent sole variable (dichotomised smoking status) and adjusted by age (smoking status multiplied by age), to established its association to age as a possible confounding factor.

4.10. Literature review

i) English and Spanish language only: time limits made the translation from any other language impractical.

ii) Synonyms were limited to Thesaurus

iii) The information sources were limited to :
- PubMed, Medline, LSE's library, Ramon y Cajal Hospital's library, Mostoles Hospital's library and Google databases. The first three are included in EndNote bibliographic software and facilitate the quick reading of abstracts and literature classification.
- Documents and grey literature located in Endothelial Pathology Unit's intranet, professionals' personal files compilation and archives of hard copies.

iv) Literature published between 1992, when Muscari et al. [70] performed a study to assess the possible involvement of humoral immunity in essential hypertension and cardiovascular pathology, and December 2016 only. Some previous references are limited to definitions of terms included in more recent publications.

v) Keywords: C3 complement OR/AND C3 convertase OR/AND metabolic syndrome OR/AND insulin resistance OR/AND cardiovascular risk OR/AND C-reactive protein OR/AND endothelial function OR/AND inflammatory cascade OR/AND atherosclerosis OR/AND complement pathways OR/AND inflammatory markers OR/AND endothelial dysfunction OR/AND C-reactive protein OR/AND Lp(a) OR/AND homocystein OR/AND fibrinogen OR/AND oxidative stress OR/AND Regicor OR/AND HOMA OR/AND multi-regression analysis OR/AND hyperlipidaemia OR/AND nitric oxide OR/AND ATPIII criteria OR/AND hypertension OR/AND T2DM

vi) After basic searches in Cochrane and Google, in September 2016, a similar recent study of C3 convertase relation to cardiovascular risk and endothelial function in Spanish population was not identified.

vii) Search strategy algorithm:

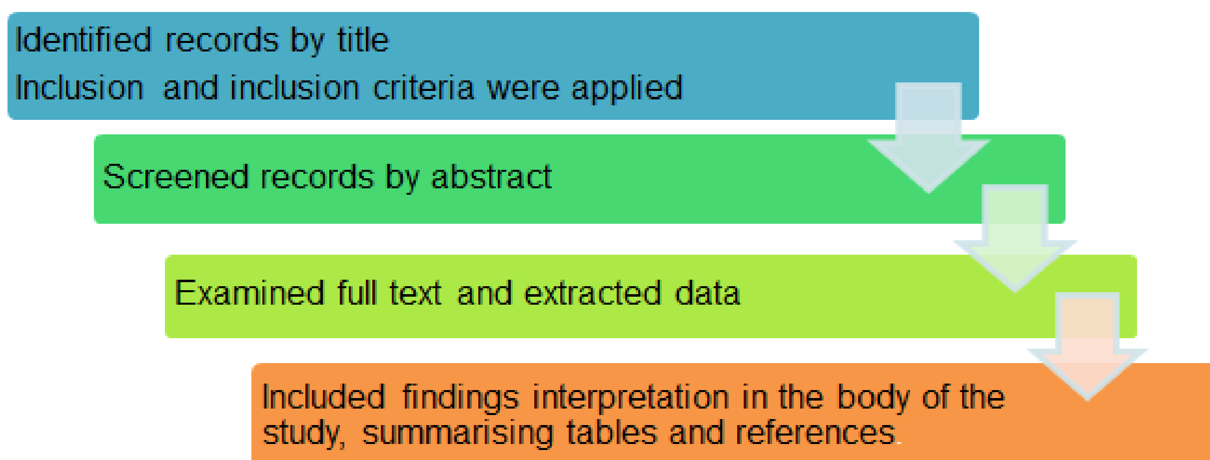

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Figure 4.8: Search strategy algorithm

4.11. Limitations

Financial and personnel constraints limited the availability of tests and reactives for a certain length of time and the number of performed tests for the study.

The overlap of active clinical routine as a physician on top of the research activity reduces the available time to develop the research and extended the timeline.

The lack of resources (human and financial) in research and development limited the number of available tests throughout the research and also affected by the availability of reactants for ECMU laboratory.

The calculation of cardiovascular risk would be more precise in treatment-free patients, but the incremented risk of cardiovascular events by removing indicated treatments in cardiometabolic patients is ethically controversial.

4.12 Study timeline

The data collection process started in August 2010, during the development of my doctoral advanced studies project until August 2013. The thesis project was submitted in October 2012. The literature, references review and writing processes were on-going routine in parallel with the thesis development. The statistical analysis progressed from October 2015 until August 2016, the results analysis between April 2016 and October 2016, and the discussion slightly overlapped from August until February 2016. Conclusions, text formatting, style review, printing and submission were performed from December 2016 until March 2017 when the final paper was submitted (figure 4.9).

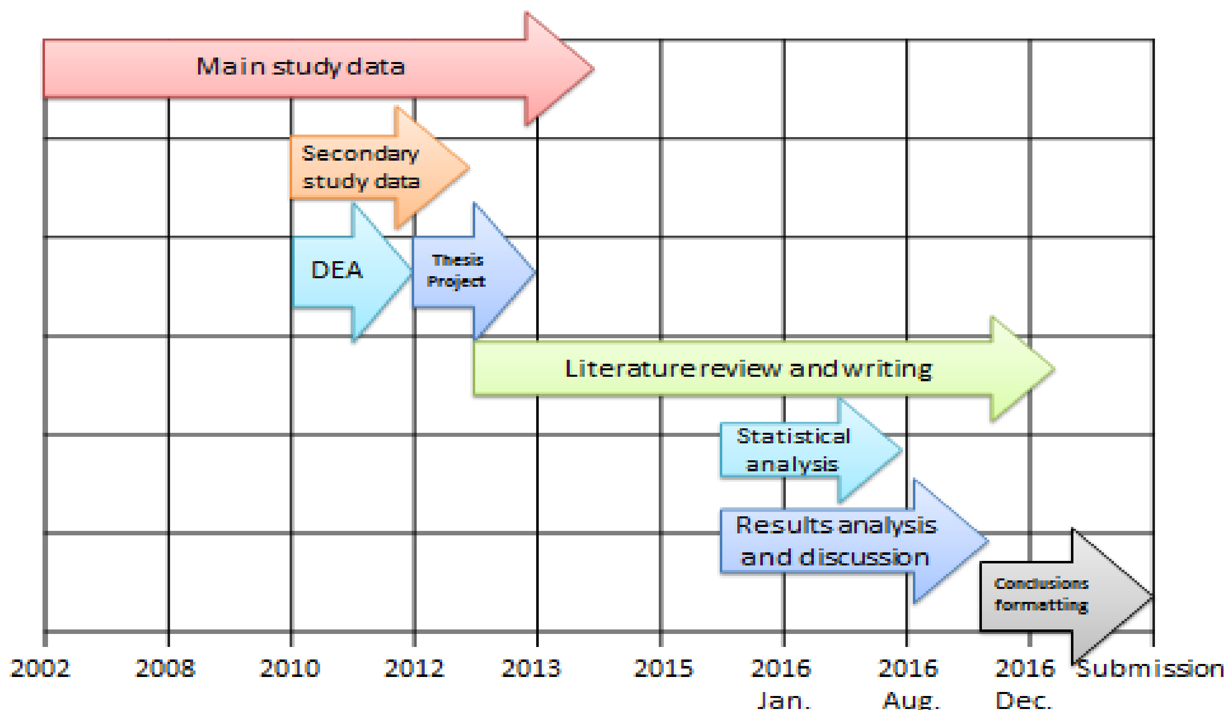


Figure 4.9: Study timeline graph from data collection to submission.

5. RESULTS

5. Results

5.1. Population description

The study population included a total of n=374 subjects, where 168 were females (44.9%). At baseline examination, the mean age of the overall population was 53.6±14.8 years.

Study sample population (n=374)		N	Percentages
Gender	Males	206	55.1%
	Females	168	44.9%

Table 5.1: Gender distribution of the study sample population.

The majority of patients were overweight (BMI≥25), dyslipidaemic (altered lipid profile) and hyperglycaemic (FG≥100 mg/dl), as seen in more detail in the table 5.2.

Variables			Population (n=374)		
			Mean ±SD	Minimum	Maximum
Demography	Age (years)		53.60 ±14.80	19.70	90.70
Anthropometry	BMI (kg/m^2)		27.10 ±4.70	17.30	49.52
	Waist (cm)	Males	95.70 ±9.70	70	139
		Females	87.70 ±13.80	59	128
	Waist/hip ratio	Males	0.95 ±0.06	0.77	1.20
		Females	0.86 ±0.09	0.66	1.09
Haemodynamic	SBP (mmHg)		133.33 ±20.13	87	205
	DBP (mmHg)		82.11 ±10.56	55	116
	Pulse Presure (mmHg)		55.22 ±16.68	21	134
	MAP (mean arterial pressure)		99.18 ±12.14	69.67	142.67
	Heart rate (bpm)		71.86 ±12.40	55	116
Metabolism	Fasting Glucose (FG) (mg/dl)		107.36 ±40.02	64	421
	A1c (%)		5.81 ±1.21	4	11.70
	Total cholesterol (mg/dl)		201.99 ±46.45	83	407
	LDLc (mg/dl)		128.09 ±41.47	25	316
	TG (mg/dl)		118,39 ±63.33	34	381
	HDLc	Males (mg/dl)	44.66 ±8.27	25	76
		Females (mg/dl)	56.76 ±13.14	29	105

Table 5.2: Summary of baseline characteristics of the study sample population. Means were calculated from their clinical examination and basic blood test data with special interest in metabolic profile. Abbreviations: body mass index, BMI; systolic blood pressure, SBP; diastolic blood pressure; A1c glycosylated haemoglobin; DBP; low-density lipoprotein cholesterol; LDLc; high-density lipoprotein cholesterol, HDLc; triglycerides, TG.

5.1.1 Personal Medical History (PMH)

The metabolic profile of our sample population showed a high prevalence of hypertension (55.1%), hypercholesterolemia (46%) and T2DM (27.8%) in their personal medical history (PMH). The distinctive feature of our sample population was the high proportion of basally diagnosed metabolic pathologies.

Furthermore, the population exhibited a tendency to obesity where 23.5% patients were obese (BMI≥30 kg/m^2) and 42% overweight (BMI ≥25-<30 kg/m2). In particular, we highlighted the prevalence of central obesity. The average waist-hip ratio in both genders (0.95 in men, 0.86 in women) overcame the upper recommended limit established by the WHO. These particular PMH characteristics of our population are displayed in more detail in table 5.3.

Regarding smoking habits, 27.8% were active smokers and 25.1% former smokers (>3 months smoking-free period). The forthcoming analysis pointed out that smokers were significantly younger than non-smokers (48.44±11.97 *vs.* 55.64±16.63 yr. old; p<0.001).

Personal medical history population variables			Population (n=374)	
			N	Percentages
PMH	Hypertension		206	55.1%
	Hypercholesterolemia (LDLc>160 mg/dl)		173	46.3%
	Low HDL-c (<40mg/dl males, <50md/dl females)		78	20.95
	Hypertriglyceridemia (TG>150mg/dl)		86	23.0%
	Type-2 diabetes mellitus		95	25.4%
	Obesity	Obese (BMI>30 Kg/m^2)	88	23.5%
		Overweight (BMI 25-30 Kg/m^2)	157	42.0%
	Smoking habit	Smokers	104	27.8%
		Ex-smokers	94	25.1%

Table 5.3: Summary of the proportions of metabolic conditions presented in the patients' personal medical history (PMH). There is a high prevalence of hypertension, dyslipidaemia, obesity and diabetes in the main sample population. Obesity degrees were calculated from BMI data. Abbreviations: low-density lipoprotein cholesterol,LDLc; high-density lipoprotein cholesterol, HDLc; triglycerides, TG; body mass index, BMI.

5.2 Metabolic Syndrome (MetS) and C3 complement

In order to classify the population sample we stratified the population into three groups according to the number of ATPIII criteria met per patient.
MetS criteria diagnosis comprised patients with ciphers over each criterion threshold and/or already diagnosed in their PMH and under treatment for each metabolic pathology. 35.3% of the sample population presented MetS (≥3 MetS-criteria), 49.7% suffered from incomplete MetS (1-2 MetS-criteria) and 15% were metabolically healthy population (MetS-criteria free). As observed in the table 5.4 and graph 5.1 the majority of patients suffered from incomplete or complete metabolic MetS.

In addition to this first finding, we distributed the population into groups, from zero to five fulfilled constitutive factors of metabolic syndrome per individual. The population were distributed among all the groups, with representatives in all the categories (graph 5.1. and table 5.4).

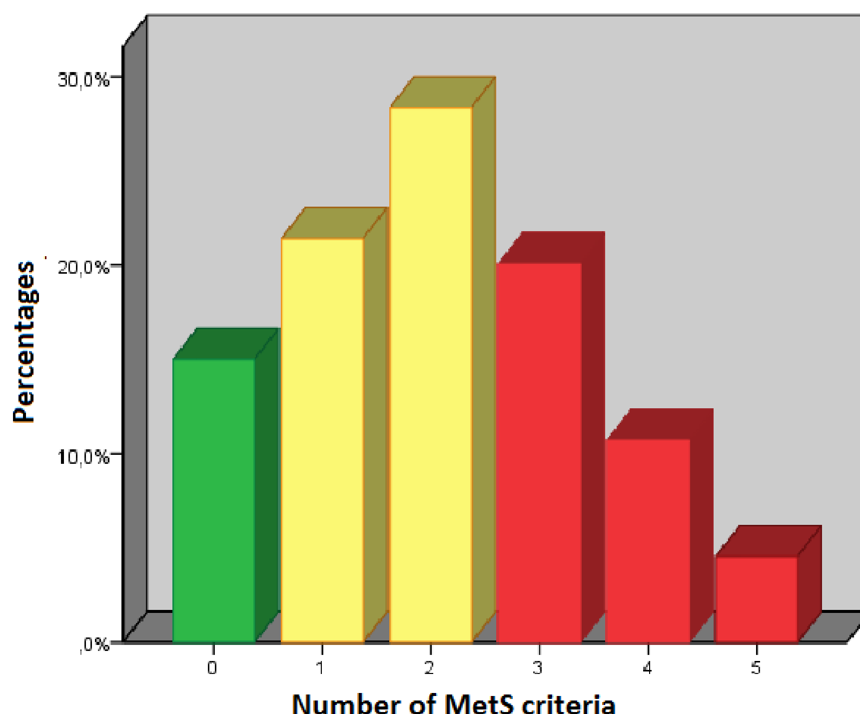


Figure 5.1: Bar graph shows the stratification of our population by number of ATP-III metabolic syndrome (MetS) criteria met per patient. Complete metabolic Syndrome is displayed in red, incomplete MetS in yellow and healthy metabolic patients in green.

Metabolic syndrome	Number of criteria	N	Percentages	
Healthy subjects	None	56	15.0%	15%
Incomplete MetS	One	80	21.4%	49.7%
	Two	106	28.3%	
Complete MetS	Three	75	20.1%	35.3%
	Four	40	10.7%	
	Five	17	4.5%	

Table 5.4: Percentages of subjects classified in healthy, incomplete and complete MetS according to the number of MetS criteria met per patient.

Hypertension (65%) and hyperglycaemia (42%) resulted the most prevalent criteria when we analysed individually the constitutive factors of the metabolic syndrome; albeit all ATP-III criteria were also presented in a significant percentagein the sample population (central obesity 36%, low HDLc 35%, hpertriglyceridemia 26%), as shown in the table 5.5.

ATP-III Metabolic Syndrome criteria (n=374)	N	Percentages
1. Hypertension (BP >135/85 mmHg or under treatment for HBP)	244	65.2%
2. Hyperglycaemia (fasting glucose >100mg/dl or under treatment for hyperglycaemia)	156	41.7%
3. Central obesity (waist ≥88cm in women and ≥102cm in men)	133	35.6%
4. Low HDLc (HDLc <40mg/dl in males, <50mg/dl in females or treated with diet and exercise)	132	35.3%
5. Hypertriglyceridemia (TG>150mg/dl and/or under treatment for hypertriglyceridemia)	97	25.9%

Table 5.5: Percentages distribution of ATP-III metabolic syndrome criteria in the study. Hypertension (65.2%) and hyperglycaemia (41.7%) were the most prevalent characteristics. TG: triglycerides.

5.2.1. C3 levels and ATPIII criteria

The first step of our study was to assess the correlation between C3 concentrations measured in blood and the presence of MetS.

C3 complement was measured in all the subjects of our population sample (n=374). The descriptive statistical values were mean 129.65 (±26.02) mg/dl, minimum 62.00 mg/dl and maximum 216.00 mg/dl respectively.

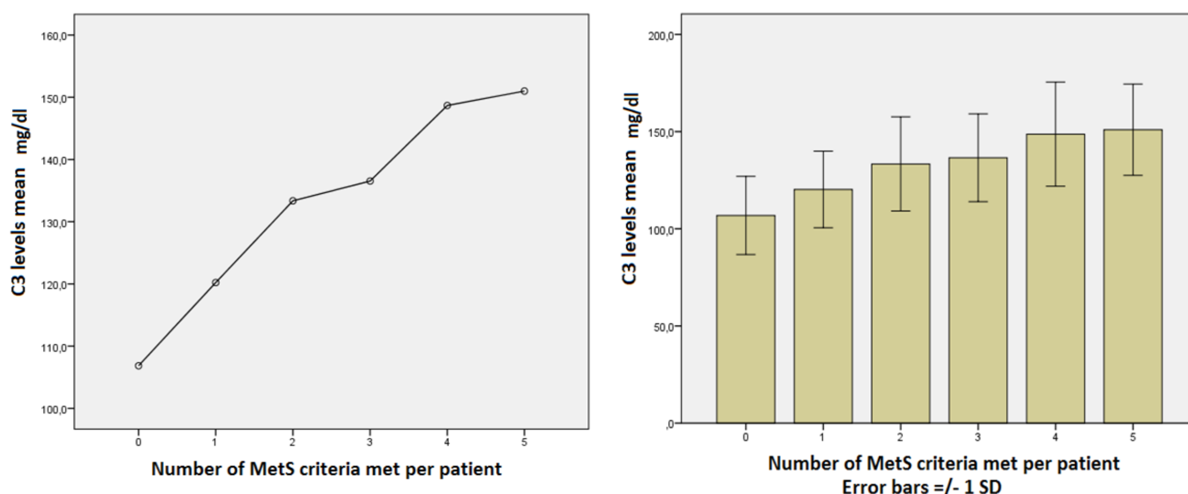
Patients who suffer from metabolic syndrome had statistically significant higher concentrations of C3 complement than metabolically healthy patients (142.10±25.47 vs. 122.88±20.68; p<0.001). Furthermore, the mean levels of C3 complement increased proportionally to the number of MetS criteria met per patient (p<0.001), as shown in table 5.6.

C3 levels (mg/dl) per number of Metabolic Syndrome criteria			
MetS criteria	N	Mean ±SD	p-value
0	56	106.85 ±20.14	<0.001
1	80	120.22 ±19.69	
2	106	133.36 ±24.22	
3	75	136.53 ±22.60	
4	40	148.67 ±26.78	
5	17	151.00(±23.49	

Table 5.6 Distribution of the population (n=374) per number of MetS criteria met per patient. The mean value of C3 complement measurements per group of number of MetS criteria met, standard deviation within each group and significant difference among groups (p<0.001)

The association between C3 and MetS criteria was barely linear, showing a strong positive linear correlation (Pearson coefficient r=+0.49, p<0.001). Linear correlation is also graphically represented in figure 5.3. Moreover, the representation of C3 complement concentrations is in correlation to the number of MetS criteria fulfilled by patient appeared almost linear as shown in the graph 5.3.

The outcome of this C3 concentrations increment was proportional to the number of MetS criteria. As seen in figure 5.2 and 5.3 the difference between the two and three criteria groups was noticeably shorter though.



Figures 5.2 and 5.3: median graphs. The first one shows the almost linear correlation between C3 complement level means and the number of C3 criteria met. In the graph on the right, the C3 complement plasma concentrations are expressed per number of MetS criteria met per patient, with error bars depicting the variation (±1 standard deviation).

Distribution of C3 complement (mg/dl) among MetS and MetS criteria								
Variable			N	C3 complement (mg/dl)				
				Mean ±SD	Mean difference	95%-CI of the mean		p-value
						Upper b.	Lower b.	
MetS		No	242	122.88 ±24.20	19.20	24.38	14.01	<0.001
		Yes	132	142.08 ±24.71				
MetS criteria	HTG	No	277	124.45 ±24.41	20.07	25.76	14.38	<0.001
		Yes	97	144.52 ±24.84				
	HBP	No	130	119.41 ±24.91	15.69	21.02	10.37	<0.001
		Yes	244	135.11 ±24.98				
	Low HDLc	No	242	125.33 ±25.70	12.25	17.65	6.84	<0.001
		Yes	132	137.58 ±24.80				
	T2DM	No	218	124.21 ±24.89	13.04	18.25	7.83	<0.001
		Yes	156	137.26 ±25.74				
	Central obesity	No	241	123,70 ±24.47	16.68	21.95	11.42	<0.001
		yes	133	140.41 ±25.38				

Table 5.7: C3 complement concentrations' means per presence or absence of MetS criterion. The presence of each Mets criterion is associated with higher C3 levels than absence. The MetS criteria presence/absence were calculated according to MetS criteria definitions described in table 5.5. Abbreviations: hypertriglyceridemia, HTG; hypertension, HBP.

The MetS group (≥3 MetS criteria, mean=142.08±24.71 mg/dl) presented higher levels of C3 complement than Mets-free group (mean=122.88±24.20 mg/dl; $p<0.001$). In addition to this, the presence of the MetS constitutive factors individually was also significantly associated ($p<0.001$) with higher concentrations of C3 complement as shown in table 5.7.

5.3. Association between C3 inflammation and coagulation

In the first place, the correlation between **inflammatory** markers and C3 complement levels in plasma was tested with C-reactive protein (CRP) concentrations. CRP measurements were obtained in all the subjects of the study population (n=374). CRP descriptive values were 2.89±2.12 mg/L), ranged from a minimum of 0.14 mg/L to a maximum of 14.90 mg/L. Subjects with CRP over 15 mg/l were excluded from the study (see methodology).

The relationship between the C3 complement and the CRP showed a strong linear correlation in our population sample ($r=0.271$, $p<0.001$), as displayed in the figure 5.4.

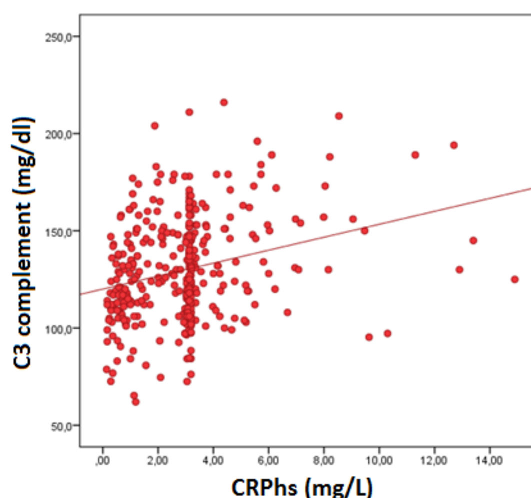


Figure 5.4: Scattered plot showing a strong statistical correlation ($p<0.001$) between CRP and C3 complement measurements. The vertical line of dots at 3mg/L represents the test sensitivity limit before a high sensitivity test was set up in the hospital general laboratory.

Coagulation was analysed in this population by three pro-thrombotic factors such as fibrinogen and homocysteine.

As main representative of pro-thrombotic marker fibrinogen was measured in our sample population (297.63±64.32 mg/dl). There was strong association (r=0.254, p<0.001) between fibrinogen and C3 complement values.

5.4. C3 complement and insulin resistance

The correlation between C3 complement levels and insulin resistance was checked by the association with HOMA variable, as explained in the methodology. Hence, as disclosed in the methodology, the sample population was reduced to n=320 patients with basal insulin measured. The division of the original sample population resulted into two cohorts, the/a non-exogenous insulin replacement group (NEIR, n=320) and an/the exogenous insulin replacement group (EIR, n=54).

Since EIR group required exogenous insulin and presented fictitious endogenous insulin levels, clinical metabolic differences were assumed between both groups. Even though this premise was assumed, both cohorts were tested for differences in their hydrocarbon metabolism. The (?) EIR group showed higher fasting glucose concentrations (130.65±66.17 mg/dl) and A1c (6.75±1.62 mmol/L) than the NEIR group (103.43±32.22 mg/dl and 5.66±1.05 mmol/L). Therefore, both groups showed significantly different glucose-metabolism characteristics as shown in table 5.8. .

Independence tests for NEIR (n=320) vs. EIR (n=54)							
Variable		N	Mean ±SD	Mean difference	95%-CI		p-value
					Lower b.	Upper b.	
C3 (mg/dL)	EIR	54	130.42 ±26.01	5.30	-2.22	12.81	0.17
	NEIR	320	125.12 ±25.87				
FG (mg/dl)	EIR	54	130.65 ±66.17	27.22	8.83	38.61	0.004
	NEIR	320	103.43 ±32.22				
A1c (mmol/L)	EIR	54	6.75 ±1.62	1.09	0.63	1.54	<0.001
	NEIR	320	5.66 ±1.05				
Age (years)	EIR	54	55.69 ±15.20	2.49	-1.80	6.77	0.225
	NEIR	320	53.20 ±14.75				

Table 5.8: Independence assessment for several non-modifiable, age, and modifiable glucose-metabolism variables, such as C3 complement, fasting glucose (C3), glycated haemoglobin (A1c) and age was performed between exogenous insulin replacement group (EIR) and non-exogenous insulin replacement group (NEIR).

On the other hand, there was no difference in age distribution between the two groups (p=0.225). In addition to age, C3 mean concentrations showed no differences between both groups (p=0.17) either. Meanwhile, gender distribution between both groups differed with borderline significance (EIR group women=57%, p=0.046), as shown in the table 5.8

For the population used in the insulin resistance sub-study, NEIR, where the basal insulin levels were measured, the calculated HOMA values (2.19±1.67%) were as shown in table 5.9.

HOMA values distribution in NEIR group (n=320)				
Variable	Mean	SD	Range	
			minimum	Maximum
HOMA (%)	2.19	1.67	0.32	10,18

Table 5.9: Summary of HOMA variable descriptive statistics in non-exogenous insulin replacement (NEIR) group.

The C3 complement concentrations showed a positive linear correlation with insulin resistance measured by HOMA (r=0.406 p<0.001). Therefore, higher levels of C3 complement were strongly associated with increasing levels of insulin resistance as shown in figure 5.5.

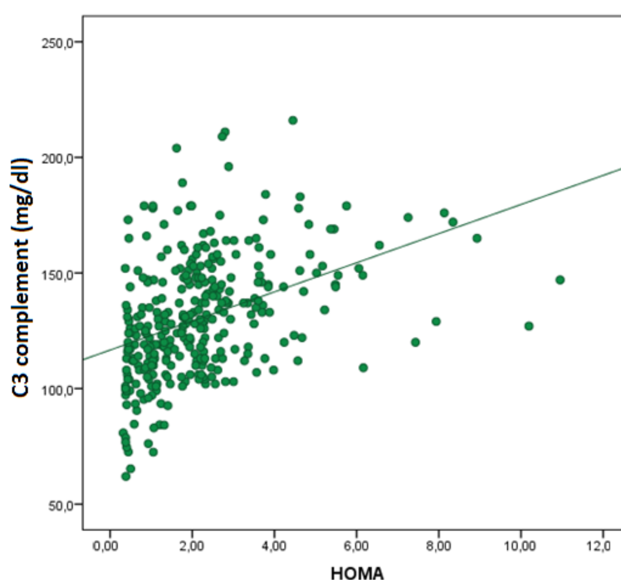


Figure 5.5: Scatter plot shows the relationship between HOMA, insulin resistance, and C3 complement plasma concentrations. A strong linear correlation was exhibited between both variables (r=0.406 p<0.001).

Since insulin plasma levels were not available in the EIR group, the glucose-metabolism was checked through the fasting glucose levels. The fasting glycaemia in the EIR group did not show a significant correlation with C3 complement levels. Thus, the difference in the metabolic characteristics between both groups was corroborated again (r=0.164, p=0.241) as shown in the figure 5.6.

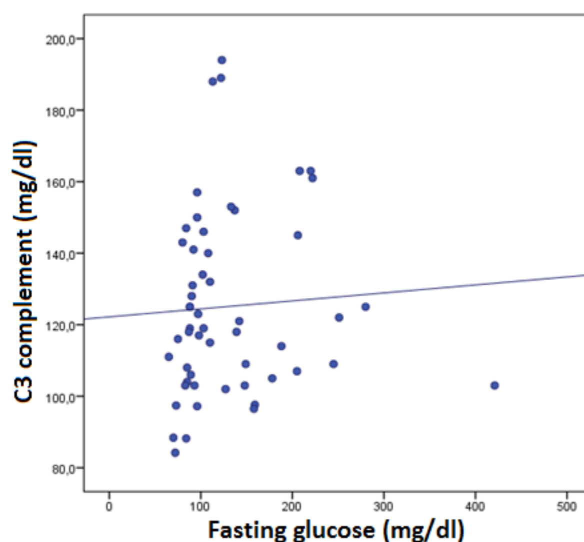


Figure 5.6: Scatter plot did not show correlation between fasting glucose measurements and C3 complement levels in exogenous insulin replacement cohort (EIR), where consequently, basal insulin was not measured (r=0.164, p=0.241).

5.5. Correlation of C3 with classical cardiovascular risk factors

Non-modifiable classical cardiovascular factors, such as gender and age, did not show statistically significant association with C3 complement levels. Age was not linearly correlated to C3 complement levels (r=0.09, p=0.090). With regard to gender, there were no significant differences in C3 levels between males and females (p=0.210), as seen in table 5.10.

There were no differences in age and gender distribution among the groups of subjects who presented each one of the modifiable risk factors and those who were factor-free, as seen in figure 5.7 and table 5.10. For instance, hypertensive and normotensive

patients had similar age and gender proportion. The exception was tobacco consumption, which was age dependant as explained in subsequent results.

C3 complement (mg/dl) and gender							
Variable Gender	N	Mean	SD	Mean difference	95%-Confidence interval		p-value
					Lower bound	Upper bound	
Male	206	131.18	24.31	3.39	-1.92	8.70	0.210
Female	168	127.78	27.93				

Table 5.10: Distribution of C3 values according to gender.

After confirming this independence, we studied the possible behavioural analogy between C3 convertase levels and the most frequent classical cardiovascular risk factors.

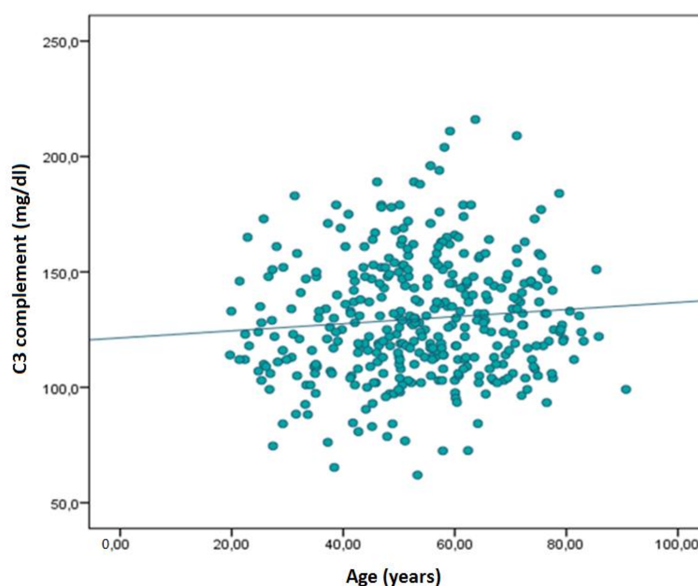


Figure 5.7: Scatter diagram representing a certain tendency but no significant correlation between age and C3 complement concentrations in blood (r=0.088 p=0.09).

As first analysed cardiometabolic risk factor, **obesity, as defined by BMI,** was measured according to BMI and showed a linear positive correlation with C3 levels. The sample population was divided into three groups: normal weight (BMI<25), overweight (BMI 25-30) and obesity (BMI>30). In both extremes, morbid obesity and anorexia, we assumed similar progressive distribution of weight values. C3 values were significantly different among the three groups (p<0.001), with higher values of C3 complement associated to degree of obesity (table 5.11). *Moreover, There was a*

positive linear correlation betweenC3 levels and increasing obesity degrees (r=0.37, p<0.001)(Figure 5.8)

C3 complement (mg/dl) and general obesity					
Variable		N	Mean	SD	P-value
BMI	Normal weight <25	129	118.31	2.23	<0.001
	Overweight 25-30	157	131.13	1.82	
	Obesity ≥30	88	143.66	2.66	

Table 5.11: Differences in C3 according to the degree of obesity. BMI cut-off points followed the obesity standards established by the WHO for Caucasians.

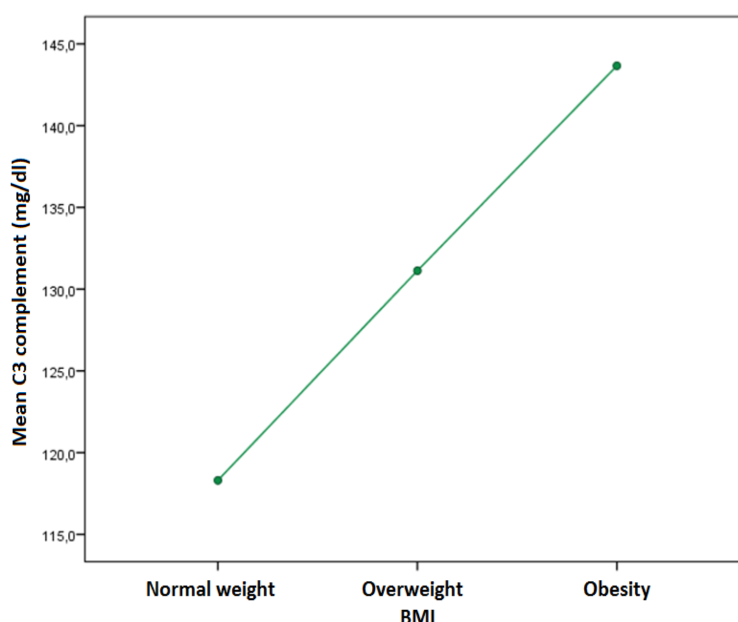


Figure 5.8: Positive linear correlation between C3 levels and increasing obesity degrees (r=0.37, p<0.001).

Following this study line, C3 concentrations were compared among subjects with and without the presence of classical risk factors other than obesity (Table 5.12).

The strongest positive correlation bound high C3 concentrations to high levels of **triglycerides** (p<0.001).

Hypertensive patients also showed higher levels of C3 complement than normotensive patients as displayed in table 5.12.

C3 complement (mg/dl) and classical cardiovascular risk factors							
Variable		N	Mean ± SD	MD	95%-CI		p-value
					Lower b.	Upper b.	
Hypertension (>135/85mmHg)	No	168	124.80±27.93	8.82	3.57	14.07	0.001
	Yes	206	133.62±23.70				
Hypercholesterolemia (LDLc >160mg/dl)	No	201	125.52±25.84	8.93	3.69	14.16	0.001
	Yes	173	134.45±25.48				
Low HDLc (<40 mg/dL males, <50mg/dL females)	No	296	127.58±26.14	9.94	3.50	16.38	0.003
	Yes	78	137.22±24.15				
Hypertriglyceridemia (TG> 150 mg/dl)	No	288	124.89±24.25	20.71	14.78	26.64	<0.001
	Yes	86	145.60±25.52				
Hyperglicaemia (FG>100 mg/dl)	No	279	127.54±26.41	8.30	2.28	14.33	0.007
	Yes	95	135.85±23.93				

Table 5.12: Summary of significant associations between presence of cardiovascular risk factors and higher levels of C3 complement.

We also analysed the association ($p<0.001$) of high concentrations of C3 with high levels of **LDLc** (>160mg/dl). In order to compare the LDLc levels in our population, a common cut-off value of >160mg/dl was established for hypercholesterolemia diagnosis in our sample population. The LDLc cut-off point for hypercholesterolemia differs from treatment threshold goal (general LDLc<130mg/dl) in the cardiovascular unit and is personalised per patient according to cardiometabolic PMH.

Diabetic patients showed significantly higher C3 concentrations than those without T2DM ($p=0.007$), as shown in the table 5.9. To confirm this fact we also tested the relationship between C3 measurements and **A1c** determinations in our population.

The T2DM ranges include pre-diabetes diagnosis for percentages of A1c of 5.7% or above until diabetes diagnosis from 6.5% or above. Both variables, C3 complement and A1c, reinforced a positive linear correlation ($r=0.112$, $p=0.03$) as shown in the figure 5.6.

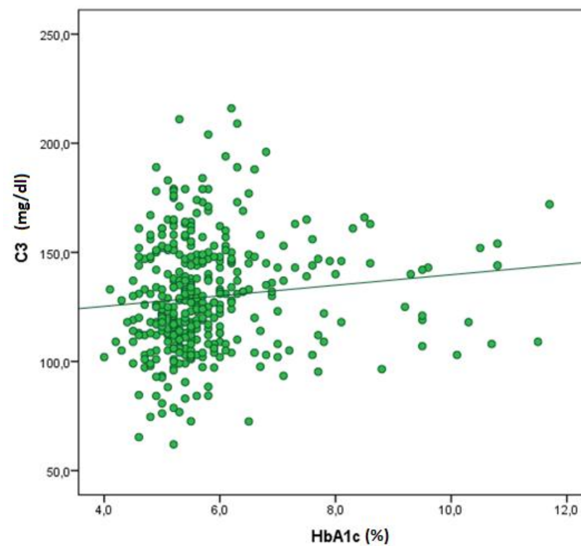
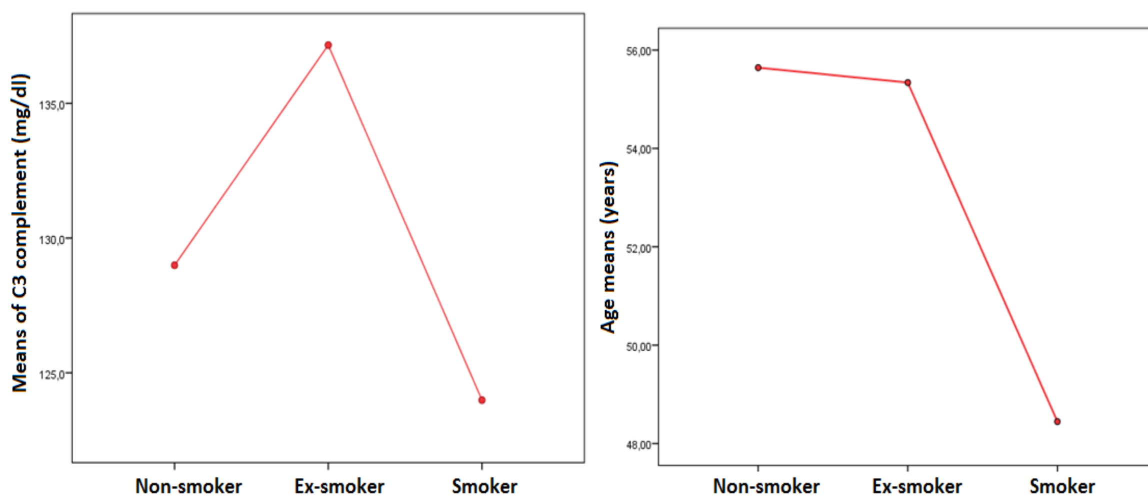


Figure 5.9: Scattered plot shows statistically significant correlation between A1c(%) measurements and C3 complement (mg/dl) concentrations (r=0.112 p=0.03).

In relation to the tobacco consumption, the association between C3 complement and **smoking habits** showed a significant inverse correlation (p=0.001). Due to the questionable implications of a possible protective effect of tobacco against atheroma formation, we analysed closely the characteristics of the population of smokers.

This examination showed that smokers (48.44±11.97 yr. old) were significantly younger than former smokers (53.34±12.72 yr. old) and non-smokers (55.64±11.97 yr. old. There was also a significant association between age and tobacco consumption (p<0.001) The association between age and smoking habit is also shown in the table 5.13 and graphs 5.10 and 5.11.



Figures 5.10, 5.11: Graphs displaying the means of C3 (first graph) and age (second graph) per tobacco consumption status.

Anova C3 complement (mg/dl) (n=374)				
Variable		N	Mean ±SD	P-value
Tobacco	Non-smoker	176	128.99 ±25.34	0.001
	Ex-smoker	94	137.16 ±25.40	
	Smoker	104	123.99 ±26.02	
Age	Non-smoker	176	55.64 ±16.63	<0.001
	Ex-smoker	94	55.34 ±12.72	
	Smoker	104	48.44 ±11.97	

Table 5.13: C3 complement means per smoking habit status which shows a negative correlation between smoking consumption and C3 complement concentrations. There is also a negative association between age and tobacco consumption, with the smokers group being the youngest and the non-smokers the oldest group (p<0.001).

5.6. Association between C3 and new cardiovascular risk markers

As a young component of the MetS, uric acid was measured in our population (5.81±1.59 mg/dl). The relationship between C3 complement and **uric acid** measurements exposed a strong linear correlation (r=0.224, p<0.001). Therefore, high levels of uric acid were associated with high levels of C3 complement as shown in the plot 5.12.

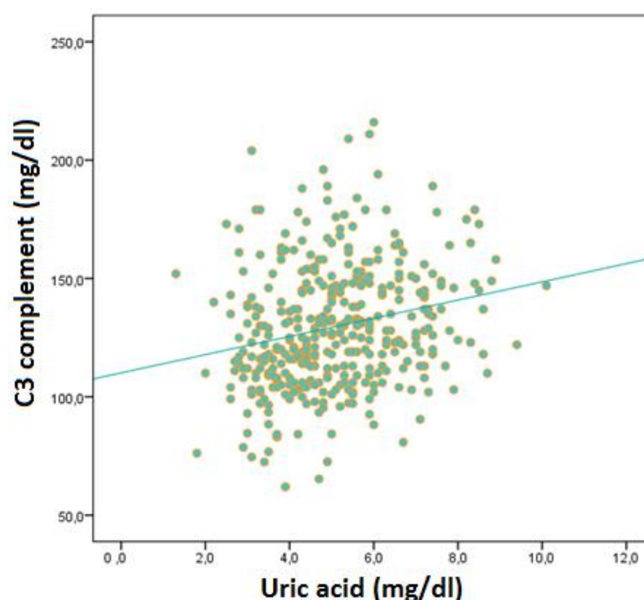


Figure 5.12: Uric acid levels and C3 complement measurements regression graph. For these results r=0.224, showing strong linear correlation (p<0.001)

Since the values of **uric acid** could be compromised by treatment with allopurinol, the relationship between C3 complement and uric acid concentrations could be also compromised. In virtue of this assumption, the correlation of C3 levels with the absence or presence of diagnosed hyperuricemia (9.8% of the study population) was also tested. Although the correlation between both variables was not significant, a positive tendency was shown, as displayed in table 5.14.

According to **ferritin** levels measured in our population sample (117.05±11.87μg/dl), there was strong linear association between ferritin blood concentrations and C3 complement levels (r=0.142, p<0.006) as shown in figure 5.13. As we did with the previous variables, in order to check the possible effect of patients treated with phlebotomies, we also compared the presence or absence of diagnosed hyperferritinemia (9.9% of the sample population) with C3 measured values. The association between both variables did not reach statistical significance, as shown in the table 5.14.

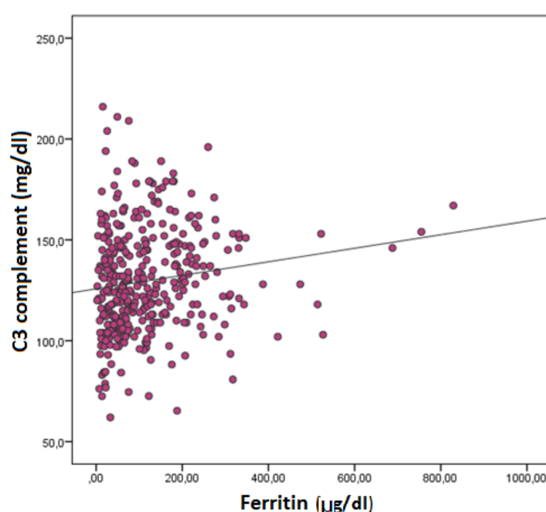


Figure 5.13: scattered plot with regression line of ferritin concentrations and C3 levels in 374 patients, both measurements are statistically positively associated (r=0.14, p=0.006). As seen in the graph outlier values have not been excluded here. Regression analysis has been done to fit a representative line of the set of data association between both variables.

With regard to lipoprotein(a) (Lp(a)) (22.77±29.64 mg/dl), new cardiovascular biomarker also related to inflammation no significant linear correlation with C3 was shown, as seen in graph 5.14. Accordingly, the concentrations of C3 were not significantly different between patients with and without presence of

hyperlipoproteinemia(a) (11.8% of the analysed population) as seen in figure and table 5.14.

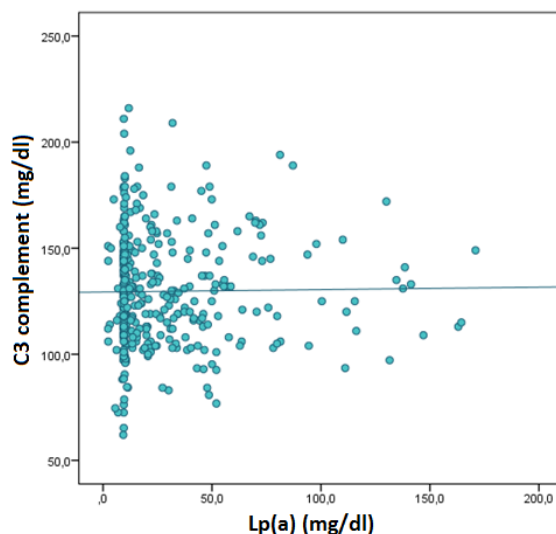


Figure 5.14: Graph showing Lp(a) and C3 complement values did not have a significant correlation (r=0.013, p=0.802). The vertical line drew by an accumulation of dots displays the Lp(a) test sensitivity limit before a second high sensitivity test, which included values close to 0, was set up in the Endothelium Pathology Unit.

T-student C3 complement (mg/dl)					Difference			
Variable		N	Mean	SD	MD	95% CI		p-value
						Lower bound	Upper bound	
Hyperuricemia (>7µg/dl)	No	337	129.33	26.27	3.31	5.56	12.18	0.463
	Yes	37	132.63	23.80				
Hyper-Lpa (>30 mg/dl)	No	330	129.10	26.47	4.69	-3.52	12.90	0.262
	Yes	44	133.79	22.17				
Hyperferritinemia (>180 µg/dl)	No	337	128.99	26.13	4.50	2.15	15.54	0.137
	Yes	37	135.69	24.52				

Table 5.14: Differences in C3 complement concentrations according to the presence or absence of hyperuricemia, hyperferritinemia and high-Lp(a),none on them showed statistical significance. .

5.7. C3 complement association with cardiovascular risk (REGICOR)

The cardiovascular risk was calculated through the REGICOR model (Framingham model adapted for Spanish population) as the risk of suffering a cardiovascular event in 10 years according to patient's age, gender and personal characteristics. In our population, REGICOR mean was 3.65±2.91% 10-year cardiovascular risk of an individual, ranged from 0.01% as minimum value to 17.65% as maximum value.

Testing the relationship between both variables, a positive linear correlation was observed between C3 concentrations and REGICOR values (Pearson $r=0.196$, $p<0.001$), as shown in graph 5.16.

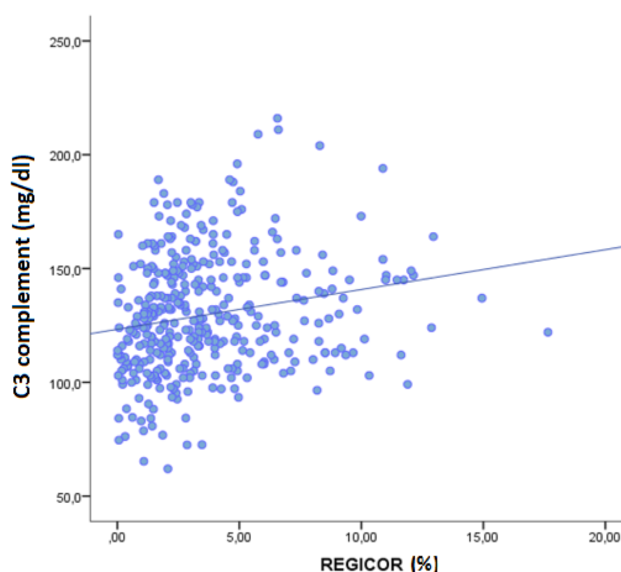
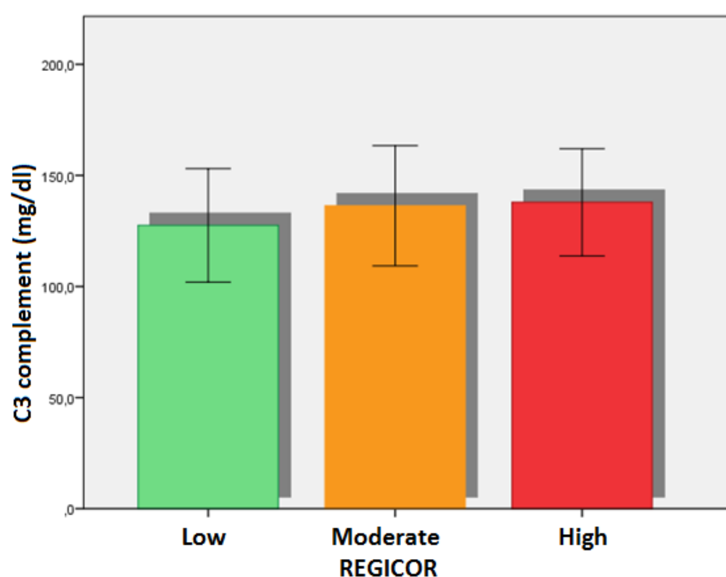


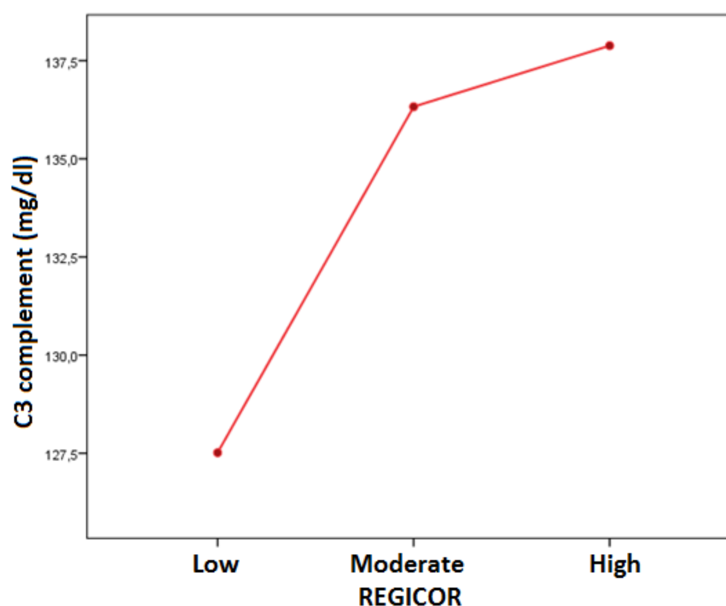
Figure 5.15: scattered plot representing the linear correlation between C3 levels in plasma and cardiovascular risk percentages in 10 years calculated by REGICOR ($r=0.196$, $p<0.001$)

As in routine clinical practice, we divided our population sample into three levels according to their cardiovascular risk. Following the REGICOR scales, as mentioned in the methodology, low risk was given to those individuals with less than a 5% possibility of suffering from a cardiovascular event in the next ten years, patients with 5-10% were considered moderate risk and those with >a 10% possibility were considered high risk as shown in table 5.15 and graphs 5.16 and 5.17.

C3 (mg/dl) and REGICOR		N	Mean	SD	P-value
REGICOR Levels (CV risk)	Low risk <5%	286	127.51	25.56	0.016
	Moderate risk 5-10%	72	136.32	27.08	
	High risk >10%	16	129.65	24.08	

Table 5.15: Distribution of C3 concentrations according to cardiovascular risk REGICOR levels (low, moderate and high cardiovascular risk).





Figures 5.16 and 5.17: representation of C3 mean concentrations per cardiovascular risk level (low, moderate, high) according to REGICOR formula.

The high risk cardiovascular group sample resulted too small (n=16) for statistical analyses and we decided to group moderate and high cardiovascular risk subjects into one moderate-high cardiovascular risk group for further calculations.

Two population groups of low cardiovascular risk <5% (n=286) and moderate-high cardiovascular risk ≥5% (n=88) were created. The relationship between C3 complement levels and REGICOR risk levels was tested again, showing that higher concentrations of C3 complement were significantly associated with higher cardiovascular risk (p=0.004), as displayed in graph 5.18 and table 5.16.

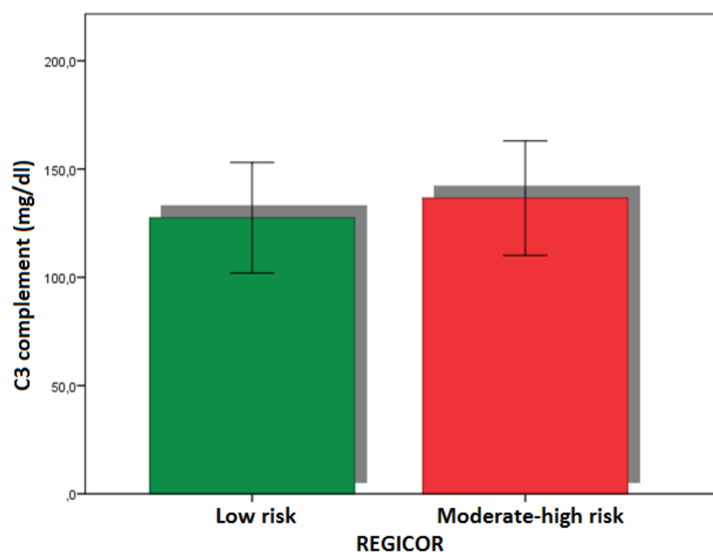


Figure 5.18: representation of C3 mean concentrations per cardiovascular risk levels (low and moderate- high) calculated according to REGICOR formula.

C3 complement (mg/dl) and REGICOR			Difference			
Coronary risk according to REGICOR model (%)	N	Mean ±SD	MD	95%-CI		p-value
				Lower b.	Upper b.	
Low risk <5%	286	127.51 ±25.56	9.09	2.91	15.27	0.004
High risk >5%	88	136.61 ±26.44				

Table 5.16: Statistically significant positive correlation (p=0.004) between C3 concentrations and cardiovascular risk levels calculated by REGICOR formula, with the C3 levels being higher for high cardiovascular risk patients (136.61±35.56 mg/dl) than for low risk subjects (127.51±26.44 mg/dl).

5.8. Emerging cardiometabolic risk and endothelial damage biomarkers

In random sub-groups of the population several detection tests were carried out, such as adiponectin, IL-10, IL6, TNF-α, VCAM-1, TAP, TAC or TBARS measurements. These tests were performed according to the availability of reactant's kits at the Endothelium and Cardiometabolic Unit's laboratory. These subgroups were selected randomly according to the accessibility to kits and reactants during their first visit. The characteristics of the multiple subgroups were not tested due to the limitations of the study and we assumed no differences with the original sample population.

5.8.1. Metainflammatory biomarkers

Adiponectin is considered an anti-inflammatory marker and recognised endothelial protector. The adiponectin levels (µg/ml) were arbitrarily measured in 254 subjects of the sample population. Adiponectin concentrations in plasma were negatively correlated to C3 complement values (r= -0.184, p= 0.003).

The distribution of adiponectin values was different across the C3 complement quartiles, with lower adiponectin levels associated with upper quartiles. (p=0.025) as shown in table 5.17 and figure 5.19.

Adiponectin levels (µg/ml) distribution between C3 quartiles							
				95% CI for Mean			
C3	N	Mean	SD ±S Error	Lower B.	Upper B.	Min.	Max.
Q1	64	10.45	6.03 ±0.75	8.94	11.95	2.36	35.78
Q2	67	9.85	5.71 ±0.69	8.46	11.24	3.80	31.54
Q3	57	9.57	5.23 ±0.69	8.18	10.96	3.15	37.41
Q4	66	7.74	3.99 ±0.49	6.76	8.72	2.38	21.70
Total	254	9.39	5.36 ±0.33	8.73	10.05	2.36	37.41

Table 5.17: Distribution of the adiponectin values across the C3 complement quartiles,.

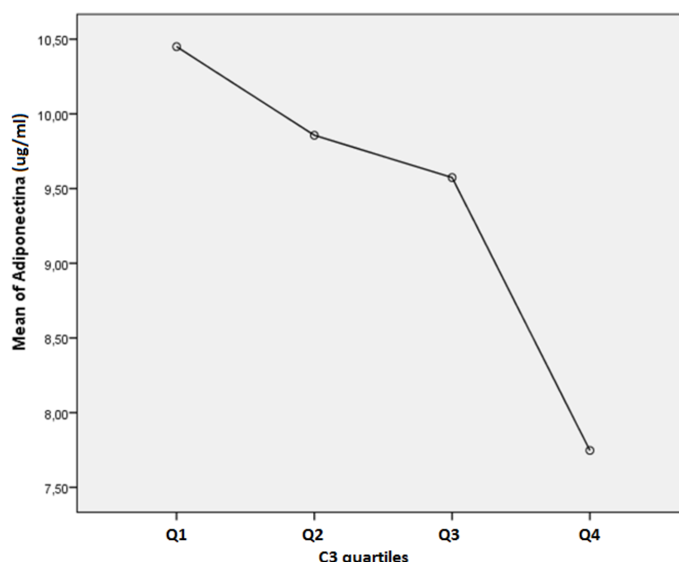


Figure 5.19: The graph represents the negative association between adiponectin leveles and C3 quartiles (p=0.025).

Interleukin-6 levels were randomly quantified in 244 patients of the population sample. Higher levels of interleukin-6 were positively correlated to C3 complement concentrations in plasma (r=0.747, p=0.021).

Interleukin-10 concentrations were randomly determined in 112 individuals of our study population. Il-10 showed an inverse correlation with C3 complement measurements, with borderline significance (r= -0.169, p=0.075). However, assessing the distribution of Il-10 between the C3 complement quadrants, a correlation was not shown (r= -0.134, p=0.262), as displayed in table 5.18 and figure 5.20 in further detail.

Distribution of Interleukin-10 (pg/ml) between C3 quartiles							
C3	N	Mean ±SD	Std. Error	95% CI for Mean		Min.	Max.
				Lower B.	Upper B.		
1,00	25	1.73 ±1.19	0.24	1.24	2.23	0.16	5.23
2,00	32	1.63 ±1.18	0.21	1.21	2.06	0.13	5.83
3,00	27	1.32 ± 0.73	0.14	1.03	1.61	0.31	3.35
4,00	28	1.25 ±1.02	0.19	.86	1.65	0.30	4.89
Total	112	1.49 ±1.05	0.10	1.29	1.68	0.13	5.83

Table 5.18: distribution of Interleukin-10 levels among across C3 quartiles (p=0.262).

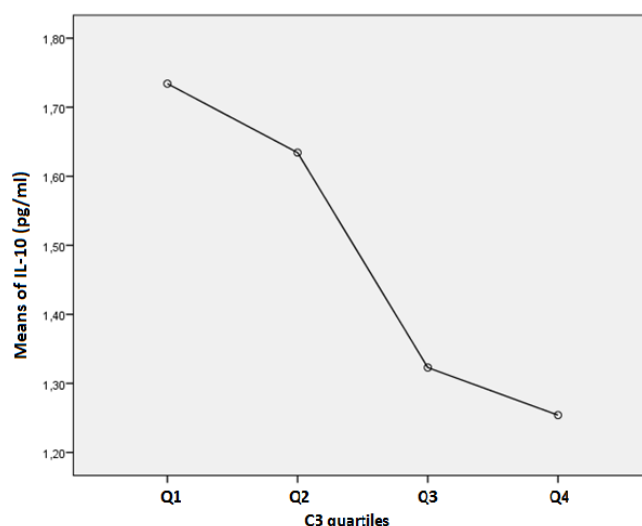


Figure 5.20: mean values of Il-10 according to C3 complement cuartiles.

Tumoral necrosis factor (TNF-α) levels were quantified randomly in 90 subjects of our study population, a considerable smaller sample. TNF-α levels displayed a mild negative behaviour with respect to C3 complement values (r=-0.03, p=0.078,), albeit this negative association was not demonstrated.

5.8.2. Endothelial damage biomarkers

Plasminogen activator inhibitor-1 (PAI-1) was measured at random in 75 subjects of our sample population. The association with C3 complement was significant (r=0.335, p=0.003,), but the distribution between C3 complement quartiles exhibited borderline association *(p=0.068) as shown in table 5.19 and figure 5.21.*

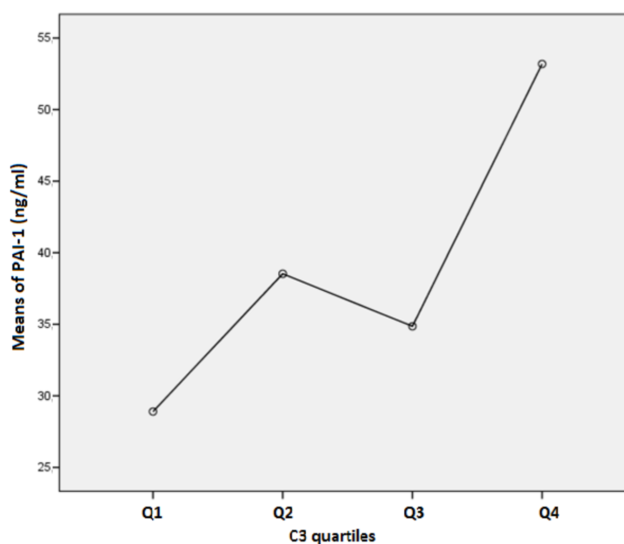


Figure 5.21: means of PAI-1 concentrations per quartile of C3.

PAI-1 mean values (ng/ml) per C3 quartiles							
			Std.	95% CI for Mean			
C3	N	Mean ±SD	Error	Lower B.	Upper B.	Min.	Max.
Q1	25	28.90 ±25.98	5.19	18.17	39.62	1.76	109.03
Q2	22	38.52 ±34.52	7.36	23.21	53.83	8.15	135.47
Q3	13	34.85 ±14.81	4.11	25.90	43.81	7.02	64.24
Q4	15	53.18 ±32.29	8.33	35.30	71.07	10.93	104.86
Total	75	37.61 ±29.43	3.39	30.84	44.38	1.76	135.47

Table 5.19: descriptive distribution of the PAI-1 means between C3 quartiles

Tissue-type plasminogen activator (TPA) levels were determined in 265 patients selected randomly. These values were compared with C3 complement levels and a strong association between both variables resulted ($r=0.263$. $p<0.001$). The distribution of the means per C3 quartiles was also studied, but the association in the distribution was not shown as seen in figure 5.22 and table 5.20.

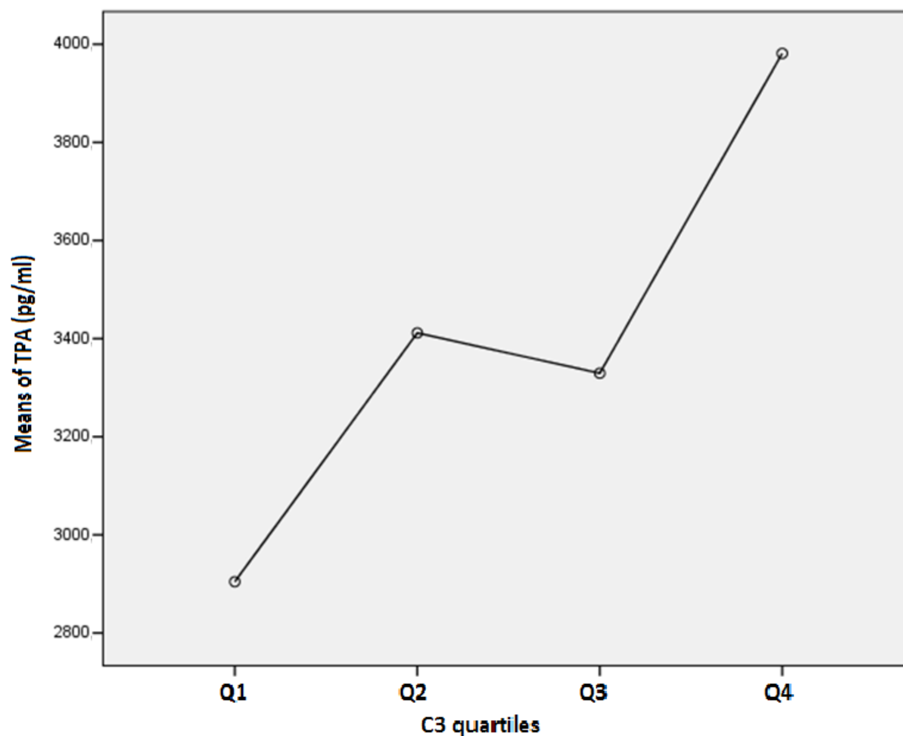


Figure 5.22: Representation of the means of TPA and quartiles of C3.

Endothelial expression of vascular cell adhesion molecule-1 **(VCAM-1)** correlates with atheroma formation. We obtained the results from 208 subjects at random. There was also a significant correlation between both variables (r=0.19, p=0.019).

Distribution of TPA means (pg/ml)							
				95% CI for Mean			
C3	N	Mean ±SD	Std. Error	Lower B.	Upper B.	Min.	Max.
Q1	71	2904.43 ±1507,50	178.91	2547.62	3261.26	730.3	8261.2
Q2	70	3411.56 ±1717.37	205.26	3002.07	3821.05	966.9	9090.0
Q3	57	3329.34 ±1467.73	194.45	2939.90	3718.78	1222.4	7746.0
Q4	67	3981.37 ±1577.25	192.69	3596.64	4366.09	1562.7	8852.7
Total	265	3402.07 ±1614.10	99.15	3206.84	3597.30	730.3	9090.0

Table 5.20: Distribution of the TPA mean values per C3 quartiles (r=0.19, p=0.019).

5.8.3. Oxidative stress biomarkers

Finally the oxidative stress biomarkers were represented by total antioxidant capacity (TAC) and thiubarrbituric acid substances (TBARS).

TAC levels (µM) were determined in 201 subjects of our sample population in an arbitrary way. The initial inverted direction of C3 convertase and TAC values, did not reach statistical significance (r=-0.50, p=0.485).

TBARS (µM/L) values were measured in 232 patients of our study population chosen randomly. TBARS levels showed marginal linear correlation with C3 complement values (r=0.114, p=0.084).

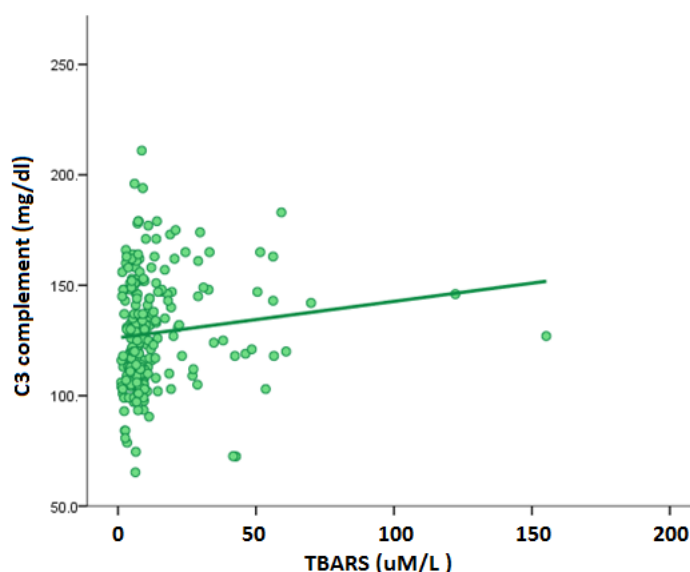


Figure 5.23: Borderline linear association between C3 complement and TBARS values (p=0.084).

5.9. Variables distribution analysis between C3 complement quartiles

To assess more exhaustively the correlation between all our variables with C3 complement we performed an analysis per C3 quartiles. The distribution of the variables per quartiles also distributed their thresholds and pathological diagnosis per quartiles.

Before proceeding with the analysis of the variables' behaviour, we assessed the distribution of the population across the C3 quartiles. The results of this analysis showed, that the number of subjects was homogenously distributed among quartiles with a similar number and percentage of subjects per quartile as shown in table 5.21.

Complement C3 quartiles	Frequency	Percentage (%)	Valid %	Cumulative %
Q1	93	24.9	24.9	24.9
Q2	92	24.6	24.6	49.5
Q3	93	24.9	24.9	74.3
Q4	96	25.7	25.7	100
Total	374	100	100	

Table 5.21: Homogenous distribution of the sample population among the quartiles of C3 complement concentrations.

5.9.1. Distribution of Sample population into C3 quartiles

In order to assess the behaviour of different variables along the whole range of C3 concentrations, we first assessed the distribution of the C3 complement variable, showing a histogram compatible with a normal distribution as shown in graph 5.24.

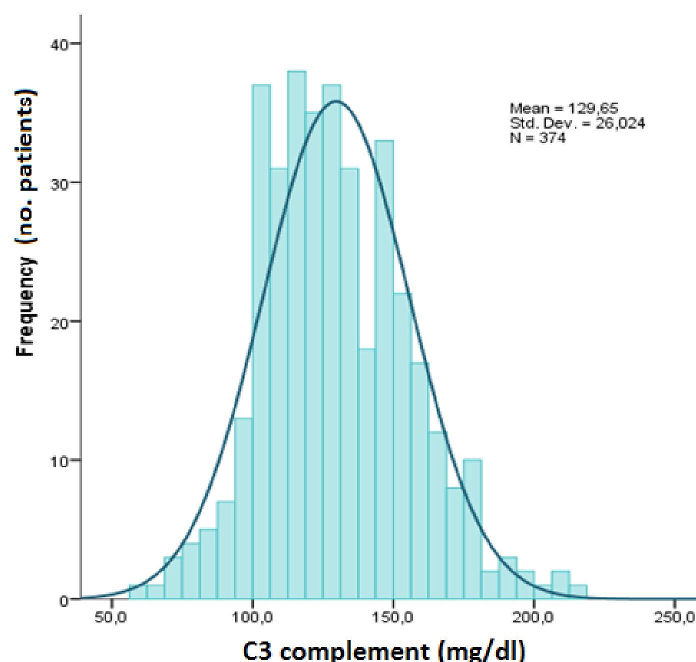


Figure 5.24: histogram of C3 complement concentrations and normal distribution curve.

Our study population (n=374) was divided into 4 quartiles based on the distribution of the C3 complement concentrations among the subjects. The division by quartiles was not affected by extreme values of non-normal distributions because the quartiles were calculated from non-parametric parameters (percentiles). In the study population the C3 complement variable (mean=129.65±26.02 mg/dl) had a median value of 127.00 mg/dl (percentile 50). C3 complement quartiles cut-off values were as follows: Q1: C3<111.75; Q2: C3 ≥ 117.5 to <127; Q3: 3 ≥127 to <147; and Q4 C3 ≥147, as shown in table 5.22.

Percentiles	25th	50th	75th
C3 (mg/dl)	111.75	127.00	147.00

Table 5.22: C3 distribution by percentiles, representing the quartiles cut-off points. Percentile 50 represents de median (127.00 mg/dl).

To check the independence of modifiable variables in our study the homogeneity of non-modifiable variables among the quartile's groups was assessed as follows.

There was no significant association between the gender distribution and C3 quartiles' groups as seen in the table 5.23 (p= 0.175).

C3 complement quartiles (N=374)		Gender		Total
		Females	Males	
Q1	N	42	51	93
	Percentage	45.2	54.8	100
Q2	N	52	40	92
	%	565	43.5	100
Q3	N	54	39	93
	%	58.1	41.9	100
Q4	N	58	38	96
	%	60.4	39.6	100
Total	N	206	168	374
	%	55.1	44.9	100

Table 5.23: Homogenous gender distribution per quartile according to the number of subjects and percentages (p=0.175).

There were not significant differences either in age among the C3 complement quartile's groups. The distribution of subjects per groups according to age was homogenous as seen in table 5.24.

Age distribution among the C3 complement quartiles (years)							
C3 quartiles	N	Mean ±SD	Std. Error	95% CI for Mean		Min.	Max.
				Lower B.	Upper B.		
Q1	93	51.08 ±15.18	1.57	47.95	54.20	24.79	90.70
Q2	92	53.73 ±16.13	1.68	50.39	57.07	19.70	85.80
Q3	93	56.26 ±14.56	1.50	53.26	59.26	19.92	82.31
Q4	96	53.20 ±13.11	1.33	50.54	55.85	22.84	85.39
Total	374	53.56 ±14.82	0.766	52.05	55.07	19.70	90.70

Table 5.24: Display of the age distribution in the sample population across the C3 quartiles. There was not a significant correlation of the mean ages among groups (p=0.12).

5.9.2. Distribution of Cardiometabolic continuous variables across the C3 quartiles

In order to assess the association between C3 complement concentrations and cardiovascular risk, the relationship between C3 values distributed by quartiles and several cardiometabolic continuous variables was evaluated.

Description of population distribution divided into C3 complement quartiles (n=374)								
Variables	C3 quartiles	N	Mean ±SD	Std. Error	95% CI for Mean		Min.	Max.
					Lower B.	Upper B.		
SBP (mmHg)	Q1	93	126.30 ±18.73	1.94	122.44	130.16	87	204
	Q2	92	134.72 ±23.20	2.41	129.91	139.52	91	205
	Q3	93	135.26 ±20.16	2.09	131.11	139.41	91	197
	Q4	96	136.94 ±16.61	1.69	133.57	140.30	101	183
	Total	374	133.33 ±20.13	1.04	131.28	135.38	87	205
DBP (mmHg)	Q1	93	78.00 ±10.57	1.09	75.82	80.18	55	112
	Q2	92	82.27 ±9.816	1.02	80.24	84.30	60	103
	Q3	93	82.59 ±9.83)	1.02	80.57	84.62	59	110
	Q4	96	85.46 ±10.74	1.09	83.28	87.64	65	116
	Total	374	82.11 ±10.55	0.54	81.03	83.18	55	116
Waist/hip ratio	Q1	93	0.87 ±0.09	0.01	0.85	0.89	0.70	1.05
	Q2	92	0.90 ±0.09	0.01	0.88	0.92	0.63	1.16
	Q3	93	0.93 ±0.07	0.01	0.92	0.95	0.70	1.09
	Q4	96	0.94 ±0.08	0.01	0.92	0.95	0.67	1.20
	Total	374	0.91 ±0.09	0.01	0.90	0.92	0.63	1.20
Waist (cm)	Q1	93	85.46 ±11.67	1.21	83.05	87.86	63	113
	Q2	92	90.43 ±12.42	1.29	87.86	93.01	59	128
	Q3	93	94.59 ±9.93	1.03	92.54	96.63	67	113
	Q4	96	97.75 ±12.05)	1.23	95.30	100.19	70	139
	Total	374	92.11 ±12.41	0.64	90.84	93.37	59	139
BMI (Kg/m²)	Q1	93	25.09 ±3.98	0.41	24.27	25.91	17.75	35.35
	Q2	92	25.91 ±4.32	0.45	25.01	26.80	18.31	47.54
	Q3	93	28.07 ±3.94	0.41	27.25	28.87	17.30	37.17
	Q4	96	29,40 ±5.24	0.53	28.33	30.45	21.14	49.52
	Total	374	27.13 ±4.71	0.24	26.65	27.61	17.30	49.52

Table 5.25: Description of the haemodynamic and anthropometric variables distribution along the C3 complement levels quartiles.

All the anthropometric cardiometabolic variables' means increased in proportionally to the C3 complement quartiles. Furthermore, all the anthropometric means, such as BMI ($p<0.001$), SPB ($p=0.001$), DPB ($p<0.001$), waist/hip ratio ($p<0.001$) and waist size ($p<0.001$) showed positive linear correlations. The variables distribution among C3 complement quartiles is displayed in table 5.25.

The number of MetS factors per patient was associated with C3 quartiles ($p<0.001$). Thus, most of the MetS factors-free or metabolically healthy patients were situated in the first C3 complement and there were no patients presenting five MetS constitutive criteria. Additionally, the percentage of patients showing positive for all the factors increased accordingly to the quartiles increment as shown in table 5.26. Furthermore, the majority of the measured numerical metabolic variables also showed a positive correlation to the C3 complement concentration per quartiles. In particular, those related to MetS.

Number of MetS criteria per C3 complement quartiles distribution								
C3 quartiles		Percentage of number of MetS criteria presented per patient						Total
		0	1	2	3	4	5	
Q1	N	31	27	23	11	1	0	93
	% within Q1	33.3	29.0	24.7	11.8	1.1	0.0	100
	% within no. of criteria	55.4	33.8	21.7	14.7	2.5	0.0	24.9
Q2	N	17	23	26	16	8	2	92
	% within Q2	18.5	25.0	28.3	17.4	8.7	2.2	100
	% within no. of criteria	30.4	28.7	24.5	21.3	20.0	11.8	24.6
Q3	N	6	20	23	24	10	10	93
	% within Q3	6.5	21.5	24.7	25.8	10.8	10.8	100
	% within no. of criteria	10.7	25.0	21.7	32.0	25.0	58.8	24.9
Q4	N	2	10	34	24	21	5	96
	% within Q4	2.1	10.4	35.4	25.0	21.9	5.2	100
	% within no. of criteria	3.6	12.5	32.1	32.0	52.5	29.4	25.7
Total	N	56	80	106	75	40	17	374
	% by no. of criteria	15.0	21.4	28.3	20.1	10.7	4.5	100
	% within no. of criteria	100	100	100	100	100	100	100

Table 5.26: Distribution of the percentage of number of metabolic criteria met per C3 quartile.

The **cardiovascular risk** calculated by the REGICOR model showed a sample population distribution with a mean risk in 10 years of 3.63 ±2.9 % cardiovascular, from a minimum of 0.01% to a maximum of 17.63%. The risk measured by REGICOR showed a statistically significant difference across the C3 complement quartiles (p=0.004), as displayed in table 5.27.

REGICOR (%) distribution by C3 complement quartiles							
Q	N	Mean ±SD	Std. error	95% CI for Mean		Min	Max
Q1	93	2.70 ±2.43	0.25	2.20	3.20	0.03	11.90
Q2	92	3.77 ±3.21	0.33	3.10	4.43	0.01	17.65
Q3	93	3.90 ±2.99	0.31	3.28	4.52	0.03	14.94
Q4	96	4.13 ±2.79	0.28	3.57	4.70	0.04	12.95
Total	374	3.63 ±2.91	0.15	3.33	3.93	0.01	17.65

Table 5.27: Differences in Cardiovascular risk measured by REGICOR formula among the C3 quartile groups (p=0.004).

Fasting glucose, a metabolic marker of **hyperglycaemia,** showed a positive tendency towards the increment of C3 quartiles. The mean values of fasting glucose (p=0.119), had an increasing trend according to the C3 quartiles, but they did not reach a statistically significant difference as seen in figure 5.25. A similar non-significant trend was also observed in the glycosylate haemoglobin values (p=0.171). Both values were measured in the whole population without exclusions.

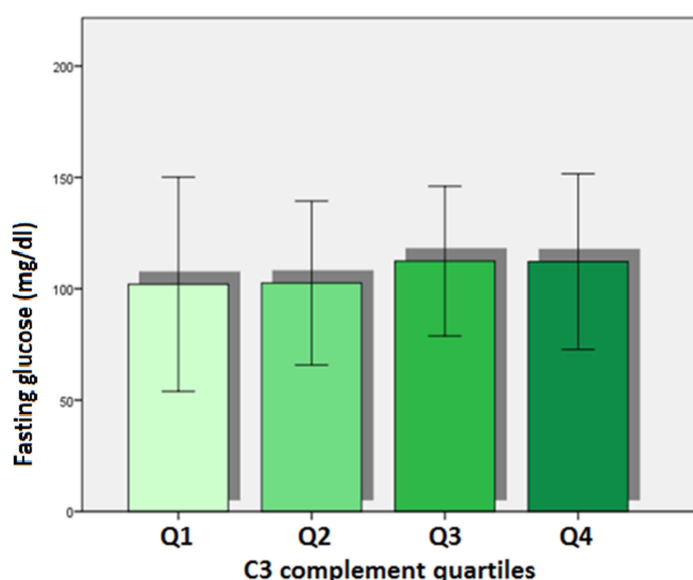


Figure 5.25: Bar chart representing the tendency incrementing the mean fasting glucose levels per C3 complement quartiles (p=0.171).

Meanwhile, the **insulin resistance** representative variable, calculated through the HOMA formula, showed a strong positive correlation with the C3 quartiles. The different sample population used to calculate insulin resistance was noted again, which excluded exogenous insulin dependent subjects (n=320) as explained earlier in this paper.

The Insulin resistance drew a clear positive correlation ($p<0.001$) with C3, HOMA incremented proportionally its percentages per quartile.

All the variables related with the glycaemic metabolism, FG, A1c and HOMA showed strong association ($p<0.001$) with C3 quartiles, as the distribution of their means along the C3 quartiles show in more detail in table 5.28.

The lipid metabolism of our population showed a more pathologic lipid profile in those patients with higher C3 quartiles as follows: Triglycerides levels were positively correlated ($p<0.001$) as showed in figure 5.26.

Meanwhile HDLc concentrations were inversely correlated as low HDLc is correlated to cardiovascular risk ($p<0.001$).

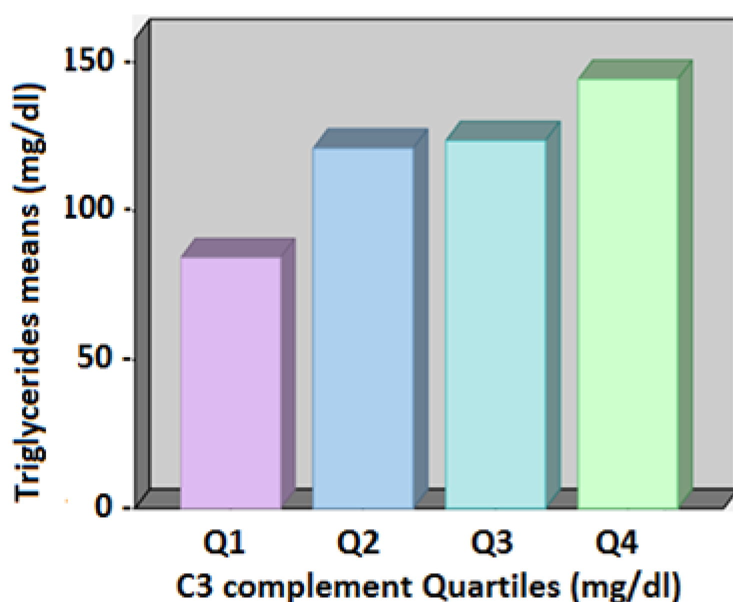


Figure 5.26: Values of triglycerides concentrations means per C3 complement quartiles.Both variables showed a strong linear correlation (p<0.001).

Description of population divided into C3 complement quartiles (n:374)								
T2DM variables		N	Mean ±SD	S error	95% CI for Mean		Min	Max
Fasting glucose (mg/dl)	Q1	93	102.03 ±48.09	4.98	92.13	111.94	64	421
	Q2	92	102.58 ±36.82	3.83	94.95	110.20	71	280
	Q3	93	112.44 ±33.60	3.48	105.52	119.36	71	241
	Q4	96	112.18 ±39.47	4.02	104.18	120.17	73	302
	Total	374	107.36 ±40.02	2.06	103.29	111.43	64	421
A1c (%)	Q1	93	5.76 ±1.29	.13	5.49	6.03	4.0	11.5
	Q2	92	5.61 ±1.08	.11	5.39	5.84	4.4	10.3
	Q3	93	5.99 ±1.17	.12	5.75	6.23	4.1	10.8
	Q4	96	5.88 ±1.24	.12	5.63	6.13	4.6	11.7
	Total	374	5.81 ±1.20	.06	5.69	5.93	4.0	11.7
HOMA (score)	Q1	73	1.31 ±1.01	.12	1.08	1.55	.32	6.16
	Q2	78	1.74 ±1.23	.14	1.46	2.02	.40	7.43
	Q3	84	2.45 ±1.58	.17	2.11	2.80	.39	10.19
	Q4	85	3.07 ±2.05	.22	2.62	3.51	.37	10.95
	Total	320	2.18 ±1.67	.09	2.00	2.37	.32	10.95

Table 5.28: Distribution of the sample population values of fasting glucose, glycosylate haemoglobin and insulin resistance (calculated with HOMA formula) per C3 complement quartiles.

On the other hand, other lipid profile parameters, such as LDLc and total cholesterol, did not show significant differences between LDLc (p=0.720) levels, total cholesterol concentrations (p=0.466) and the C3 complement quartiles as showed in table 5.29. None of these parameters are MetS criteria. Therefore, they are not directly involved in the cardiovascular risk score accepted in this study (REGICOR),

Description of population divided into C3 complement quartiles (n:374)								
Lipid profile		N	Mean ±SD	Std. error	95% CI for Mean		Min	Max
Total cholesterol (mg/dl)	Q1	93	199.85 ±43.58	4.519	190.87	208.82	130	365
	Q2	92	203.99 ±45.63	4.757	194.54	213.44	83	407
	Q3	93	196.99 ±46.25	4.796	187.46	206.51	115	389
	Q4	96	207.00 ±50.07	5.110	196.85	217.15	122	367
	Total	374	201.99 ±46.45	2.402	197.27	206.71	83	407
LDLc (mg/dl)	Q1	93	127.86 ±39.19	4.064	119.79	135.93	55	274
	Q2	92	128.15 ±40.65	4.238	119.73	136.57	25	316
	Q3	93	124.61 ±40.46	4.195	116.28	132.94	61	315
	Q4	96	131.60 ±45.53	4.647	122.38	140.83	59	280
	Total	374	128.09 ±41.47	2.144	123.87	132.30	25	316
Triglycerides (mg/dl)	Q1	93	84.14 ±35.94	3.727	76.74	91.54	34	233
	Q2	92	120.92 ±65.79	6.859	107.30	134.55	41	354
	Q3	93	123.53 ±61.01	6.327	110.96	136.09	40	273
	Q4	96	144.16 ±69.95	7.140	129.98	158.33	44	381
	Total	374	118.39 ±63.33	3.275	111.95	124.83	34	381
HDLc (mg/dl)	Q1	93	54.62 ±13.56	1.406	51.83	57.42	32	101
	Q2	92	51.34 ±13.37	1.393	48.57	54.10	25	105
	Q3	93	48.16 ±11.32	1.174	45.83	50.49	29	80
	Q4	96	46.39 ±9.03	.922	44.56	48.21	29	71
	Total	374	50.09 ±12.30	.636	48.84	51.34	25	105

Table 5.29: Distribution of the means lipid profile parameters among the C3 complement quartiles.

5.9.3. C3 quartiles association with inflammation and coagulation.

The **inflammation** biomarker, CRP, increased its values accordingly to increasing C3 complement quartiles. High sensitivity CRP showed a statistically positive correlation with the C3 complement levels divided in quartiles ($p<0.001$). **Coagulation** was represented in this analysis by three pro-thrombotic factors such as fibrinogen, homocysteine and lipoprotein(a).

Description of the sample population divided into C3 complement quartiles (n:374)								
Biomarkers		N	Mean ±SD	Std. Error	95% CI Mean		Min	Max
CRPhs (mg/L)	Q1	93	2.50 ±1.80	0.18	2.12	2.87	0.4	10.30
	Q2	92	2.45 ±1.92	0.20	2.05	2.85	0.16	14.90
	Q3	93	2.90 ±2.17	0.22	2.45	3.35	0.33	13.40
	Q4	96	3.6.5 ±2.32	0.23	3.18	4.12	0.29	12.70
	Total	374	2.88 ±2.17	0.10	2.67	3.10	0.14	14.90
Fibrinogen* (mg/dl)	Q1	62	275.42 ±56.20	7.13	261.15	289.69	181.7	464.0
	Q2	68	291.22 ±63.63	7.71	275.82	306.63	159.9	517.2
	Q3	79	299.48 ±59.76	6.72	286.10	312.87	161.0	448.2
	Q4	75	319.83 ±69.50	8.02	303.84	335.82	153.4	522.9
	Total	284	297.63 ±64.31	3.81	290.11	305.14	153.4	522.9
Homocysteine (μmol/L)	Q1	93	12.28 ±4.37	0.45	11.38	13.18	6.0	30.0
	Q2	92	11.96 ±3.36	0.35	11.27	12.66	4.5	21.6
	Q3	93	11.52 ±3.02	0.31	10.90	12.15	4.9	21.4
	Q4	96	11.69 ±2.79	0.28	11.12	12.26	5.9	21.8
	Total	374	11.86 ±3.43	0.17	11.51	12.21	4.5	30.0
Lp(a) (mg/dl)	Q1	93	27.92 ±28.88	2.99	21.97	33.87	2.4	147.0
	Q2	92	27.90 ±30.56	3.18	21.58	34.23	2.3	164.4
	Q3	93	26.50 ±29.36	3.04	20.45	32.55	2.4	141.3
	Q4	96	28.71 ±30.17	3.08	22.60	34.83	2.3	170.9
	Total	374	27.77 ±29.64	1.53	24.75	30.78	2.3	170.9
Ferritin (μg/dl)	Q1	93	91.15 ±93.24	9.66	71.95	110.36	7.20	526.65
	Q2	92	109.13 ±92.33	9.62	90.05	128.29	2.42	514.06
	Q3	93	123.22 ±109.56	11.36	100.65	145.78	2.95	687.80
	Q4	96	143.70 ±134.22	13.69	116.50	170.89	4.43	829.14
	Total	374	117.04 ±110.22	5.69	105.84	128.25	2.42	829.14

Table 5.30: Description of the distribution of levels of inflammation and coagulation markers in the sample population divided by C3 complement concentration levels.$p<0.05$.*

As a first coagulation representative, fibrinogen was distributed in the sample population from a minimum of 153.4 mg/dl to a maximum of 522.9 mg/dl, with a mean of 2 97.63 ±69.50 mg/dl.

Second representative, homocysteine (Hcy) values ranged from 4.5 µmol/L to 30 µmol/L, with a mean of 11.86 ±2.79 µmol/L.

Finally, Lpa measurements varied from 2.3 mg/dl to 170 mg/dl and the mean was 29.64±27.77 mg/dl (table 5.30). Values of Lipoprotein-a (Lp(a)) over 30 mg/dl indicated Lp(a) overload. There was not a significant correlation shown between hyperlipoproteinemia(a) and higher quartiles of C3 complement (p=0.504).

None of both variables, Lpa (p=0.966) nor Hcy (p=0.464), showed a correlation with C3 complement quartiles. Only fibrinogen values were significantly different among the C3 quartiles, as shown in table 5.30 (p=0.034).

5.9.4. Metabolic pathologies distribution between C3 quartiles

The sample population was distributed according to the presence or absence of cardiometabolic pathologies into two dichotomous variables. The percentages allocated per yes/no diagnosis and differences per quartile were assessed by cross-tabulation and chi squared.

A uric acid level over 6µg/dl was defined as iron overload, hyperuricemia. The sample population was divided into hyperuricemic and non-hyperuricemic groups. There was not significant association between the presence of **hyperuricemia** and higher values of C3 complement in plasma divided into quartiles (p=0.812).

On the other hand, **Hyperferritinemia** threshold was set at ferritin values in plasma over 180µg/L. The study population was split in two groups and correlated to C3 complement quartiles. The population was also segmented into hyperferritinemic and normal values of ferritin patients. There was no significant association between both variables (p=0.386). Both associations are shown in more detail in table 5.31.

C3 quartiles cross-tabulation with uric acid and lipoprotein(a) overloads							
C3 quartiles		Hyperuricemia			Hyperferritinemia		
		No	Yes	Total	No	Yes	Total
Q1	N	86	7	93	86	7	93
	% Q1	92.5	7.5	100	92.5	7.5	100
	% Variable	25.5	18.9	24.9	26.1	15.9	24.9
Q2	N	82	10	92	81	11	92
	% Q2	89.1	10.9	100	88.0	12.0	100
	% Variable	24.3	27.0	24.6	24.5	25.0	24.6
Q3	N	84	9	93	80	13	93
	% Q3	90.3	9.7	100	86.0	14.0	100
	% Variable	24.9	24.3	24.9	24.2	29.5	24.9
Q4	N	85	11	96	83	13	96
	% Q4	88.5	11.5	100	86.5	13.5	100
	% Variable	25.2	29.7	25.7	25.2	29.5	25.7
Total	N	337	37	374	330	44	374
	% Total	90.1	9.9	100	88.2	11.8	100
	% Variable	100	100	100	100	100	100

Table 5.31: Cross-tabulations between c3 quartiles and presence of hyperuricemia or hyperferritinemia.

The same analysis was performed with CRP and homocysteine. A CRP level over 2mg/L, according to Jupiter study, was defined as high CRP. Likewise, a correlation between both variables was not demonstrated (p=0.622). Homocystein overload hyperhomocyteinemia, was conventionally described as above 15 µmol/L. A statistical association was demonstrated between high C3 complement quartiles and a diagnosis of **hyperhomocysteinemia** (p=0.025). These three cross-tabulations are shown in table 5.32.

C3 quartiles cross-tabulation with:										
C3 complement Quartiles		Iron excess			High C-reactive protein			Hyperhomocysteinemia		
		No	Yes	Total	No	Yes	Total	No	Yes	Total
Q1	N	88	5	93	92	1	93	81	12	93
	% Q1	94.6	5.4	100	98.9	1.1	100	87.1	12.9	100
	% Variable	26.1	13.5	24,9	24.9	20.0%	24.9	28.2	13.8	24.9
Q2	N	82	10	92	91	1	92	72	20	92
	% Q2	89.1	10.9	100	98.9	1.1	100	78.3	21.7	100
	% Variable	24.3	27.0	24,6	24.7	20.0	24.6	25.1	23.0	24.6
Q3	N	83	10	93	91	2	93	66	27	93
	% Q3	89.2	10.8	100	97.8	2.2	100	71.0	29.0	100
	% Variable	24.6	27.0	24,9	24.7	40.0	24.9	23.0	31.0	24.9
Q4	N	84	12	96	95	1	96	68	28	96
	% Q4	87.5	12.5	100	99.0	1.0	100	70.8	29.2	100
	% Variable	24.9	32.4	25,7	25.7	20.0	25.7	23.7	32.2	25.7
Total	N	337	37	374	369	5	374	287	87	374
	% Total	90.1	9.9	100	98.7	1.3	100	76.7	23.3	100
	% Variable	100	100	100	100	100	100	100	100	100

Table 5.32: Cross-tabulation between C3 complement quartiles and iron excess, CRP overload and Hyperhomocysteinemia.

Because of their particular relevance, the classical cardiovascular factors were studied with more attention. The association of C3 complement quartiles with **hypertension** was doubly examined. First, we analysed the association with previous clinical diagnoses of hypertension (BP>135/85), second, with HBP MetS criterion (BP >135/85 mmHg or under treatment for HBP).

This double analysis showed significant association between the prevalence of hypertension and its incremental distribution per higher C3 complement quartiles. However, the association was stronger with HBP MetS criterion ($p<0.001$) than with hypertension diagnosis ($p=0.011$), as shown in table 5.33.

Cross tabulation between							
C3 complement Quartiles		Hypertension			HBP MetS criterion		
		No	Yes	Total	No	Yes	Total
Q1	N	55	38	93	52	41	93
	% Q1	59.1	40.9	100	55.9	44.1	100
	% Variable	32.7	18.4	24.9	40.0	16.8	24.9
Q2	N	41	51	92	33	59	92
	% Q2	44.6	55.4	100	35.9	64.1	100
	% Variable	24.4	24.8	24.6	25.4	24.2	24.6
Q3	N	36	57	93	22	71	93
	% Q3	38.7	61.3	100	23.7	76.3	100
	% Variable	21.4	27.7	24.9	16.9	29.1	24.9
Q4	N	36	60	96	23	73	96
	% Q4	37.5	62.5	100	24.0	76.0	100
	% Vatriable	21.4	29.1	25.7	17.7	29.9	25.7
Total	N	168	206	374	130	244	374
	% Total	44.9	55.1	100	34.8	65.2	100
	% Variable	100	100	100	100	100	100

Table 5.33: Cross-tabulation between hypertension, HBP MetS criteria and C3 complement quartiles.

The distribution of hypercholesterolemia (LDLc>160mg/dl) per C3 complement quartiles also showed a significant association (p=0.006). A closer examination of the patients with a presence of **low HDLc** (men<40mg/dl, women<50mg/dl) also showed a significant association between both variables (p=0.007).

This association was even stronger when C3 complement quartiles were crossed with low HDLc MetS criteria (low cyphers or under treatment, p=0.001) as displayed in more detail in table 5.34.

Cross tabulation between							
Quartiles		Low HDLc			Low HDLc MetS criterion		
		No	Yes	Total	No	Yes	Total
Q1	N	No	Yes	Total	75	18	93
	% Q1	85	8	93	80.6	19.4	100
	% Variable	91.4	8.6	100	31.0	13.6	24.9
Q2	N	28.7	10.3	24.9	60	32	92
	% Q2	70	22	92	65.2	34.8	100
	% Variable	76.1	23.9	100	24.8	24.2	24.6
Q3	N	23.6	28.2	24.6	57	36	93
	% Q3	72	21	93	61.3	38.7	100
	% Variable	77.4	22.6	100	23.6	27.3	24.9
Q4	N	24.3	26.9	24.9	50	46	96
	% Q4	69	27	96	52.1	47.9	100
	% Variable	71.9	28.1	100	20.7	34.8	25.7
Total	N	23.3	34.6	25.7	242	132	374
	% Total	296	78	374	64.7	35.3	100
	% Variable	79.1	20.9	100	100	100	100

Table 5.34: The distribution of population with low HDLc diagnosed by plasma concentrations and by MetS criteria among the C3 complement quartiles showed strong association.

The allocation of patients diagnosed with **hyperglycaemia** per C3 complement quartile showed a positive association. Again the diagnosis of hyperglycaemia MetS criterion ($p<0.001$) was stronger than the single fasting glucose levels measurement (FG>100mg/dl, $p=0.01$) as is shown in table 5.35.

Assuring the aforementioned statistical analysis, the distribution of hypertriglyceridemic patients among the C3 complement quartiles showed the strongest association. The majority of the hypertriglyceridemic subjects was allocated in the higher quartiles, inversely to the non-hyperglyceridemic individuals. Both the diagnosis of **hypertriglyceridemia** according to triglycerides plasma levels (TG>150mg/dl, $p<0.001$) and hypertriglyceridemia MetS criterion ($p<0.001$) showed a significant association with C3 quartiles, as displayed in more detail in table 5.36.

Cross tabulation between							
		Hyperglycaemia			Hyperglycaemia MetS criteria		
Quartiles		No	Yes	Total	No	Yes	Total
Q1	N	75	18	93	66	27	93
	% Q1	80.6	19.4	100	71.0	29.0	100
	% Variable	26.9	18.9	24.9	30.3	17.3	24.9
Q2	N	76	16	92	62	30	92
	% Q2	82.6	17.4	100	67.4	32.6	100
	% Variable	27.2	16.8	24.6	28.4	19.2	24.6
Q3	N	59	34	93	44	49	93
	% Q3	63.4	36.6	100	47.3	52.7	100
	% Variable	21.1	35.8	24.9	20.2	31.4	24.9
Q4	N	69	27	96	46	50	96
	% Q4	719	28.1	100	47.9	52.1	100
	% Variable	24.7	28.4	25.7	21.1	32.1	25.7
Total	N	279	95	374	218	156	374
	% Total	74.6	25.4	100	58.3	41.7	100
	% Variable	100	100	100	100	100	100

Table 5.35: distribution of the sample population classified by presence/absence of hyperglycaemia and C3 complement quartiles.

Cross tabulation between							
		HTG			HTG MetS Criterion		
Quartiles		No	Yes	Total	No	Yes	Total
Q1	N	89	4	93	89	4	93
	% Q1	95.7	4.3	100	95.7	4.3	100
	% Variable	30.9	4.7	24.9	32.1	4.1	24.9
Q2	N	73	19	92	71	21	92
	% Q2	79.3	20.7	100	77.2	22.8	100
	% Variable	25.3	22.1	24.6	25.6	21.6	24.6
Q3	N	71	22	93	61	32	93
	% Q3	76.3	23.7	100	65.6	34.4	100
	% Variable	24.7	25.6	24.9	22.0	33.0	24.9
Q4	N	55	41	96	56	40	96
	% Q4	57.3	42.7	100	58.3	41	100
	% Variable	19.1	47.7	25.7	20.2	41.2	25.7
Total	N	288	86	374	277	97	374
	% Total	77.0	23.0	100	74.1	25.9	100
	% Variable	100	100	100	100	100	100

Table 5.36: The distribution of the hypertrygliceridemic and non-hypertrigliceridemic populations by C3 quartiles showed a statistical correlation (p<0.001).

As described in the methodology, the sample population was stratified by degrees of obesity as follows, normal healthy weight (BMI=18-25), overweight (BMI>25-30), obese (BMI>30). The distribution of these three groups among the C3 complement quartiles was positively associated (p<0.001). The obese and overweight groups were more prevalent in the upper quartiles. Abdominal obesity (waist >102 cm in males, >88 cm in females) was also significantly associated with increased C3 complement levels, and more prevalent in superior quartiles (p<0.001) as shown in table 5.37.

Cross tabulation between								
Quartiles		Waist			BMI			
		Normal	MetS waist	Total	Normal	Overweight	Obese	Total
Q1	N	73	20	93	49	34	10	93
	% Q1	78.5	21.5	100	52.7	36.6	10.8	100
	% Variable	30.3	15	24.9	38.0	21.7	11.4	24.9
Q2	N	69	23	92	42	38	12	92
	% Q2	75	25	100	45.7	41.3	13	100
	% Variable	28.6	17.3	24.6	32.6	24.2	13.6	24.6
Q3	N	53	40	93	19	44	30	93
	% Q3	57	43	100	20.4	47.3	32.3	100
	% Variable	22	30.1	24.9	14.7	28	34.1	24.9
Q4	N	46	50	96	19	41	36	96
	% Q4	47.9	52.1	100	19.8	42.7	37.5	100
	% Variable	19.1	37.6	25.7	14.7	26.1	40.9	25.7
Total	N	241	133	374	129	157	88	374
	% Total	64.4	35.6	100	34.5	42	23.5	100
	% Variable	100	100	100	100	100	100	100

Table 5.37: Cross-tabulation distribution of BMI and central obesity across the C3 complement quartiles.

However, there was not a demonstrated association between gender and distribution by C3 complement quartiles either, showing that C3 levels were independent of gender(p=0.157).

As explained further above, due to the scarce number of high cardiovascular risk subjects, the sample population was stratified in low (<5%) and moderate-high (≥5%) cardiovascular risk. Moderate-high CV risk showed a trend of association with C3 complement quartiles (p=0.092). Meanwhile, C3 complement quartiles exhibited a positive association with diagnosis of MetS (≥3 MetS criteria), as the number of metabolic patients increased proportionally to the C3 quartiles (p<0.001), as can be seen in table 5.38 in more detail.

Cross tabulation between										
Quartiles		Gender			MetS			REGICOR CV risk		
		Male	Female	Total	No	Yes	Total	<5%	>5%	Total
Q1	N	42	51	93	81	12	93	80	13	93
	% Q1	45.2	54.8	100	87.1	12.9	100	86	14.0	100
	% Variable	20.4	30.4	24,9	33.5	9.1	24.9	28	14.8	24.9
Q2	N	52	40	92	66	26	92	68	24	92
	% Q2	56.5	43.5	100	71.7	28.3	100	73.9	26.1	100
	% Variable	25.2	23.8	24,6	27.3	19.7	24.6	23.8	27.3	24.6
Q3	N	54	39	93	49	44	93	69	24	93
	% Q3	58.1	41.9	100	52.7	47.3	100	74.2	25.8	100
	% Variable	26.2	23.2	24,9	20.2	33.3	24.9	24.1	27.3	24.9
Q4	N	58	38	96	46	50	96	69	27	96
	% Q4	60.4	39.6	100	47.9	52.1	100	71.9	28.1	100
	% Variable	28.2	22.6	25,7	19	37.9	25.7	24.1	30.7	25.7
Total	N	206	168	374	242	132	374	286	88	374
	% Total	55.1	44.9	100	64.7	35.3	100	76.5	23.5	100
	% Variable	100	100	100	100	100	100	100	100	100

Table 5.38: Cross-tabulation distribution of gender, presence of MetS and cardiovascular risk among C3 quartiles. C3 complement quartiles were independent of gender (0.157). Reversely, moderate-high cardiovascular risk was correlated (p=0.092) and metabolic syndrome was strongly associated (p<0.001) to C3 complement quartiles' distribution.

Finally, the presence of active smokers was higher in the lower quartiles and inversely distributed. A smoking habit was not statistically associated with C3 complement quartiles (p=0.59). When we separated non-smokers into ex-smokers and never-smokers the inverse correlation was not statistically significant either (p=0.10), table 5.39.

Cross tabulation between smoking habit and C3									
Quartiles		Smoking			Smoking				
		No	Yes	Total	No	Former	Yes	Total
Q1	N	61	32	93	50	11	32	93
	% Q1	65.6	34.4	100	53.8	11.8	34.4	100
	% Variable	22.6	30.8	24.9	28.4	11.7	30.8	24.9
Q2	N	62	30	92	38	24	30	92
	% Q2	67.4	32.6	100	41.3	26.1	32.6	100
	% Variable	23.0	28.8	24.6	21.6	25.5	28.8	24.6
Q3	N	76	17	93	47	29	17	93
	% Q3	81.7	18.3	100	50.5	31.2	18.3	100
	% Variable	28.1	16.3	24.9	26.7	30.9	16.3	24.9
Q4	N	71	25	96	41	30	25	96
	% Q4	74	26	100	42.7	31.3	26	100
	% Variable	26.3	24	25.7	23.3	31.9	24	25.7
Total	N	270	104	374	176	94	104	374
	% Total	72.2	27.8	100	47.1	25.1	27.8	100
	% Variable	100	100	100	100	100	100	100

Table 5.39: Association between smoking status classifications (smoker/non-smoker vs. smoker/ex-smoker/non-smoker) and C3 levels.

5.10. Multivariate analysis of factors associated to C3 complement concentrations

The multivariate model of lineal regression was performed as described in the methodology. With this model, we analysed the constitutive factors of MetS and certain laboratory variables as possible predictors of C3 complement levels.

Our maximum model initially comprised 17 variables, which explain the 34% of the C3 complement values variability (co-linearity coefficient=34), as described in the table 5.40.

The non-modifiable variables, age and gender, remained in the model to adjust interaction of the modifiable variables, although none of them had statistical significance in the initial model. Co-linearity between age and gender was associated.

Tobacco consumption variable was adjusted to age. To avoid co-linearity problems, quantitative variables were centred at their mean before including them in the multivariate models.

We initially suspected interaction between age and smoking habit variables in our sample population. Therefore, the association between C3 and smoking habit would have been influenced by the patient's age. Consequently, we introduced a variable to model the interaction, age per smoking habit. By doing so, the relation of smokers and C3 levels was differentiated from non-smokers.

Descriptive Statistics C3 multivariate model population				
	Mean ±SD	N	Pearson Correlation	Sig. (1-tailed)
C3	130.42 ±26.01	320	1.000	.
Age	53.21 ±14.75	320	0.07	0.120
Gender	0.43 ±.50	320	-.104	0.032
CPR	2.72 ±1.90	320	0.277	<0.001
MetS	0.33 ±.47	320	0.362	<0.001
REGICOR high risk	0.23 ±.42	320	0.161	0.002
Hypertriglyceridaemia	0.26 ±.44	320	0.379	<0.001
Hypertension	0.64 ±.48	320	0.282	<0.001
Low HDLc	0.34 ±.47	320	0.209	<0.001
Hyperglycaemia	0.40 ±.49	320	0.252	<0.001
Waist	0.32 ±.47	320	0.310	<0.001
HOMA	2.19 ±1.68	320	0.406	<0.001
Hypercholesrerolemia	0.47 ±.50	320	0.187	<0.001
Hyperhomocysteinemia	11.63 ±3.36	320	-0.069	0.110
Lipoprotein(a)	27.51 ±29.79	320	-0.016	0.385
Smoking	0.28 ±.45	320	-0.131	0.010
Smoking-age	13.24 ±22.44	320	-0.115	0.020

Table 5.40: Multivariate model for C3 complement population description. N=320, Patients under exogenous insulin treatment were excluded as explained in insulin resistance analysis.

Gender, hyperhomocysteinemia and lipoprotein(a) diagnosis variables according to cut-off points were not significantly associated to C3 complement conforming to the multivariate analysis ($p>0.050$). Gender was inversely correlated to C3 levels (males have higher C3 values). Smoking habit negatively correlated to C3 concentrations and, as mentioned earlier, was associated to age.

According to beta coefficient's significance the first less significant variables removed from the maximum model were smoking and interaction of smoking habit and age. When we adjusted tobacco to age there was not significant association any longer. At the same moment or directly after removing smoking habit, the next less influential variables Table 5.41 presents data of the multivariable model.

Initial maximum multivariate model							
	Unstandardized Coefficients		Standard Coefficient			95% CI for B	
Variable	B	Std. Error	Beta	t	Sig.	Lower B	Upper B
C3	121.67	7.44		16.36	.000	107.04	136.31
Age	-0.19	011	-0.11	-1.66	0.10	-0.41	0.04
Gender	-3.76	2.89	-0.07	-1.30	0.19	-9.44	1.93
CPR	2.10	.65	0.15	3.23	0.00	0.82	3.39
MetS	-7.78	4.62	-0.14	-1.69	0.09	-16.87	1.31
REGICOR	-2.72	3.45	-0.04	-.79	0.43	-9.51	4.06
Hypertriglicerida emia	15.73	3.31	0.26	4.75	0.00	9.21	22.25
HBP	10.85	3.05	0.20	3.56	0.00	4.84	16.86
Low HDLc	5.74	3.10	0.11	1.85	0.07	-0.36	11.83
Hyperglycaemia	8.59	3.33	0.16	2.58	0.01	2.03	15.14
Waist	12.10	3.30	0.22	3.65	0.00	5.57	18.63
HOMA	2.06	0.90	0.13	2.29	0.02	0.29	3.83
Hypercholesterol emia	6.21	2.53	0.12	2.46	0.02	1.23	11.18
Hyperhomocyste inemia	-0.59	0.40	-0.08	-1.50	0.14	-1.37	0.19
Lipoprotein(a)	-0.02	0.04	-0.03	-0.53	0.59	-0.10	0.06
Smoking	-4.56	10.95	-0.08	-0.48	0.68	-26.10	16.99
Smoking-age	-0.07	0.21	-0.06	-0.34	0.73	-0.49	0.35

Table 5.41: initial analysis of the maximum model variables. The less significant variables and first to be removed were smoking (p=0.677) and smoking adjusted to age (smoking-age, p=0.733)

The remaining variables after regression analysis were considered predictors of C3 complement concentrations. The regression analysis finished when all the variables included in the maximum model reached statistical significance ($p \leq 0.05$), excluding age and gender as fixed variables (table 5.42).

Predictive factors of C3 complement concentrations by co-linearity diagnosis Dependable variable: C3							
	Unstandardized Coefficients		Standard Coef.			95.0% CI Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower B.	Upper B.
Age	-0.19	0.1	-0.11	-1.94	0.05	-0.39	0.00
Gender	-1.95	2.62	-0.04	-0.74	0.46	-7.12	3.20
CRP	2.06	0.66	0.15	3.12	0.00	0.76	3.36
HTG	14.34	3.01	0.24	4.76	0.00	8.41	20.27
HTA	8.70	2.81	0.16	3.10	0.00	3.18	14.22
Hyperglycaemia	6.35	3.02	0.12	2.10	0.04	0.41	12.29
Waist	9.14	2.86	0.16	3.20	0.00	3.51	14.76
HOMA	2.39	0.90	0.15	2.67	0.01	0.63	4.15
High LDLc	4.90	2.49	0.09	1.97	0.05	0.01	9.79

Table 5.42: Final results of the multivariate linear correlation model. Inflammation (CRP), MetS criteria (HTG, HBP, FG and waist) and IR (HOMA) were the predictor variables of the 32% variance of the C3 complement.

Some confounding factors, such as hyperglycaemia, and HOMA, were predictive of the C3 levels. However, the most significant covariates for C3 were hypertriglyceridemia ($p<0.001$), waist, hypertension, and CRP ($p=0.002$) in our multivariate linear model.

The predictive value of hypercholesterolemia in the C3 model turned out to be borderline significance ($p=0.050$). Age ($p=0.52$) and gender ($p=0.456$) remained not statistically significant to predict C3 values in the final outcome measure model. Together, those variables could explain more than 32% of the variance of C3 levels ($R^2=0.316$).

Patients with vascular inflammation (higher CRP levels), insulin resistance (higher HOMA results) and/or fulfilling MetS criteria, such as central obesity (waist), HBP or hypertriglyceridemia will have higher C3 complement levels with equal age and gender.

6. DISCUSSION

6. Discussion

In this observational cross-sectional study, representative of population from Madrid referred to secondary cardiometabolic care, the majority of our population was metabolically unhealthy, as expected for a cardiometabolic unit, where 49.7% suffered from incomplete MetS and 35% from MetS, while the prevalence of the MetS in Spain for adults was 31% according to DARIOS study [218]. Furthermore, hypertension (65%) and hyperglycaemia (42%) resulted as the most prevalent pathologies when we analysed individually the constitutive factors of MetS. This finding was contrary to expectations in literature where central obesity plays the main role [219].

Our study demonstrated that circulating C3 strongly predicts the cluster of MetS (Adult Treatment Panel III–defined) independently of the MetS components. In previous research in South Asians but not in Caucasians, C3 levels were found to be independently associated with MetS [220]. In preceding studies that did not fully adjust for the MetS components, C3 was found to be a predictor of complete MetS [68, 221, 222].

The presence of each MetS criteria was strongly associated to higher concentrations of C3 complement individually in our population. So, the degree of obesity, high levels of triglycerides, low HDL, hypertensive and hyperglycaemic status, were foreseen by C3 level variations. These findings were not previously known and we considered them particularly relevant. Thus, these new insights indicate that complement cascade activation would be within the first physiologic alterations leading to MetS independently of its aetiology as pre-diabetic and cardiovascular condition.

Besides this, the C3 complement values increased proportionally to the number of MetS criteria. This is the first documented study in which C3 complement predicted the number of MetS criteria. Even more reassuringly, the association between C3 levels and ATP-III criteria was almost linear. Adding extra evidence, the percentage of patients positive for all the factors increased in parallel to increment of the quartiles and there were no patients presenting five MetS constitutive criteria in the first C3 quartile and most of the MetS factors-free or metabolically healthy patients were situated in the

first C3 complement quartiles. Thus, C3 concentrations would be able to be considered a predictor of MetS and stratification by number of MetS criteria.

Even more interesting, there was a change in the slope inclination between two and three criteria met. Levels of C3 complement, although higher for three criteria, were similar. This fact made us consider the possible arbitrary threshold of three criteria for MetS diagnosis, not sustained in clinical evidence. This fact has already been described by Lemieux in relation to the hypertriglyceridaemic waist [223]. However this slope has not been described for other combination of MetS components and it should call expert's attention for further MetS actualisations. Moreover, the graph correlating C3 complement means and cardiovascular risk also changed the inclination of the slope between moderate and high risk, as explained later in the text. If we can simplify MetS diagnosis, through equal risk, we will be able to benefit a larger number of patients with preventive strategies.

The association between obesity, general obesity and central obesity with C3 values was confirmed in this study by four ways: firstly quantitatively, correlating values of BMI and waist size with C3 high concentrations; secondly qualitatively, associating diagnosis of overweight and obesity by BMI thresholds and central obesity by WHO cut-off points for Caucasians with high levels of C3 complement; thirdly, there was statistical correlation between BMI and central obesity with C3 complement quartiles; fourthly, waist as one of the variables remaining as predictor of C3 values variability by multivariate analysis as will be shown below. The central role of C3 in obesity-induce inflammation has been described in the literature according to general obesity [224, 225] but not stratified by central obesity and C3 quartiles.

Carrying on with the firm association between C3 levels and MetS, hyperglycaemic diagnosis showed a significant correlation with high C3 concentrations. To confirm this fact we also tested the relationship between C3 measurements and glycated haemoglobin (A1c) determinations in our population. Both variables reinforced a positive linear correlation. This positive association was emphasised when C3 complement quartiles were crossed with the diagnosis of hyperglycaemia MetS criterion that showed a linear distribution.

Low HDLc MetS criterion was also associated with C3 complement levels and this distribution was even more significant when C3 complement quartiles were crossed. As

expected due to its inverse correlation to cardiovascular risk broadly accepted, HDLc were also negatively correlated to C3 concentrations. With this correlation we confirmed the negative association between HDLc, cardiovascular protector, and C3 complement levels already described in the literature [222, 226].

The readings of diastolic and systolic blood pressure also counterpartyed C3 concentrations in plasma as seen previously in the literature [227]. Besides this, the diagnosis of hypertension as a qualitative variable demonstrated parallel correlation with the C3 quartiles, with high C3 complement concentrations being a predictor of hypertension in our study. When C3 complement quartiles were crossed the correlation was stronger with hypertension MetS criterion than with single hypertension readings, but both were statistically significant. We preferred the qualitative than the quantitative hypertension variable because the blood pressure values could be distorted in treated patients and removing treatments is considered an ethical limitation in our study.

Qualitatively, hypertriglyceridemia diagnosis represented the most robust interrelation with C3 values in our study. Moreover, both diagnosis of hypertriglyceridemia, triglycerides plasma levels and hypertriglyceridemia MetS criterion, showed an increasing parallel correlation when C3 complement quartiles were crossed. Ultimately, hypertriglyceridemia was the most influential factor determining C3 complement values according to multivariate analysis as explained below. Along these lines, C3 complement would be a predictor of hypertriglyceridemia [228, 229].

Following the thread, we mention the two often forgotten important companions of the MetS. Firstly hyperuricemia [19], which has been a long term accompanist of obesity and secondly hyperglycaemia. Hyperuricemia has been found to predict the development of both obesity and T2DM [230]. Furthermore, it has been described hyperuricemia association with MetS in multiple papers [231, 232], but the association between C3 complement and uric acid has not been further investigated. The results of this study showed an intense association between both variables, linear distribution by numbers, strong association with hyperuricemia diagnosis and reinforcing proportional distributions along quartiles, an association not previously demonstrated in the literature.

On the other hand, raised levels of ferritin have been found relevant to central obesity and metabolically obese normal weight individuals, hypertension, dyslipidaemia and MetS in several studies of Western populations [233]. In our study, high values of

ferritin were strongly associated with high values of C3 complement. Meanwhile the correlation of hyperferritinemia diagnosis and C3 complement was not significant because of the inference effect of phlebotomies in our population.

One of the key features of our study was represented by the fact that levels of complement C3 were independent of non-modifiable factors, such as age and gender when we correlated quantitatively and by C3 quartiles. Therefore, none of the previous findings analysed above in this discussion can be attributed to these factors. This correlation was checked with C3 concentrations and with the population distributed per quartile. However, a slight influence between age factor and C3 levels showed as a tendency but not significant association in the scatter diagram between the values of both variables. This impact of age in the C3 concentrations behaviour was definitely discarded in the multivariate analysis mentioned below.

Finally within the MetS entity, the association with Insulin Resistance (IR) was proven with a strong positive correlation of C3 plasma levels and C3 quartiles with HOMA values, influencing all of the above unlike age and gender [144, 224, 234]. This strong link between HOMA and C3 convertase activity independent of non-modifiable factors has been fully supported by statistical evidence in our study by association between HOMA and C3 concentrations, C3 quartiles, diagnosis of insulin resistance and as bidirectional predictive factor of C3 concentrations with multiple regression analysis at the end of our statistical analysis, this original finding was not found in the literature

Cardiovascular risk factors not included among the MetS components and their association with C3 will be considered below prior to analysing the cardiovascular risk, named LDLc, smoking habit, Lp(a) and Hcy.

In our study, LDLc levels were also strongly correlated with C3 complement levels, a finding not demonstrated for the general population in the literature [8], which has been only previously demonstrated in familiar hypercholesterolemia [9, 10]. As with the obesity-LDLc relationship [235], the possible correlation of LDLc with C3 via VLDLc, which belongs to MetS as a broadly accepted component [236], and hypertriglyceridemia, is not excluded. Only small dense LDLc, instead of total LDLc, are considered as part of the MetS and were not measured in this study which also comprised treated patients [237, 238].

With respect to smoking habit and C3, the inverse association represented an unexpected finding, as also happened previously in the literature in the negative association between tobacco and Alzheimer's and other examples [239, 240], but can be explained because the smokers group was significantly younger than that of non-smokers and ex-smokers. We proved in this study that in our population tobacco consumption was influenced by age. When adjusted to age, smoking habit did not show a correlation with C3 complement. Despite this, we can not obviate that tobacco consumption can activate the complement alternative pathway in vitro by modifying the third component of complement [241].

As a mention of the cardiovascular risk factors not associated to MetS but emerging factors, lipoprotein(a) and homocystein have been related to cardiovascular pathology [193, 242]. None of them showed association with C3 concentrations in our study population. Therefore our study confirmed in humans the correlation findings previously found in rabbits, where crossed inmuno-electrophoresis analysis indicated that Lp(a) apo(a) portion retarded the migration of C3b in complement activated serum, but had no effects on complement C3 activation[243, 244].

With the above results at hand, regarding the correlation of C3 with MetS and LDLc, a statistically significant correspondence between C3 concentrations and cardiovascular risk levels calculated by REGICOR formula did not surprise us, with the C3 levels being higher for high cardiovascular risk patients than for low risk subjects. Moreover, reinforcing this close association, a robust interrelationship was found between C3 concentrations and cardiovascular risk scales, C3 levels and REGICOR treated as a numerical variable and C3 complement quartiles and REGICOR values.

The relationship between cardiovascular risk measure by Framingham score was previously described in the literature [245-247] and C3 was established as a cardiovascular risk associate in both cross-sectional and in longitudinal studies in the CODAM study [248], but there are no previous publications correlating C3 complement levels with REGICOR, just suggested in a medical conference abstract written by ourselves [249]. With this research we confirm our previous suspicion and the hypothesis of this paper associating C3 levels with REGICOR CV score.

The Framingham risk and REGICOR cardiovascular risk scores predict development only of cardiovascular disease whereas the presence of the MetS, which has been also correlated to C3 complement, predicts both diabetes and cardiovascular disease. Subsequently, C3 convertase can become a cardiometabolic biomarker of both pathologies.

A step further, C3 convertase was also associated with the more complex idea of cardiometabolic syndrome (CMS), an amplified version of MetS, as explained in the knowledge review chapter. Thus, active components like inflammation (CRP, fibrinogen) and metaflammation (adipokines), oxidative stress and endothelial dysfunction are included in the complex CMS equation.

Inflammation in the cardiometabolic syndrome (CMS), where its low-grade inflammation is led by the CRP and followed by Lysosomal phospholipase A2 (LPLA2) [250], amyloid protein a (SAA: serum amiloide A) [251] and fibrinogen [252] participated, is continued by metaflammation (in Greek: μετά, along with), that should also be added to this process. This metabolic triggered inflammation has been described as a process of neighbourhood (highly vascularised abdominal fat) and a process of distance (intestinal dysbacteriosis, periodontal disease) [253]. Metaflammation would amplify the basic inflammation which characterises the atherothrombotic disease [254].

As a first finding, we showed a close relationship between C3 and C-reactive protein levels in our population sample. The cardiovascular chronic inflammatory status was established according to the threshold of CRP resulting from the JUPITER study [255, 256]. Both, inflammatory status diagnosis and CRP numerical values were heavily correlated to C3 complement levels. Moreover, CRPhs increased its values in parallel with the C3 complement levels according to the behaviour of its means along the C3 complement quartiles. Thus, high sensitivity CRP showed a firm correlation to the C3 complement levels divided in segments and C3 convertase activity became a solid predictor of inflammatory status in our population. This strong association confirms the central role of C3 convertase in the inflammation process as seen in the literature [257, 258].

The intense relationship between fibrinogen, the other evaluated inflammatory marker, and C3 complement levels and quartiles has been demonstrated in our population.

Fibrinogen has proaggregant properties besides its role as an inflammatory marker, thence, C3 convertase activity may represent a barometer of the pro-coagulant state degree in our population plasma, not previously found in the literature.

A step further, we looked for the links of C3 with metaflammation related to highly vascularised abdominal fat tissue actively participating in the low-grade inflammation involving the vascular endothelium [94, 259]. Consequently, adipose tissue is now considered a dynamic endocrine organ that releases adipokines. Multiple adipokines are involved in this complex process [89, 253, 260-263], from all of them we measured two pairs, pro-inflammatory adopokines (TNF-α and IL-6) and anti-inflammatory adipokines (adiponectin and IL-10). In our study it was mandatory to further examine the possible interrelation between both activities, pro and anti-inflammatory, and C3 levels. As an expected result of its recognised protective role, adiponectin was clearly inversely correlated with C3 complement concentrations in our population by direct correlation and distributed into quartiles. Adiponectin is considered a protective cytokine produced in healthy adipose tissue and its levels decline in inflammation and oxidative stress. There are a scarce number of publications correlating C3 convertase with adiponectin in population subgroups [235, 264, 265]. Together, these results may partly contribute to explain the role of adiponectin linking obesity with atherosclerosis, as a dependent dose-response of this hormone and C3 complement concentration. Likewise, IL-10 concentrations, another protective adipokine, resulted borderline inversely correlated to C3 plasmatic levels. Although this marginal correlation could not be confirmed by quartiles distribution, it represents a new input in the cardiometabolic literature where the correlation between IL-10 and C3 levels only has been confirmed in vitro studies in acute inflammation [266-268]

On the opposite side, IL-6 y la TNF-alpha behaved in the expected direction. Both cytokines values grew in parallel with C3 values, contrarily to the protective adipokines previously observed, although only IL-6 reached statistical signification. Thus, IL-6 pro-inflammatory marker levels were correlated positively with C3 convertase values in our study. Recently, it was demonstrated that complement C3 (C3) synthesis can be up-regulated by pro-inflammatory cytokines, such as IL-6, but the authors were not able to relate C3 levels with IL-6 [245]. IL-6/C3 association was demonstrated in burned patients with classical acute inflammation, correlating both variables levels with the severity of injury and development of infection [269]. In the same manner, these parameters could be used in metaflammation to measure the severity of vascular injury

and prediction of CV risk. Unfortunately, tumoral necrosis factor (TNF-α) levels were only quantified in a small random subgroup of 90 subjects following the same direction although the scarce number of results did not demonstrate a correlation with C3 values. Platel and al. suggested in vitro the possible action of TNF-α as u-regulator of C3 production in a dose-dependent manner but it has been not confirmed in vivo [270].

On the grounds of the aforementioned low-grade inflammation, our results showed that C3 complement circulatory concentration levels were a useful inflammatory and metaflammatory measurer. This finding alone would deserve further studies, in vivo and in vitro, specifically designed to clarify if there is also a physiologic interaction on top of its biomarker action. This physiologic interaction should be understood as active participation and interaction in the low-grade inflammation which characterises the atherothrombotic vascular disease from early stages.

Inflammation and metaflammation form along with endothelial dysfunction and oxidative stress a pathogenic triad of great vascular impact [94]. After the analysis of the results obtained with pro and anti-inflammatory adipokines and with CRP, we continued evaluating the C3 interaction with biomarkers of endothelial dysfunction and oxidative stress.

Within the endothelial markers, we have chosen the most liable ones, which are represented by VCAM, tPA and PAI-1. VCAM-1 ligand is expressed in endothelial cells after cytokines stimulation and plays an important role in the recruitment of mononuclear leukocytes to inflammatory sites in vivo [271]. Endothelial dysfunction leads to rapid induction of VCAM-1 expression and increased monocyte adhesion. The significant association between both variables in our research, VCAM-1 and C3, reaffirms our initial hypothesis of C3 convertase as a marker of endothelial damage. Even more interesting, C3a activates the phosphorylation of protein kinases (MAPK) and induces the up-regulation of vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) [272]. Therefore, as a destructive endothelial cascade C3 convertase up-regulates VCAM-1, which promotes cellular adhesion, endothelial and vascular damage. In contempt of this logical thinking, the correlation of these two proteins with endothelial damage is in short supply in the literature. This finding about the VCAM-1 is considered of enormous interest by us because of contrary findings in the literature about its relationship with the C3 as

endothelial dysfunction marker and also causative entity, with potential to amplify all the CMS cascade.

In our study, despite of the small number of subjects tested the correlation between plasminogen activator inhibitor-1 (PAI-1), not only as an adipokine but as an excellent endothelial marker [19], and C3 complement was strongly significant. Raised PAI-1 is responsible for hypofibrinolysis in specific diseases, and it has been specifically described in MetS and diabetes [273]. This down-regulation of fibrinolysis and pro-aggregating factors, such as raised Von Willebrand factor (fvW) in endothelial dysfunction, leads to a prothrombotic state responsible of a high percentage of cardiovascular events. Even more interesting, today we know that PAI-1 also promotes formation of endothelial microparticles and reduces transmembrane asymmetry of phospholipids increasing in vitro thrombin generation [274].

As the PAI-1, tPA levels were also strongly correlated with C3 complement values. tPA is a serine protease secreted by the endothelial cell and considered the quintessential fibrinolytic factor, which binds to fibrin and converts the inactive plasminogen into plasmin, widely considered standard of care in acute ischemic stroke [275]. Recently it has been demonstrated that tPA activates the classic complement pathway from C3 to terminal components [144]. Moreover, tPA is produced and released from endothelial cells and is responsible for the removal of intravascular fibrin deposits coordinated with the complement cascade [276]. tPA and PAI-1 activate the complement cascade but only the tPA activates the kinin system in plasma [268].

C3 can represent a marker of both factors, tPA and PAI-1, and the final balance between both of them will decide the resulting status whether anti-thrombotic or pro-thrombotic in relation to haemostasis. Because of the dual association of C3 with both of them, C3 would assume a neutral role.

Entering more into detail of the analysed markers, the association between tPA and VCAM- 1 with C3 quartiles showed a significant association, meanwhile C3 quartiles and PAI-1values showed a borderline positive correlation.

As the third axis of the vascular triad, inflammation, endothelial dysfunction and oxidative stress, oxidative stress joined the party, although in our study it appeared as borderline signification. So, oxidative stress is a key feature in atherogenesis, involved

in the whole process from endothelial dysfunction to atheromatic plaque formation and rupture. In this direction, disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the endothelial cell and represent the earliest stage of the endothelial dysfunction [277, 278]. As a byproduct of lipid peroxidation, TBARS (Thiobarbituric acid reactive substance) has been considered a marker of oxidative stress and the TBARS assay has been applied in clinical studies linking oxidative stress response with cardiovascular risk. Although TBARS levels showed marginal association with C3 complement values, the small sample analysed indicated that we pointed in the right direction [279]. There is no available literature correlating both variables and further studies should be designed to establish the specific role of C3 in oxidative stress as predictor, marker or participant factor.

Total antioxidant capacity in human plasma (TAC) is a useful technique to evaluate individual responses to antioxidant treatment, but with low liability within a population context [280, 281], as occurred in our study that then explained our no statistically significant result.

As a final statistical test in this research, in order to distinguish between the relations of C3 complement (dependant variable) with others independent variables, we used the multivariable analysis technique. This tool evaluated the strength of associations between age, gender, various components of the MetS, inflammation, insulin resistance, cardiovascular risk and C3 complement. With the final multivariate analysis we initially explained the 35% of the variability of the C3 complement dependant variable in our sample population. With this statistical tool we simultaneously analysed multiple independent variables, those previously found significantly associated with C3 and measured in all the non-exogenous insulin dependent subjects in the study, with our dependent variable, C3 complement quartiles, using matrix correlational algebra. Although C3 complement was previously defined in our study independent from the non-modifiable factors age and gender, they both remained in our multivariable model of regression analysis on purpose to dismiss any possible cofounding in the association assessment with other modifiable variables in this study [218].

The resulted cluster of most influential variables in their multivariable interrelationship with C3 complement was hypertriglyceridemia in first place followed by hypertension, CRP, waist, insulin resistance (HOMA) and hyperglycaemia in descending order

according to signification, independently of age and gender. The factor analysis yielded this cluster of six variables that alone accounted for almost 34% of the total C3 complement variance. Thus, HOMA can have an influence but does not explain all the findings related to MetS, CMS and C3.

Our study provided to triglycerides the leading role as factor with the biggest impact over C3 levels and deserves an additional comment. The role of triglycerides in cardiometabolic pathology grew in the last two decades since the overexpression receptors for VLDL (VLDLr) by unhealthy endothelium is known [19]. These receptors are present in small amounts in the healthy endothelium to play a key role in three actions: a) LPL stimulation, an enzyme of endothelial origin that is essential in the metabolism of triglycerides [282]. b) PAI-1 expression, as endothelial synthesis product, that in physiological amounts plays a determining role in haemostatic control but which excess leads to hypofibrinolytic status [283]. c) VLDLr, besides its action recognising VLDL, is intrinsically a potent endogenous inhibitor that negatively regulates the angiogenic properties of RVECs (retinal vascular endothelial cells). Therefore, the VLDLR activates RVECs and significantly enhances angiogenesis in vivo and in vitro [284]. Its three beneficial actions at endothelium are achieved with low degrees of expression. Ideally, the organism, in the presence of hypertriglyceridemia, would be able to overexpress VLDLr at the liver level to improve the plasma clearance of VLDL. Thus, VLDLr overexpression would indirectly reduce the rate of plasma LDLc (formed in the bloodstream itself from VLDL by the CEPT, cholesterol ester transfer protein) [285]. Unfortunately, this overexpression at hepatic level is only possible through genetic therapies [286]. Contrarily, the overexpression of VLDLr in MetS and diabetes occurs at vascular level and participates in endothelial dysfunction as Saban-Ruiz et al. described. From all the above in respect to VLDLr, there is an association described in the literature between overexpressed VLDLr in presence on high triglycerides and PAI levels, because of the main role of hypretrygliceridemina in our study this relationship acquires a bigger dimension.

As a final comment related to hypertriglyceridaemia, I would like to stress the importance of its association with C3 complement that should be a factor taken into account for treatment purposes. Hypertryglyceridaemia has recently been forgotten in clinical practice guidelines and in daily practice by doctors in general. These results made us recommended to measure C3 plasma concentrations in Hypertriglyceridemic patients. Doing so we would be aware of the importance of the high levels of

triglycerides effects and treat in consequence. The treatment of hypertriglyceridemia seems more justified when taking into account its relation with VLDLr and PAI-1 as described above.

Summarising our research, C convertase value goes beyond its role as cardiovascular risk predictor and our most interesting results described C3 complement as predictor of classical MetS, insulin resistance and newer Cardiometabolic Syndrome (CMS) with insulin resistance swinging between both syndromes.

In relation to MetS the robust relation with each of the criteria and the proportional relation to the number of criteria called our attention and made us to consider the presence two MetS criteria enough to initiate preventive treatment strategies. In this direction, based on these findings, subgroups of incomplete MetS patients with higher C3 complement values may be identified that are at a disproportionally high risk of developing insulin resistance and cardiovascular disease, with possibly therapeutic consequences. Therefore, C3 convertase down-regulation may become the target for new biological treatments to reduce cardiovascular events directly and indirectly, but this final question will be answered in future specific studies designed to target C3 convertase activity. According to Cardiomentabolic Syndrome, C3 has demonstrated its role as witness of the vascular triad which comprises inflammation, endothelial dysfunction and possibly oxidative stress, and its role as a somehow active factor interacting with all of them. Future studies will provide us with the degree and importance of our findings.

Finally, The C3 complement seems to go beyond CRP in several aspects, moreover in relation to MetS/CMS. None of the multiple studies performed in relation to CPR obtained as many relevant findings as our study. Besides this, C3 seems to have higher specificity than CRP, which is raised in multiple situations where C3 complement is not. We suggest determining both variables in Cardiometabolic Units until specifically designed studies compare specificity and cost-effectiveness between both variables.

While awaiting definitive results, the simultaneous evaluation of C3 and CRP would provide the intrinsic value of CRP more liability and both variables, jointly analysed, would result in a high predictive tool as contemporary precise Cardiometabolic Medicine requires.

7. CONCLUSIONS

7. Conclusions

1. The studied population was characterised by high prevalence of Metabolic Syndrome (MetS). Besides this, hypertension and hyperglycaemia were the most prevalent MetS criteria contrary to expectations in the literature, where central obesity plays the main role. These findings matched the characteristics of a population referred to a Cardiometabolic Risk Unit.

2. The MetS was strongly correlated to high concentrations of C3 complement, meanwhile low C3 levels were associated with metabolically healthy patients free of MetS criteria. Furthermore, C3 complement levels in plasma increased proportionally to the number of diagnosed MetS criteria per subject, a finding not previously known. There was also a change in the slope inclination between two and three criteria, so levels of three criteria were similar for two and three criteria. This fact made us to consider the possible arbitrary threshold of three criteria for MetS diagnosis. Besides this, the graph representing the relation between C3 complement and cardiovascular risk also changed the inclination of the slope between moderate and high risk. Both findings could be correlated and MetS diagnosis could be simplified to two criteria through equal risk.

3. The presence of each single MetS criterion was also strongly associated with C3 complement values and quartiles, named hypertriglyceridemia, central obesity, hyperglycaemia (measured by A1c), hypertension and Low-HDLc criteria individually. The strongest association between C3 values and MetS criteria was displayed by triglycerides. High levels of C3 complement were significantly correlated with high levels of triglycerides, hypertriglyceridemia diagnosed by cut-off point, hypertriglyceridemia MetS criterion and linear association between hypertriglyceridemia and C3 quartiles. Our study provides to hyperthiglyceridemia great impact on the association C3/MetS.

4. Besides the aforementioned Metabolic Syndrome classical components, C3 complement levels were also associated with uric acid and ferritin usually forgotten in the literature.

5. Age and gender were not associated with C3 in our sample population and none of the previous findings in this paper can be explained by them. Any impact of age in the C3 concentrations behaviour was definitely discarded in the multivariate analysis.

6. Strong positive correlation between C3 plasma levels and C3 quartiles with HOMA values was proven. Insulin resistance can influence but not explain all the previous findings.

7. Even the LDLc (not a MetS criterion) was also correlated with C3 levels. As with the obesity-LDLc relationship, the possible correlation of LDLc with C3 via VLDLc is not excluded.

8. The correlation between high C3 concentrations and high cardiovascular risk diagnosed by REGICOR formula was statistically demonstrated between both variables, between numerical variables and quartiles. Therefore, C3 represented a cardiovascular biomarker for our population.

9. Our interest went beyond C3 complement as a cardiovascular biomarker to the amplified version of MetS, named Cardiometabolic Syndrome (CMS). CMS sums up to the classic MetS criteria these three axis: inflammation and metaflammation, endothelial dysfunction and oxidative stress. We obtained a positive result of C3 correlation with each one of the three interconnected axes:

- CRP and fibrinogen values, representatives of acute inflammation, increased in parallel with C3 complement levels. With regard to metaflammation, the adipokines, behaved as expected in respect to the C3 complement: positively associated IL-6 and TNF-α and inversely associated IL-10 and adiponectine.
- From the endothelial point of view, VCAM showed significant association with C3, a finding of important interest due to contrary results in the literature about the C3-VCAM association. Therefore, in our study C3 acts as endothelial dysfunction marker and causal agent, with potential to amplify the entire CMS lesional cascade. Two additional endothelial markers, tPA and PAI-1, were correlated in the limit of the statistical signification with C3 concentrations. On the other hand, a relation between overexpressed VLDLr in the CMS and the PAI levels has been described in the literature, but in our study reached a higher dimension because of the main role of hypertriglyceridemia.
- With respect to oxidative stress, a marginal association between TBARS and C3 was shown. The small sample analysed showed us that we are on the right track.

10. In this order, hypertriglyceridemia, CRP, HTA, waist, HOMA and hyperglycaemia resulted predictors of the 34% of C3 complement variability by multivariate analysis. HOMA, with less significance than the four cited elements, reached the end of the multivariate analysis to stay as an influential factor.

11. C3 seems to go beyond CRP because none of the multiples studies performed with CRP in relation to MetS/CMS obtained as many relevant findings as shown in this study. Specifically designed studies should compare C3 and CRP cost-effectiveness/specificity in the future. Meanwhile, we suggest the simultaneous evaluation of CRP and C3 determination in plasma to increase the intrinsic value liability of CRP as required in Cardiometabolic Medicine. This new medicine is predictive, personalised and anticipative, being an integral part of the Precision Medicine, the medicine of the future.

8. Resumen en Español

Resumen en Español

Introducción: La enfermedad aterotrombótica es la primera causa de muerte en el mundo occidental y su principal causa no es la hipercolesterolemia sino el Síndrome Metabólico. Éste ha pasado en las tres últimas décadas de ser un quinteto (hiperglucemia, hipertensión, hipertrigliceridemia, HDL bajo, aumento de la cintura) a ser un octeto (Síndrome Cardiometabólico) donde la triada formada por la inflamación/metainflamación, estrés oxidativo y disfunción endotelial es esencial para comprender su impacto a nivel vascular. La resistencia a la insulina está en el eje de ambos síndromes. Desde el punto de vista inflamatorio el marcador más utilizado ha sido la PCR con el inconveniente de ser altamente inespecífico y se ha puesto en marcha desde hace años una búsqueda de nuevos marcadores. El presente trabajo confirma que la C3 convertasa en sangre puede ser útil como marcador y posiblemente como factor patogénico, lo que, de confirmarse lo postularía como diana de futuras estrategias preventivas.

Objetivos: Este estudio está dirigido a analizar el comportamiento de la C3 convertasa, enzima clave y central de la cascada de complemento, en relación con el riesgo cardiovascular incluyendo la disfunción endotelial, resistencia a la insulina y riesgo cardiovascular en pacientes derivados a medicina cardiovascular especializada.

Metodologia: Fue realizado un estudio retrospectivo transversal sobre una muestra aleatoria de población adulta de Madrid derivada a medicina cardiometabólica especializada para prevención primaria de riesgo cardiovascular. La analítica convencional fue realizada en el laboratorio central del hospital mientras que los factores específicos cardiometabólicos fueron medidos en el laboratorio de la Unidad de Medicina Cardiometabólica y Daño Endotelial como parte de su rutina clínica. El riesgo cardiovascular fue calculado por la fórmula de REGICOR para población española. La resistencia a la insulina fue estimada según la fórmula de HOMA en un grupo de pacientes no dependientes de insulina exógena. La población a estudio fue estratificada en cuartiles de complemento C3 y se analizó la distribución de las variables cardiometabólicas entre los cuartiles. El análisis de regresión multivariable fue utilizado para identificar predictores de la variabilidad de las concentraciones de complemento C3.

Resultados: Fueron seleccionados un total de n=374 sujetos (53.60±14.80 años, 44.9% mujeres), donde el 65% fueron hipertensos, 42% hiperglucémicos y 35% padecían Síndrome Metabólico. Los niveles de complemento C3 fueron asociados con: 1. Síndrome Metabólico: diagnóstico de Síndrome Metabólico, cada uno de sus criterios, proporcional al número de sus criterios por paciente y con los nuevos criterios de síndrome metabolico ($p<0.001$), 2. Síndrome Cardiometabólico: inflamación (CPR y

fibrinógeno, p<0.001) y metainflamación (adiponectina e IL-6, p≤0.001), disfunción endotelial (TPA, PAI-1, p≤0.050) y el estrés oxidativo mostro tendencia (TBARS, p=0.084). 3. Resistencia a la insulina: el HOMA (p<0.001). 4. Factores de riesgo cardiovascular clásicos y con el riesgo cardiovascular calculado por REGICOR (p<0.001).Todas estas correlaciones fueron independientes de sexo y edad. La mayoría de las variables anteriores también mostraron asociación con los cuartiles de complemento C3. Hipertrigliceridemia ostentó el mayor impacto sobre el comportamiento de las concentraciones de complemento C3 según los resultados del análisis multivariable (p<0.001).

Conclusiones: En relación con el quinteto de criterios del Síndrome Metabólico, el complemento C3 resultó ser predictor del diagnóstico de Síndrome Metabólico, de cada uno de sus criterios por separado y fue proporcional al número de criterios diagnosticados por paciente, describiendo un posible umbral arbitrario entre dos y tres criterios y sugiriendo una más temprana intervención para la prevención cardiovascular ante una posible igualdad de riesgo. En relación con el octeto de Síndrome Cardiometabólico, el complemento C3 tambien se mostró predictor de inflamación, metainflamación, daño endotelial y mostró tendencia con estrés oxidativo. En el siguiente orden: hipertrigliceridemia, PCR, HTA, cintura, HOMA e hiperglucemia resultaron predictores del 34% de la variabilidad de las concentraciones de complemento. La asociación significativa de C3 con HOMA puede influir en los resultados anteriores, pero difícilmente explicaría todas las correlaciones encontradas en este trabajo. Este estudio sugirió que la evaluación simultanea de C3 y PCR podría aumentar la validez intrínseca de la PCR, como es requerido en Medicina Cardiometabólica precisa.

Palabras clave: C3 convertasa, complemento C3, aterotrombosis, aterosclerosis, Síndrome Metabólico, Síndrome Cardiometabólico, resistencia a la insulina, riesgo cardiovascular, PCR, disfunción endotelial, inflamación, metainflamación, estrés oxidativo, REGICOR, HOMA, análisis multivariable.

Áreas de Clasificación de la UNESCO

	Áreas Clasificación de la UNESCO	UNESCO International Nomenclature
3207.04	Patología Cardiovascular	Cardio-vascular pathology
3207.02	Aterosclerosis	Atherosclerosis
3201.01	Patología Clínica	Clinical Pathology

10. REFERENCES

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