Impact of adipose tissue on endothelial cells: effect of metabolic disorders

Katerina Papageorgiou

UNIVERSITAT DE BARCELONA

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Katerina Papageorgiou

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Aikaterini Papageorgiou

The co-director and tutor

Dr. Ramon Gomis de Barbarà

The co-director

Dr. Felicia Alexandra Hanzu

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Acta est fabula!!

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Abbreviations

ADA	American Diabetes Association
AGEs	Advanced glycosylation end products
AGT	Angiotensinogen
AMPK	AMP-activated protein kinase
AT	Adipose tissue
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCAA	Branched-chain amino acids
BMI	Body Mass Index
BSA	Bovine serum albumin
CAM(s)	Cellular adhesion molecule(s)
CBA	Cytometric Bead Array
cDNA	Complementary DNA
CHD	Coronary heart disease
CM	Control médium
CRP	C-reactive protein
CT	Computed tomography
CV(D)	Cardiovascular (disease)
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMO	Obese subjects with typ2 diabetes mellitus
DTT	Dithiothreitol
dNTPs	Deoxyribonucleotide triphosphate
EC(s)	Endothelial cell(s)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ER	Endoplasmic reticulum
FFA(s)	Free fatty acid(s)
FP	Fat pads
FPG	Fasting plasma glucose
G	Gentamycin
GC	Gas chromatography
HBSS	Hanks' balanced salt solution
HDL	High-density lipoprotein
HOMA	Homeostatic Model Assessment
hs-CRP	High sensitive C-reactive protein
HUVEC(s)	Human umbilical vein endothelial cell(s)
ICAM-1	Intercellular adhesion molecule-1
IDF	International Diabetes Federation
IF	Immunofluorescence
IFG	Impaired fasting glucosa
Ig	Immunoglobulin

IG	Immunogold
IGT	Impaired glucose tolerante
IL	Interleukin
IR	Insulin resistance
LC	Liquid chromatography
LDL	Low-density lipoprotein
LepR	Leptin receptor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
MCP-1	Monocyte chemoattractant protein-1
MMPs	Metalloproteinases
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MS	Mass spectrometry
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NO	Nitric oxide
Nob	Nonobese subjects
Ctr	Control
Ob	Obese
OGTT	Oral glucose tolerance test
P/S	Penicillin/streptomycin
PAI-1	plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase Caín reaction
PE	Phycoerythrin
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PFA	Paraformaldehyde
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
qPCR	Quantitative PCR
ROS	Reactive oxygen species
SC	Subcutaneous
SEM	Standard error of the mean
TAGs	Triacylglycerides
TF	Tissue factor
TGs	Triglycerides
TNF-α	Tumor necrosis factor-α
UCP-1	Uncoupling protein-1
UPLC	Ultra-high-pressure LC
VCAM-1	Vascular cell adhesion molecule-1
VIS	Visceral
VWF	von Willebrand factor
WAT	White adipose tissue
WC	Waist circumference
WHO	World Health Organization

I. Introduction

1. Obesity: a world-wide disease

1.1 Historical view

In the comedies of the ancient Greeks a fat character was perceived as a flawed and glutton figure, and was a subject of mocking. Hippocrates recognized corpulence as a disease but also a harbinger of others, health being the greatest human blessing. Together with his quote that "*What is food to one man is bitter poison to others*", the meaning of obesity has changed throughout history. About three centuries later, the Roman emperor Julius Caesar suggested that higher body weight correlated with a well-balanced mental disposition [1]. Back then, and until the Middle Age and the Renaissance, obesity was not considered a medical risk factor, but was rather viewed as a sign of wealth and prosperity.

It was not until the end of the 19th century and the onset of the industrial revolution that food scarcity was considered to play a major role in the economic development of industrialized societies, contributing to low survival, strength and productivity of the young generations, where the soldiers and workers were drawn from. Thus, weight, together with height, increased progressively in the developed world [2,3].

By the 21st century was achieved a historical milestone in human evolution, according to which the number of adults with excess weight equaled the number of those who were undernutritioned [3]. Spare body weight is nowadays widely recognized as one of the most important risk factors in health, increasing the social and economic burden worldwide. WHO ranks obesity and overweight in the fifth highest position for global deaths, contributing moreover to other major health burdens, such as diabetes, hypertension, ischaemic heart disease and cancer. In 2008, more than one in ten of the world's adult population was obese. It is a fact that overweight and obesity are rapidly upsurging/emerging in low- and middle- income countries, particularly in urban settings, and are linked to more deaths worldwide than underweight. Nevertheless, obesity is preventable and as a medical disorder that also leads to many comorbidities should not be neglected [4].

1.2 Definition and causes of obesity

Obesity is defined as abnormal or excessive adipose tissue accumulation that may impair health [4]. The obese state designates a disproportionate increase in weight with respect to height, sex and age. A simple quantification of this relationship commonly used to classify overweight and obesity is the body mass index (BMI), calculated by dividing a person's weight in kilograms (kg) by the square of his height in meters (m).

$$\mathbf{BMI = kg / m^2}$$

Undoubtedly, BMI provides the most useful population-level measure of overweight and obesity as it is the same for both sexes and for all ages of adults. Nevertheless, it should be

considered a rough guide because it may not correspond to the same degree of fatness in different individuals.

The World Health Organization (WHO) has established a series of criteria according to which high or low body weight in humans is classified as follows:

Classification	BMI (kg / m^2)
Underweight	<18.5
Normal	18.5 -24.9
Overweight	25 – 29.9
Obesity class I	30.0 – 34.9
Obesity class II	35.0 – 39.9
Obesity class III	≥ 40.0
Extreme obesity	> 50

After the WHO classification, there are a number of causes for obesity, being the fundamental one the energy imbalance between calories consumed and calories expended. Globally, there has been an increased intake of energy-dense foods that are high in fat, salt and sugars, but low in vitamins, minerals and other micronutrients, as well as a decrease in physical activity due to the augmented sedentary nature of many forms of work, changing modes of transportation and increasing urbanization.

1.3 Just a matter of genes?

Scientists acknowledge that multiple factors contribute to obesity including dietary, economic, psychosocial, reproductive, cultural and pharmacologic impacts. The contribution of each of those elements has been subject of intensive studies.

Genetic heritability of BMI or body fat phenotype has been validated by different research techniques, recognizing more that 50 genes (LPL, UCP's, PPAR, Ob, LepR, POMC, etc.) linked straightforwardly or not with obesity heritability [5]. As an example, monozygotic twin pairs showed a higher obesity concordance rate than dizygotic pairs (~ 0.68 vs ~ 0.28) [6,7,8,9]. In cases of adoption, the adopted subjects exhibited a BMI closer to the one of their biological parents, supplementing the role of the genetic factors over a shared familial environment [10,11].

Some forms of monogenic obesity (mutations in obesogenic genes as leptin, POMC, Prohormone Convertase 1, Neurotrophin Receptor TrkB, melanocortin 4 receptor deficiency [12] and endocrine forms of secondary obesity (i.e. Cushing syndrome) exist in humans. But obesity remains predominantly a complex polygenic disease, that, under the influence of an obesogenic environment (food excess, lack of exercise), modifies signals of caloric intake and energy expenditure. One hypothesis for that is that genes that once provided an evolutionary advantage (by allowing maximum efficiency of nutrient storage) are severely challenged when exposed to obesogenic environments [13], although this remains to be formally tested and proved. There is also the case of the pleiotropic syndromes, where obesity forms part of a variety of physical and developmental anomalies within the clinical syndromes (Prader Willi, Bardet-Biedl syndromes etc.), for the moment reported around 30 of them. A plausible cause of those syndromes is an intracellular protein produced by the defective gene, which is present throughout the body and has an unidentified function [12].

Therefore, the impulse to eat, that at extremity can result in hyperphagia, should not be considered a fully voluntarily controllable phenomenon and as the sin of gluttony, but rather as a genetic defect driven by biological signals. Many patients who develop early-onset obesity inherit it in a Mendelian way, or present subphenotypes that are similar to those of monogenic obesity characteristics. This proves the fact that a lot more genes still need to be identified in order to understand the roots of the disease completely. It is also very frequent in consanguineous families to have obese members in whom severe obesity segregates in an autosomal recessive manner and where mutations in known obesity genes are not found [14].

Moreover, many complex diseases are nowadays attributable to epigenetic changes, translated into inherited changes in gene expression that take place without changes in the DNA sequence. Epigenetic events like a prolonged, inappropriate diet determine cell fate by introducing strong environmental influences to the genome. In this sense recent researches have demonstrated that obesity is associated with an impaired adipogenesis. Thus, epigenetic mechanisms could explain the alterations that undergoes a preadipocyte when growing under a hypertrophic environment, generated by his already hypersized companions. The contribution of this mechanism in the development of obesity is still under study.

1.4 Epidemiology

Awareness on obesity has been attracted by the mass media during the last decades. It is a fact though, that the prevalence of obesity in industrialized countries began to increase gradually early in the last century.

Starting from the 1950s and continuing to the 1970s and on, surveys in the U.S. population emphasized alarming trends in obesity rates [15,16]. By the year 2000, 65 percent of the same adult population had a BMI above 25, 30 percent were obese and 5 percent were extremely obese [17]. Nowadays the U.S. belongs to the countries with the highest obesity rates in the world.

Undeniably, obesity started as a public health problem in societies with high socioeconomic status, primarily the United States and Europe in the developed world. But as soon as dietary changes in developing countries underwent significant changes (particularly in respect to fat, caloric sweeteners and animal source foods) and physical activity was shifted away from high-energy expenditure activities (like farming, mining and forestry), the burden of obesity moved

towards the poor. Countries as diverse as Mexico, China, Thailand and South Africa are shown with dramatic increases in obesity [18]. The WHO statistics pointed out 400 million obese people in the world, estimating an increase to 700 million by 2015. The transition stage of nutrition linked with a high level of obesity is finding most lower- and middle-income countries unprepared [19].

The biggest large-scale study conducted for **Europe** was the WHO MONICA (Multinational MONItoring of trends and determinants in CArdiovascular disease) project. In this study were collected data from 34 populations in 21 countries from the early 1980s to the mid-1990s. Participating subjects aged 35-64 years. According to the results obtained, Mediterranean countries, Spain, Italy, and France had lowest energy intake proportion from all sweeteners [20].

Other studies revealed obesity prevalence up to 28.3% in men and 36.5% in women. Overall obesity prevalence rates were higher in the central, eastern, and southern regions of Europe than in the western or northern ones, a geographic pattern partly explained by the negative impact of new lifestyle factors on the traditional Mediterranean diet [21].

Regarding **Spain**, the most recent data come from a study conducted by the Spanish Society for the Study of Obesity (SEEDO), that incorporate diverse epidemiologic studies performed in the country at the time period 1990-2000. Data base analysis of the DORICA study for people 25 to 60 years old revealed a total obesity prevalence of 15.5%, which appears to be more prominent in women (17.5%) than in men (13.2%). Overweight frequency is though more elevated in men at 46.4% in respect to 32.9% of the women, summing up a 39.2% of the population [22].

Obesity is not only a trend of the adults, but also of the **young**. Youth overweight has increased in the 1990s to 25.6% in the United States, 14.3% in Brazil and 7.7% in China, with annual increasing rates from 0.2-0.5% [18]. Overweight differs across children (ages 7–11 years) from Spain (34%), Italy (36%), and Malta (35%) in Southern Europe [23] and France (19%), the United Kingdom (20%), Sweden (18%), and Denmark (15%) in Western and Northern Europe, to Russia (10%) and Poland (18%) in the Eastern bloc. Adolescent (14–17 years) overweight is similar, with particularly high levels in the United Kingdom (21%) and Southern Europe (Spain (21%), Greece (22%), and Cyprus (23%)) [18]. According to the DORICA study about children and young people in Spain, overweight and obesity is related to absence of breastfeeding, low consumption of fruit and vegetables, high consumption of cakes, soft drinks and butchery products, low physical activity levels and a positive association with time spent watching TV [24].

1.5 Clinical diagnosis and Measurement of fat distribution

Fat distribution is an important factor to be taken on account, and measuring visceral fat is a better prediction factor of health risks than total body fat. Vague [25], in 1948, came first to the notion that android or male fat distribution was connected to diabetes and heart disease, a perception nowadays widely accepted. Until the present, plenty of techniques have been established for the solid measurement of body fat distribution [26]. The employment of a concrete technique relies on the equilibrium between practical and financial considerations and the aim of the study [27].

Body Mass Index

BMI has traditionally been the preferred index by which to measure body size and composition, and to diagnose underweight and overweight [4]. Generally, the BMI correlates with the accumulation of fat mass. However, this is not the case in severely obese patients due to the central accumulation of adipose tissue, which leads to a greater accumulation of adipose tissue for the same BMI.

Nonetheless, BMI measurement underlies the important limitation of being unable to distinguish between fat mass and fat-free mass, a relationship opposed with health risk [28]. Moreover, BMI is a restrictive method also in the case of elderly people [29], that have decreased fat-free mass even without a change in overall weight [30].

Alternative measures that reflect abdominal adiposity, such as waist circumference, waist–hip ratio and waist–height ratio, have been suggested as being superior to BMI in predicting CVD risk[4].

Waist Circumference

In contrast, waist circumference (WC) represents a measure of adiposity that takes into account the accumulation of abdominal fat. WC is simple to measure and interpret and it is highly correlated with visceral fat as assessed by computed tomography [31]. The potentially greater relevance of WC than BMI for predicting adverse health conditions in the elderly is underscored by the observation that fat mass accumulates intra-abdominally with age [32]. Older age groups have experienced a greater increase in abdominal obesity than younger age groups [33].

The ratio of waist circumference to hip circumference (WHR) was started to get used in the 1980s as another way to assess risk development of heart disease, diabetes, and other chronic problems associated with obesity [34-36], proceeding from central fat location. However, the preferred method remained waist circumference, measured according to National Heart, Lung, and Blood Institute/North American Association for the Study of Obesity (NHLBI/NAASO) guidelines [37].

Other Anthropometric Techniques

Central fat estimation has been accomplished in some epidemiologic studies by other phenotypical measurements getting subscapular skinfold values, a method not clinically valuable. Moreover, techniques like bone dimensions and limb circumference are based on the assumption that the subcutaneous adipose layer reflects total body fat, but this association may vary with age and gender.

Bioelectrical Impedance

Bioelectrical impedance analysis (BIA) is an indirect technique that estimates body composition via comparison of fat-free mass and total body water [38], by release of a low electrical current through the body, which passes through the fluids contained in muscle tissue more easily than through fat tissue. The resistance encountered in the latter is termed 'bioelectrical impedance', and is characteristic for a person's height, gender and weight. Adipose tissue (AT) mass is calculated by the subtraction of the free AT mass from the total body weight [38]. BIA can evaluate the AT content better than the BMI, but loses sensitivity for a BMI>35kg/m^2, overestimating AT free mass and underestimating AT [39].

Hydrostatic Weighing (HW) and Air Displacement Plethysmography (BOD-POD)

Hydrodensitometry, or hydrostatic weighing (HW), is considered to be the gold standard of the densitometric methods [40]. Based on the Archimedes principle, body volume is determined by measuring the difference between a subject's weight in water and that in air and thus determining whole-body density. A prerequisite for this technique is to submerge the subject completely underwater while exhaling maximally [41]. As a consequence, several limitations apply with this method, such as time and labor intensity, subject discomfort, and inaccessibility for many special populations.

Plethysmography refers to the measurement of size, usually volume. In air-displacement plethysmography, the volume of an object is measured indirectly by measuring the volume of air it displaces inside an enclosed chamber (plethysmograph). BOD-POD (Life Measurement, Inc, Concord, CA) is the only commercially available system for air-displacement plethysmography [42]. This technique offers established reference methods, including a quick, comfortable, automated, noninvasive, and safe measurement process, and accommodation of various subject types (e.g. children, obese, elderly, and disabled persons). Moreover, it is reasonably precise, accurate, and easy to use, but still expensive [43].

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI)

The primary advantage of these imaging modalities is the ability to quantify adipose tissue within distinct regions of the body. Computed tomography can provide information about the spatial arrangement of tissues within specific regions of the body based on how those tissues attenuate the energy from the X-ray. Fat and muscle can be primarily distinguished because of their widely different attenuation characteristics. Adipose tissue is less dense than water and displays attenuation values in the negative range (-190 to -30 HU) whereas muscle is denser than water and has a positive attenuation (0 to 100 HU), thus muscle and fat are in contrast on the computed tomography image.

Although its application to regional fat distribution is more common, MRI can be used to quantify whole body adipose tissue through an electromagnetic field. An advantage of whole-body MRI over more conventional methods for determining whole body fat content or body composition is that regional differences or changes in fat or muscle can be assessed. The disadvantage of a whole body magnetic resonance scan is that considerable and fairly sophisticated image analysis software is required [44].

MRI does not expose patients to ionizing radiation as CT does, but both techniques are difficult to employ in the case of severe obesity due to the extended time of probing and the limitations regarding the explored surface. CT and MRI allow us to calculate the correlation between clinical parameters of cardiometabolic risk and the distribution of the adipose tissue, and thus are important tools for use in subjects at cardiometabolic risk [27,44].

Dual Energy X-ray Absorptiometry (DEXA)

The principle of DEXA consists in the generation of X-rays at dual energies, whose differential attenuation in a tissue-specific manner is used to estimate three body compartments with one measurement: adipose tissue, bone and other non-adipose soft tissue (lean) [45]. The method is quick, safe, noninvasive, easy to use and has low operational costs, advantages that make it viable for clinical settings, clinical research and even large-scale population-based studies [44,46]. For that, DEXA use for assessment of body fat and body composition has become

progressively extensive, despite the fact that it does not provide information about the visceral or subcutaneous distribution of the adipose tissue, and the negative critics about the accuracy of the method for body-composition assessment and the different results exported by the different providers [47].

1.6 Health consequences associated to obesity

As obesity and overweight have reached unprecedented levels globally with a rise projected to continue, relevantly increscent have been associated-to them-comorbidities. Those are not rarely responsible for declining life quality of subjects dealing with those side effects and boosting economical and social costs, causing widespread concern.

Obesity-associated comorbidities are due to the excess of adipose tissue itself, and to metabolic or/and psychosomatic alterations induced by the surfeit of adipose mass. A short classification of them is juxtaposed in Table 1 [48,49]. Since in chapter 4 we will provide more insights into some of them, subsequent aspects are briefly portrayed.

Reduced life expectancy

In an analysis of the effects of obesity on longevity in 16 countries it was estimated that obesity reduced longevity in all countries ranging from half a year for females in Switzerland to more than a year and a half for U.S. males. These effects have been more severe in the United States than in other countries, featuring a BMI distribution that distinguishes it from the comparison countries: an unusually high rate of obesity in younger age groups and significantly higher rates of severe obesity [50].

Arterial Hypertension

Hypertension is due to increased intravascular volume and secondary to an increase in peripheral vascular resistance and activation of the rennin-angiotensin system [51]. Obesity most commonly leads to hypertension, where it appears up to five times higher than in normoweight people [52].

Cardiovascular Diseases (CVD)

Obesity contributes importantly to the appearance of CVD. CVDs, including heart disease, vascular disease and atherosclerosis, are the most critical global health threat, contributing to more than one-third of the global morbidity. At the present time, systemic atherothrombosis is recognized as a chronic condition determined by the interplay between the chronic inflammation of the vessel wall and lipid overcharge. It is likely that inflammation induced by obesity accelerates atherosclerosis.

Alteration of carbohydrates and lipid homeostasis

Impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and furthermore type 2 diabetes are associated with obesity (especially abdominal or visceral obesity). The risk of diabetes is greatly increased by weight gain. These alterations are frequently associated to various

forms of dyslipidemia with high triglycerides and/or low high-density lipoprotein (HDL) cholesterol or high low-density lipoprotein (LDL) cholesterol and hypertension.

Moreover, obese individuals commonly have elevated plasma free fatty acids (FFAs, probably due to increased FFA release from expansion of the fat mass) and so do type 2 diabetic people [53].

Table 1. *Obesity-associated comorbidities*

Cardiometabolic alterations	• Atherothrombotic coronary and cerebral disease • Arterial hypertension • Congestive heart failure • Dyslipidemia • Diabetes mellitus • Hyperuricemia
Digestive alterations	• Non-alcoholic steatohepatitis • Gastroesophageal reflux disease • Colelithiasis
Renal alterations	• Microalbuminuria
Respiratory alterations	• Asthma • Sleep apnea • Restrictive respiratory insufficiency
Neoplasms/Cancers	• Breast • Endometrium • Prostate • Colon
Others	• Daytime somnolence • Depression • Polycystic ovary syndrome • Peripheral venous insufficiency • Arthritis • Gallbladder disease • Binge-eating disorder

Psychological impacts

In today's western societies, beauty standards praise cachectic bodies to a level that individuals not fulfilling those standards, especially women, tend to feel anxious, margined in interpersonal relationships and depressed, being prone to develop obsessive behaviors as they attempt to deal with their excess weight. Thus, discrimination and bias spread unrestrained [54]. Special attention has to be applied in order to distinguish the social from the pathophysiological consequences of weight gain. Stigma and discrimination toward obese persons are pervasive and pose numerous consequences for their psychological and physical health.

2. Type 2 Diabetes Mellitus

2.1 Definition of Diabetes Mellitus

According to the American Diabetes Association (ADA), Diabetes mellitus (DM) is defined as a group of metabolic disorders characterized by chronic hyperglycemia resulting from defects in insulin secretion and/or action. The prolonged hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the heart, vascular and nervous systems and eyes, kidneys.

Table 2. *Criteria for the diagnosis of diabetes*

1	A1C ≥ 6.5%. The test should be performed in a laboratory using a method that is standardized to the DCCT assay.*
OR	
2	FPG ≥ 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.*
OR	
3	Two-hour plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR	
4	In a patient with classic symptoms of hyperglycemia or hyperglycaemic crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l).

*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing.

Adapted from the *American Diabetes Association, Diabetes Care 2011* [55].

The origin and the development of diabetes is attributable to multiple causes, starting from autoimmune destruction of the insulin producing pancreatic β-cells and the consequent insulin deficit, to abnormalities in the metabolism of carbohydrates, fat and proteins resulting from insufficient insulin secretion and/or a diminished insulin response in the target tissue (muscle, adipose tissue, liver, etc).

The principal symptoms of overt diabetes are excess thirst, polyuria, hunger, unexplained weight loss, fatigue and blurring of vision.

Diabetes can be diagnosed clinically or by the application of laboratory methods. The current diagnostic criteria for diabetes are summarized in Table 2.

2.2 Classification of Diabetes Mellitus

The classification of diabetes includes four clinical classes [55]:

- Type 1 Diabetes Mellitus
- Type 2 Diabetes Mellitus
- Gestational Diabetes Mellitus
- Other Specific Types of Diabetes

I. Type 1 Diabetes [55]

Type 1 DM results from destruction of the beta cells in the pancreas and usually leads to absolute insulin deficiency. Although it can occur at any age, type 1 DM is more common in persons less than 30 years of age. The rate of pancreatic destruction is variable and is generally more rapid in infants and children and slower in adults. Patients tend to be acutely symptomatic at onset. The primary characteristic of type 1 DM is absolute dependence on exogenous insulin to prevent ketoacidosis [56].

There are two forms of type 1 DM:

a) Immune-mediated diabetes

This form of diabetes is a result of the autoimmune destruction of the pancreatic β-cells and accounts for only 5–10% of the diabetes cases and for the majority of cases of type 1 diabetes. Previously termed as insulin-dependent diabetes or juvenile-onset diabetes, it appears with variable β-cell destruction rates: rapid mainly in young individuals (infants, children and adolescents) and slow in adults. Commonly it is diagnosed in the first category of patients, but subjects in their latter years can also be affected. Autoimmune destruction of β-cells is based on

multiple genetic predispositions, as well as on environmental factors, yet poorly defined. Patients tend also to develop other autoimmune disorders such as Graves' disease, vitiligo, autoimmune hepatitis, myasthenia gravis, and pernicious anemia.

b) Idiopathic diabetes

The existence of some forms of type 1 diabetes has no known etiologies. Some patients belonging to this category undergo permanent insulinopenia and are prone to ketoacidosis without any evidence of autoimmunity. Patients falling in this category of type 1 diabetes are of African or Asian ancestry, accounting on total for a minority of patients. Episodic ketoacidosis and varying degrees of insulin deficiency between episodes are frequently present in those individuals. Inheritance seems to be the main reason for the transmission of this form of diabetes, which lacks immunological evidence for β-cell autoimmunity. Affected patients might depend on insulin replacement therapy with a con-coherent frequency.

II. Type 2 Diabetes [55,56]

Type 2 diabetes, which accounts for about 90–95% of those with diabetes, presents an increasing prevalence. There are probably many different causes of this form of diabetes. Although the specific etiologies are not known, autoimmune destruction of β-cells does not occur. Ketoacidosis seldom occurs spontaneously in this type of diabetes; when seen, it usually arises in association with the stress of another illness such as infection. Previously referred to as non-insulin-dependent diabetes, type II diabetes, or adult-onset diabetes, underlies defects that can vary from predominant insulin resistance with relative insulin deficiency to a predominant insulin secretory defect of beta cells with insulin resistance [55].

Type 2 DM occurs more frequently in adults than in children, and the incidence increases with age (especially after age 40), obesity, lack of physical activity, in women with prior gestational diabetes mellitus and in individuals with hypertension, dyslipidemia or of different racial/ethnic subgroups. However, the prevalence of type 2 DM in children is increasing, especially in the high-risk ethnic groups, such as Native Americans, Hispanic Americans, African Americans, and Asian Americans. There have been studied many candidate genes as possibly implicated in the etiology of DM2. Recent research reported interesting results regarding the effect of TCF7L2 as a primary type 2 diabetes genetic risk mediator in African Americans [57], but the actual evidence indicates that type 2 DM is a complex genetic disease.

Because the onset is frequently insidious (hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes), many patients with type 2 DM are asymptomatic and remain undiagnosed for years. Nevertheless, such patients are at increased risk of developing macrovascular and less microvascular complications. At least initially, and often throughout their lifetime, patients do not require insulin therapy to survive.

Among type 2 DM diagnosed patients 80% are overweight [58]. Obesity itself causes some degree of insulin resistance. Patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region. Generally, upper body obesity is a recognized risk factor because it results in a significantly increased peripheral muscle and hepatic insulin resistance. Initially, beta cells compensate for this resistance

by increasing insulin secretion, inducing a relative hyperinsulinemia and maintaining normal glucose tolerance. In time, maybe due to a genetic predisposition in some individuals, beta cells fail to maintain an increased insulin secretion. This leads to the instauration of chronic hyperglycemia and overt diabetes. With the evolution of the disease, the hyperglycemia worsens, glucose toxicity ensues, and insulin secretion and action decrease. Ultimately, the loss of beta cell mass can lead to insulin dependency. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycemia, but is seldom restored to normal.

Abdominal obesity, as depicted in chapter 4, associates with a broad range of metabolic and cardiovascular complications. Besides the toxic effect of hyperglycemia per se, the instauration of chronic hyperglycemia and diabetes exponentially potentiates the traditional visceral obesity associated cardiometabolic risk factors like hypertension; increase of the plasminogen activator inhibitor (PAI-1), dyslipidemia and increase and maintain systemic inflammatory state in obese subjects. Therefore the evolution of type 2 DM is marked by macrovascular disease, atherosclerosis and CVD and in a lesser extent by the microvascular angiopathy.

III. Gestational Diabetes Mellitus (GDM) [55,56]

GDM is defined as any degree of glucose intolerance with onset or first diagnosis during pregnancy, regardless of whether it persists after giving birth and can be a predictor of late dysfunction of beta cells. It occurs in approximately 4% of pregnancies in the U.S.

IV. Other specific types of diabetes due to other causes

DM can also occur secondary to monogenetic defects in β-cell function or insulin action, pancreatic diseases or other endocrinopathies, medications, toxic chemicals, or uncommon forms of immune-mediated diabetes, e.g., "stiff man syndrome" or the presence of anti-insulin receptor antibodies. The defects in beta cell function are better characterized as follows [59]:

a) Genetic defects of the β-cell

Frequently existing is onset of hyperglycemia at an early age (generally before age 25), referred to as maturity-onset diabetes of the young (MODY), which is characterized by impaired insulin secretion with minimal or no defects in insulin action. There are various forms of MODY described at the time. Mostly, the inheritance here happens in an autosomal dominant pattern, commonly associated with mutations of hepatocyte nuclear factor (HNF)-1α and in the glucokinase gene. Some families have been identified with the inability to convert proinsulin to insulin and others with mutant insulin molecules, presenting mild glucose intolerance.

b) Genetic defects in insulin action

Rarely diabetes can result from genetically determined abnormalities of insulin action, as mutations of the insulin receptor may range from hyperinsulinemia and modest hyperglycemia to

severe diabetes. Individuals may have acanthosis nigricans and adult women enlarged, cystic ovaries. Leprechaunism and the Rabson-Mendenhall syndrome are two pediatric syndromes that have mutations in the insulin receptor gene with subsequent alterations in insulin receptor function and extreme insulin resistance.

c) Diseases of the exocrine pancreas

Any process that diffusely injures the pancreas can cause diabetes. Acquired processes include pancreatitis, trauma, infection, pancreatectomy, cystic fibrosis and hemochromatosis and pancreatic carcinoma. With the exception of that caused by cancer, damage to the pancreas must be extensive for diabetes to occur. This implies a mechanism other than simple reduction in β-cell mass.

d) Endocrinopathies

Several hormones (e.g., growth hormone, cortisol, glucagon, epinephrine) antagonize insulin action. Excess amounts of these hormones (e.g., acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma, respectively) can cause diabetes. This generally occurs in individuals with preexisting defects in insulin secretion, and hyperglycemia typically resolves when the hormone excess is resolved. Somatostatinoma- and aldosteronoma-induced hypokalemia can cause diabetes, at least in part, by inhibiting insulin secretion. Hyperglycemia generally resolves after successful removal of the tumor.

e) Drug- or chemical-induced diabetes

Many drugs can impair insulin secretion, precipitating diabetes in individuals with insulin resistance. Certain toxins such as Vacor (a rat poison) and intravenous pentamidine can destroy pancreatic β-cells. There are also many drugs and hormones that can impair insulin action like nicotinic acid and glucocorticoids. Patients receiving α-interferon have been reported to develop diabetes associated with islet cell antibodies and, in certain instances, severe insulin deficiency.

f) Infections

Certain viruses as congenital rubella coxsackievirus B, cytomegalovirus, adenovirus, and mumps have been associated with β-cell destruction or autoimmunity.

g) Uncommon forms of immune-mediated diabetes

The stiff-man syndrome is an autoimmune disorder of the central nervous system characterized by stiffness of the axial muscles with painful spasms. Patients usually have high titers of the glutamic acid decarboxylase (GAD) autoantibodies, and approximately one-third will develop diabetes.

Anti–insulin receptor antibodies syndrome (type B insulin resistance) can cause mostly diabetes by binding to the insulin receptor, thereby blocking the binding of insulin to its receptor in target tissues.

h) Other genetic syndromes sometimes associated with diabetes

Many genetic syndromes, like Down's syndrome, Klinefelter's syndrome, and Turner's syndrome are accompanied by an increased incidence of diabetes mellitus. Wolfram's syndrome is

an autosomal recessive disorder characterized by insulin-deficient diabetes and the absence of β-cells at autopsy.

2.3 Epidemiology of Type 2 Diabetes Mellitus

Over the last three decades the number of people with diabetes mellitus worldwide has more than doubled [60]. Data for 2010 estimated 285 million people worldwide with diabetes mellitus, out of which 90% with type 2 DM [61,62]. By 2030 the prediction of global diabetes mellitus rises to 439 million, representing a 7.7% of the world's total adult population aged 20–79 years [62] (Fig.1). In many zones of the World DM2 appears in epidemic proportions. The global prevalence is rapidly increasing in populations that have been industrialized in a short period of time, or, in other words, where the traditional lifestyle has given way to the occidental one. Factors that contribute to the increase in type 2 DM are therefore the increase in obesity urbanization and consequent lifestyle changes with decreased physical activity and hypercaloric diets, aging, urbanization/ lifestyle changes, and aging. Other risk factor for type 2 DM are the pertinence to particular ethnic race, family history, longevity, migration and fetal/neonatal nutrition.

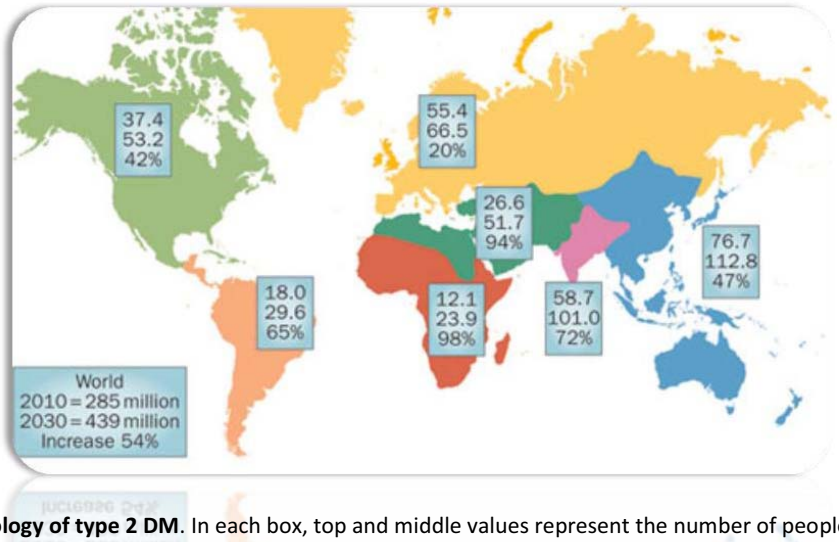


Fig.1 Epidemiology of type 2 DM. In each box, top and middle values represent the number of people with DM (in millions) for 2010 and 2030, respectively. Bottom value is the percentage increase from 2010 to 2030. Bigger left box: worldwide the number of people with DM is estimated to rise from 285 million in 2010 to 439 million by 2030, a 54% increase. Adapted from *Chen et al*, *Nature Reviews Endocrinology* 2012 [69].

Besides age and sex linked to diabetes, people with a low educational level were 28% more prone to develop it than the ones that had received better schooling throughout their lives. That was also the case in people with low socioeconomic level and poorer state of health, which moreover presented higher rates of mortality and cardiovascular disease [63]. Abdominal obesity, high blood pressure, low HDL cholesterol, high triacylglycerols and a family history of diabetes were accompanied by the presence of diabetes.

There are also particular cases regarding the diabetes incidence in some populations. The groups known to have the highest prevalence are the Pima Indians and the inhabitants of Nauru

(located in the Pacific Ocean), where diabetes affects 20% of the settlements. The basis for the genetic susceptibility to obesity and DM2 has been unclear but could be the result of an evolutionary advantageous "thrifty" gene, which promoted fat deposition and storage of calories in times of abundance and provided a positive selective advantage during periods of food shortage and starvation, but due to environmental changes during early development the selected trajectory became inappropriate and resulted in adverse effects on health [64]. Besides the racial propensity to DM2, there are also ethnic groups relatively protected from diabetes, such as the Melanesians (subregion of Oceania). Since DM2 occurrence reaches a maximum in higher age groups, ethnic groups with short life expectancy may present falsely low diabetes prevalence. There is a racial predisposition to DM2 with some ethnicities relatively protected, like the Melanesians [65].

Type 2 DM was relatively rare in developing countries some decades ago. However, the major burden of diabetes mellitus is now taking place in developing rather than in developed countries, representing an 80% of cases of diabetes mellitus worldwide [62]. Asia has emerged as the 'diabetes epicenter' in the world, as a result of rapid economic development, urbanization and nutrition transition over a relatively short period of time [66]. Among the 10 countries with the largest numbers of people predicted to have diabetes mellitus in 2030, five are in Asia (China, India, Pakistan, Indonesia and Bangladesh) [62]. In addition to Asia, the Gulf region in the Middle East [62] and Africa [67,68] are other hot spots for diabetes mellitus. Compared with developed countries, the proportion of young to middle-aged individuals with type 2 DM is higher in developing countries [62].

In the last two decades, type 2 diabetes, once thought to be a metabolic disorder exclusively of adulthood, has become increasingly more frequent in obese adolescents [70].

In this sense, although a very high prevalence of type 2 diabetes has been observed in non-Caucasian groups (African Americans, Native Americans, Hispanics), type 2 diabetes occurs in all races [71,72]. In the SEARCH study [72], the incidence rate (per 100,000 person/year) of type 2 diabetes among children and adolescents varies greatly by ethnicity, with the highest rates observed among youths aged 15–19 years in minority populations. In particular, the reported incidence rate was 49.4 for Native Americans, 22.7 for Asian/Pacific Islanders, 19.4 for African Americans, 17 for Hispanics, and 5.6 for non-Hispanic whites.

The increased prevalence of type 2 diabetes in the obese pediatric population is paralleled by an increased prevalence of the prediabetes conditions. In particular, 25% of children and 21% adolescents with severe degree of obesity, irrespective of ethnicity, were found to have IGT [73]. Similar high prevalence rates in Hispanic obese children and adolescents were subsequently reported by Goran *et al* [74]. Surprisingly, very high prevalence rates of IFG were reported in children from the Studies to Treat or Prevent Pediatric Type 2 Diabetes [75]. In Europe the scenery is not encouraging either. The prevalence of DM in European countries is estimated to rise in the next 10 years (Table 3).

Table 3. *Prevalence estimates of DM in the European Region*

	DM prevalence 2003	DM prevalence 2025
Country	%	%
Andorra	7.7	9.5
Austria	9.6	11.9
Bulgaria	10.0	11.6
Croatia	5.8	6.7
Cyprus	5.1	6.3
Finland	7.2	10.0
France	6.2	7.3
Germany	10.2	11.9
Greece	6.1	7.3
Ireland, Republic of	3.4	4.1
Italy	6.6	7.9
Netherlands	3.7	5.1
Norway	6.7	8.2
Portugal	7.8	9.5
Romania	9.3	10.6
Russian Federation	9.2	10.9
Slovenia	9.6	12.0
Spain	9.9	10.1
Sweden	7.3	8.6
Switzerland	9.5	12.6
Turkey	7.0	9.1
United Kingdom	3.9	4.7

Modified after the *Diabetes Atlas, International Diabetes Federation, 2003*.

Spain is one of the European countries with the lowest prevalence increase within a time period of 20 years. According to the very recent cross-sectional population-based study published in 2012, being the first national one, almost 30% of the study population had any kind of glucose disturbance. The total prevalence of DM was shown under age and sex adjustments at 13.8%, out of which 6.8% were not aware of it until the moment of the study. DM and impaired glucose regulation was amplified with age ($p < 0.0001$) and essentially higher in men than women ($p < 0.001$). Obesity was more probable to appear in people with DM and impaired glucose regulation; so were high blood pressure, raised triacylglycerol and low HDL cholesterol levels. Besides age and sex linked to diabetes, people with a low educational level were 28% more prone to develop it than the ones that had received better schooling throughout their lives. That was also the case in people with low socioeconomic level and poorer state of health, which moreover presented higher rates of mortality and cardiovascular disease [63]. Abdominal obesity, high blood pressure, low HDL cholesterol, high triacylglycerols and a family history of diabetes were accompanied by the presence of diabetes.

Though this study was based on a representative sample of the whole national territory and the diagnosis of diabetes was mostly made by OGTT, it lacked of high number of participants (around half of the selected population attended for examination) and of a better sex and age distribution (more women and older people took part), making age and sex corrections inevitable for the analysis. Another limitation was the people's self-report on the information, something that is common in large epidemiological surveys though [76].

2.4 Principal complications and treatment of Type 2 Diabetes Mellitus

Diabetes mellitus (DM) type 2 is a chronic disease with long-term macrovascular and microvascular as well as neuropatic complications (Fig.2) [56]**.** Yet, patients undergoing regular checkups and correct treatments are often able to minimize the consequences.

Whilst type 1 DM complications are marked from the evolution of the microangiopathy, type 2 DM patients are prone to developing **macrovascular complications** (depicted in chapter 3), respectively **disseminated atherothrombosis**. Cardiovascular morbidity and mortality is the first line complication in type 2 DM. Importantly, risk factors for coronary heart disease have to be looked into regularly. A1C, regular capillary glucose measurements, blood pressure and cholesterol levels reflect the possibilities of a stroke, cardiomyopathy and congestive heart failure. Macrovascular complications are definitely dominating the clinical picture of the disease by conditioning acute and chronic disease prognosis, life expectancy and quality of life.

Moreover, patients with type 2 diabetes are at increased risk of diabetes-associated **eye complications**. Patients are 40% more likely to suffer from glaucoma than healthy people (the longer the diabetic status, the more common the glaucoma is), and 60% from cataracts. Diabetic retinopathy, although less frequent than in type 1 diabetes, can also occur. **Microalbuminuria** and an **impaired renal function** can also be a complication of type 2 diabetes, amplifying the renal effects of hypertension.

Many different foot problems can appear in diabetic people, starting from **neuropathy**, **skin changes**, **ulcers** and calluses, ending to poor circulation and eventual amputation of the affected member. Skin complications like bacterial and fungal infections, itching, dermopathies,

allergic reactions and acanthosis nigricans are very common in diabetic patients, appearing at a rate of 33% among those.

Not neglectable is the **mental health** of people with diabetes, which can vary between anger, denial and depression. Other associated complications comprise of oral problems, loss of hearing, ketoacidosis, nephropathy, stress, etc [77].

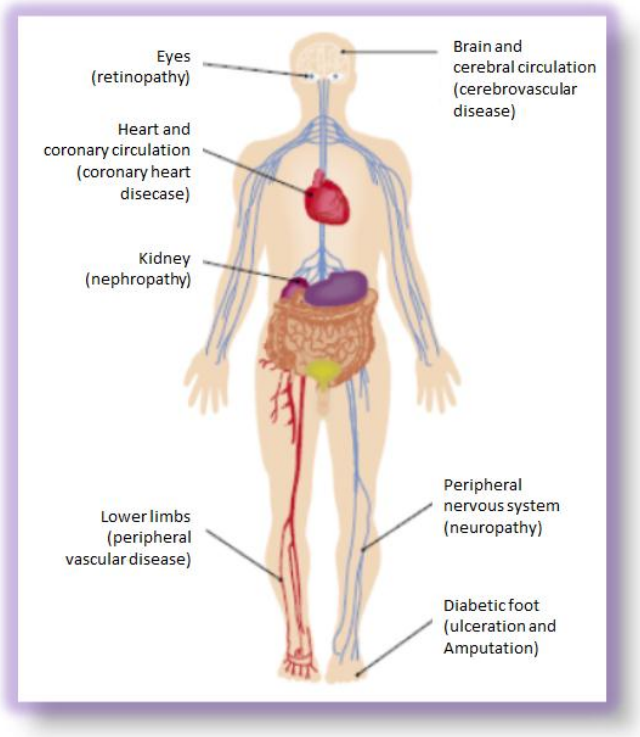


Fig.2 Major diabetes- associated complications.
Adapted from the *Diabetes Atlas, International Diabetes Federation, 2003*.

Treatment of type 2 DM apart from medication requires the patient's devotion in changing daily habits. Medical nutrition therapy, exercise and weight loss are at the onset of the disease and for the further control as essential as a correct insulin and noninsulin treatment. Patients with improperly controlled type 2 DM or unresponsive to diet and medications require insulin therapy, as type 1 DM patients do. The therapy's aim is to maintain as normal as possible the blood glucose levels throughout the day.

For the treatment of type 2 DM, six classes of oral agents are available on the international market [78]:

• Biguanides, such as metformin, block hepatic glucose production and are thought to have insulin sensitizer proprieties (effects of metformin are detailed in chapter 5). Metformin is worldwide employed as first line agent in the treatment of type 2 diabetes after the failure of the diet.

• The sulfonylurea compounds do mainly stimulate the secretion of insulin from the pancreatic islets. Their use also results in reduction of hepatic glucose production, and diminution of insulin

resistance through reversal of the post-receptor defect, and increase in the number of insulin receptors.

• The nonsulfonylurea insulin secretagogues repaglinide and nateglinide bind to a specific site on the sulfonylurea receptor and increase insulin secretion.

• Glucosidase inhibitors, as acarbose and miglitol, block starch, sucrose, and maltose absorption.

• Insulin sensitizer: Thiazolidinediones, like pioglitazone and rosiglitazone, have the capacity to decrease insulin resistance by enhancing insulin-mediated glucose disposal by muscle. Because of idiosyncratic liver damage and liver failure, the Food and Drug Administration (FDA) removed one of the thiazolidinediones, troglitazone, from clinical use in the United States in 2000. Another agent, rosiglitazone, has been associated in clinical studies with an increased risk of cardiac insufficiency, angina or myocardial infarction, but others have excluded this risk, rendering the conclusions uncertain [79].

• Incretin mimetic agents: are either inhibitors of dipeptidyl peptidase-IV (DPP-IV) like sitagliptin, vildagliptin, linagliptide, saxagliptin or analogous of glucagon-like peptide-I (GLP-I) like exenatide, liraglutide. Generally, incretin mimetic agents are increasing insulin secretion, suppress hyperglucagonemia, delay gastric emptying, and depress appetite.

In a study published in 2008 in the Journal of the American Medical Association were presented data analyzing medications prescribed between 1994 and 2007 for all U.S. office visits among patients 35 years and older with type 2 diabetes. The source was the U.S. National Disease and Therapeutic Index and the researchers were lead to the conclusions that apart from the increasing diabetes population, a great rise was applied in the complexity and the cost of the diabetes treatments. New data are awaited with respect to the period after the introduction of incretin mimetics [80].

The actual golden standard in diabetes treatment represents the achievement, following the treatment strategies presented above, of an A1C of ~7%, a blood pressure of ~130/80 mmHg and total cholesterol of below 175 mg/dl [55].

3. Atherothrombosis

3.1 The endothelium

The vascular endothelium forms the inner cellular lining of the blood vessels and is considered the largest organ of the human body. It has emerged as a key regulator of vascular homeostasis, since endothelial cells are not inert and don't function only as a barrier between the blood and the tissues, but rather present a vast metabolic activity.

Thereby it plays an important role in many physiological functions, including the control of vascular tone, blood cell trafficking, haemostatic balance, the interchange of fluids and cells between blood and tissues, the development and resolution of inflammatory processes as well as

innate and adaptive immunity. Moreover, it acts as a signal transducer for metabolic, hemodynamic and inflammatory factors that modify the function and morphology of the vessel wall.

Due to its great activity the endothelium either is a primary determinant of a disease state or a victim of a collateral damage. Alterations in the function of the endothelial cell can lead to dysfunctions like atherosclerotic changes and the progression of vascular lesions [81,82].

3.2 Endothelial and subendothelial activation

The subendothelium is the connective tissue between the endothelium and the inner elastic membrane in the intima of arteries. It involves the extracellular matrix synthesized by the endothelial cells, which contains numerous proteins, like collagen, von Willebrand factor (VWF), laminin, metalloproteinases (MMPs) and tissue factor (TF). The subendothelium acts as a supporter of the endothelial cells and as a secondary barrier if the endothelium is disrupted. When the subendothelium is exposed to rapidly flowing blood upon vessel damage, platelets adhere rapidly to the exposed surface, decelerate, and aggregate to arrest bleeding. Platelets are the major components of the blood responsible for initiating the coagulation cascade and the formation of the repairing thrombus at sites of blood vessel damage [83].

Under the term endothelial cell activation are understood the changes that the endothelium undergoes in order to respond to inflammatory stimuli proceeding by agents, such as interleukins, chemokines, etc. The terms *activated* and *active* do not indicate a condition alike. Generally, the endothelial cells are highly active as mentioned before [82].

Five principal changes occur during endothelial cell activation:

a) **Loss of vascular integrity**: the subendothelium gets exposed and the efflux of fluids from the intravascular space is possible to occur.
b) **Expression of leukocyte adhesion molecules**: adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 when upregulated adhere to the endothelium and move into the tissues [84].
c) **Phenotypic change from antithrombotic to prothrombotic**: loss of surface anticoagulant molecules, reduced fibrinolytic capacity, loss of the platelet antiaggregatory effects, production of platelet activating factor, nitric oxide, and expression of tissue factor are included [85].
d) **Cytokine production**: Local cytokines are synthesized, including interleukin 6 which regulates the acute phase response, as well as chemoattractants such as interleukin 8 and monocyte chemoattractant protein 1 [86].
e) **Upregulation of human leukocyte antigen (HLA) molecules**: their expression allows endothelial cells to act as antigen presenting cells, especially important in transplant rejection [87].

There are two stages of endothelial cell activation [85]. The first one takes place rapidly since it does not require gene upregulation or *de novo* protein synthesis. Effects include the retraction of endothelial cells, expression of P-selectin, and release of von Willebrand factor. During the second response are stimulated gene transcription and protein synthesis, therefore it is a time requiring stage. The genes involved are those for adhesion molecules, cytokines and tissue factor.

On the other hand, endothelial cell dysfunction is by definition maladaptive. Under an environmental persistence of severe damaging stimuli, the dysfunction of endothelial cells evolves irreversibly with loss of cell integrity and the damage of the underlying tissue. Endothelial cell dysfunction has been implicated in advanced atherosclerosis following an abnormally hyperadhesive endothelial cell surface [82].

Nevertheless, an adaptive reaction can be beneficial in one case but deleterious in another, like in the case of monocyte adhesion that leads to atherosclerosis when it takes place in the coronary artery, but is a potent defense in pulmonary arteries [82].

Cell adhesion is an essential process required for the correct functioning of multicellular organisms. Cellular adhesion molecules (CAMs) are involved in a variety of processes: cell-cell and cell-matrix adhesion, cell migration [88], activation of lymphocytes and initiation of the immune response. Inflammatory stimuli inducing transmigration towards the subendothelial tissues lead to the expression of CAMs by the endothelium, or at the surface level, or in the matrix, or as soluble receptors in the bloodstream [89,90].

Five families of adhesion receptors have been identified: 1) the selectins**,** 2) the immunoglobulins (Ig), 3) the cadherins**,** 4) the integrins**,** 5) the homing receptors [91].

Out of those families, the selectins and the immunoglobulins have an expression relevant to the presence of an inflammatory environment.

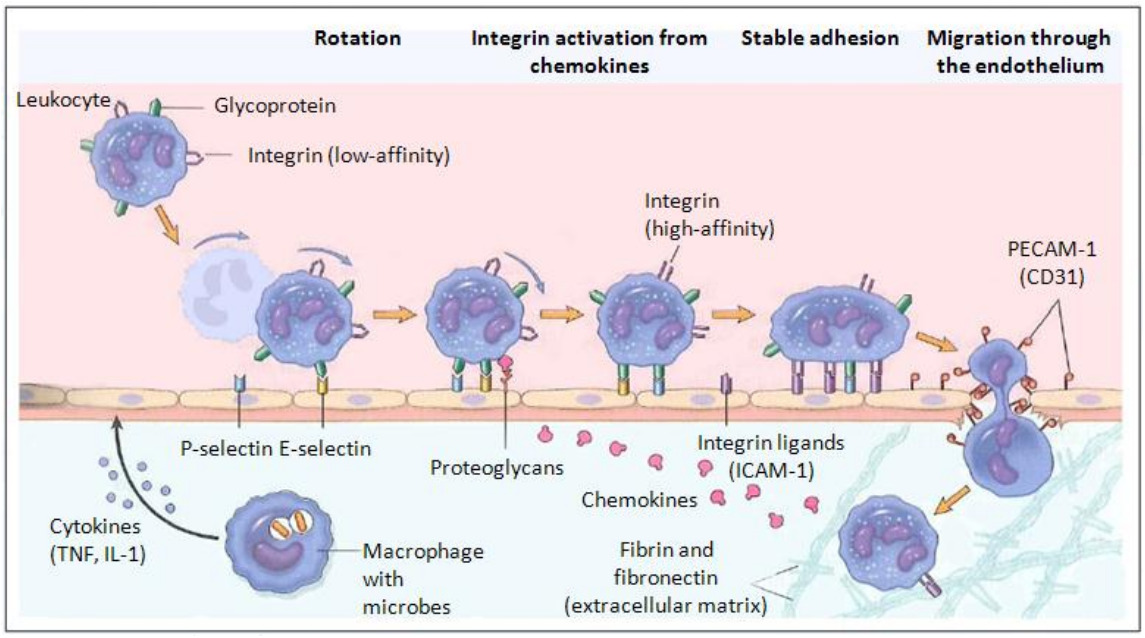


Fig.3 Leukocyte migration through the blood vessels. Leukocytes initially rotate, become activated, adhere to the endothelium and migrate by crossing it. Adapted from *Robbins and Cotran, Pathologic Basis of Disease 2005* [93].

The **selectins** are responsible for the initial interaction of circulating leukocytes with the activated vascular endothelium. Leukocytes roll along the vessel wall before firm adhesion and diapedesis at sites of tissue injury and inflammation. The selectin family includes three members with nomenclature according to the cell type on which it was originally identified: E-selectin (endothelium), P-selectin (platelets), and L-selectin (leukocytes) [92].

The **Ig** gene superfamily consists of cell-surface proteins that are involved in antigen recognition, complement-binding or cellular adhesion, and are implicated in the final phase of the cellular adhesion of granulocytes and monocytes to the endothelium. Five members of this family expressed by endothelial cells are involved in leukocyte adhesion: intercellular adhesion molecules -I and –II (ICAM-1= CD54, ICAM-2= CD102), vascular cell adhesion molecule-I (VCAM-1= CD106), platelet-endothelial cell adhesion molecule-I (PECAM-1= CD31) and the mucosal addressin (MAdCAM-1) [94].

3.3 The endothelium in arterial thrombosis

The human coronary artery consists of **endothelial cells** that form the layer in contact with the blood. The endothelium is based on the **intimal** layer that contains smooth muscle cells diffused within the intimal extracellular matrix. The next adjacent stratum is the **media**, comprising of multiple layers of smooth muscle cells much more densely present than in the intima, and surrounded by a matrix rich in elastin and collagen.

Atherothrombotic arterial disease, atherosclerosis, consists of the progressive diminution of the caliber of the vessel wall as a consequence of the local accumulation of cholesterol and formation of the atherosclerotic plaques. It occurs mostly at bifurcations of arteries, regions characterized by a disturbed blood flow [95,96].

A lesion begins as a fatty streak and can develop into an intermediate lesion and then into a lesion that is vulnerable to rupture and, finally, into an advanced obstructive lesion. In the first stage it is characterized by the progressive accumulation of atherogenic lipoproteins such as low-density lipoproteins (LDLs), constituting the asymptomatic “fatty streaks” within regions of the arterial intimae. The evolution of these lesions depends on further activation and dysfunction of the endothelium accompanied by inflammation of the vessel wall; increased lipoprotein oxidation and accumulation of lipid-rich material in activated macrophages (the so called foam-cells). Although they cause no clinical pathology, fatty streaks are widely considered to be the initial lesion leading to the development of complex atherosclerotic lesions [95]. Further development of the true atherosclerotic plaques with diminution of the vessel diameter (stenosis) is the consequence of fibrous tissue accumulation, smooth cell proliferation and extracellular matrix deposition around foam cells. In addition to monocytes, other types of leukocytes, particularly T-cells, are recruited to atherosclerotic lesions and help to perpetuate a state of chronic inflammation. As the plaque grows, compensatory remodelling takes place, such that the size of the lumen is preserved, while its overall diameter increases. Smooth muscle cells form a fibrous cap beneath the endothelium, and these walls off the plaque from the blood. This process contributes to the formation of a necrotic core within the plaque and further promotes the recruitment of inflammatory cells [95,97,98,99].

This nonobstructive plaque can abruptly rupture or the endothelium can erode, resulting in the exposure of thrombogenic material, including VWF and TF, and the formation of a thrombus in the lumen (Fig.4). If the thrombus (clot) is large enough, it blocks the artery finally inducing an acute coronary syndrome or myocardial infarction or a stroke. Ultimately, if the plaque does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and consequently the quantity of blood supplied to the adjacent tissue is reduced, leading to symptomatic hypoxia of the adjacent tissue when the stenosis is >75% of the vessel diameter or in conditions of increased

oxygen consumption [95,98,100]. Initiation and progression of the atherothrombotic lesions are highly complex processes, and many aspects of atherogenesis are still under study.

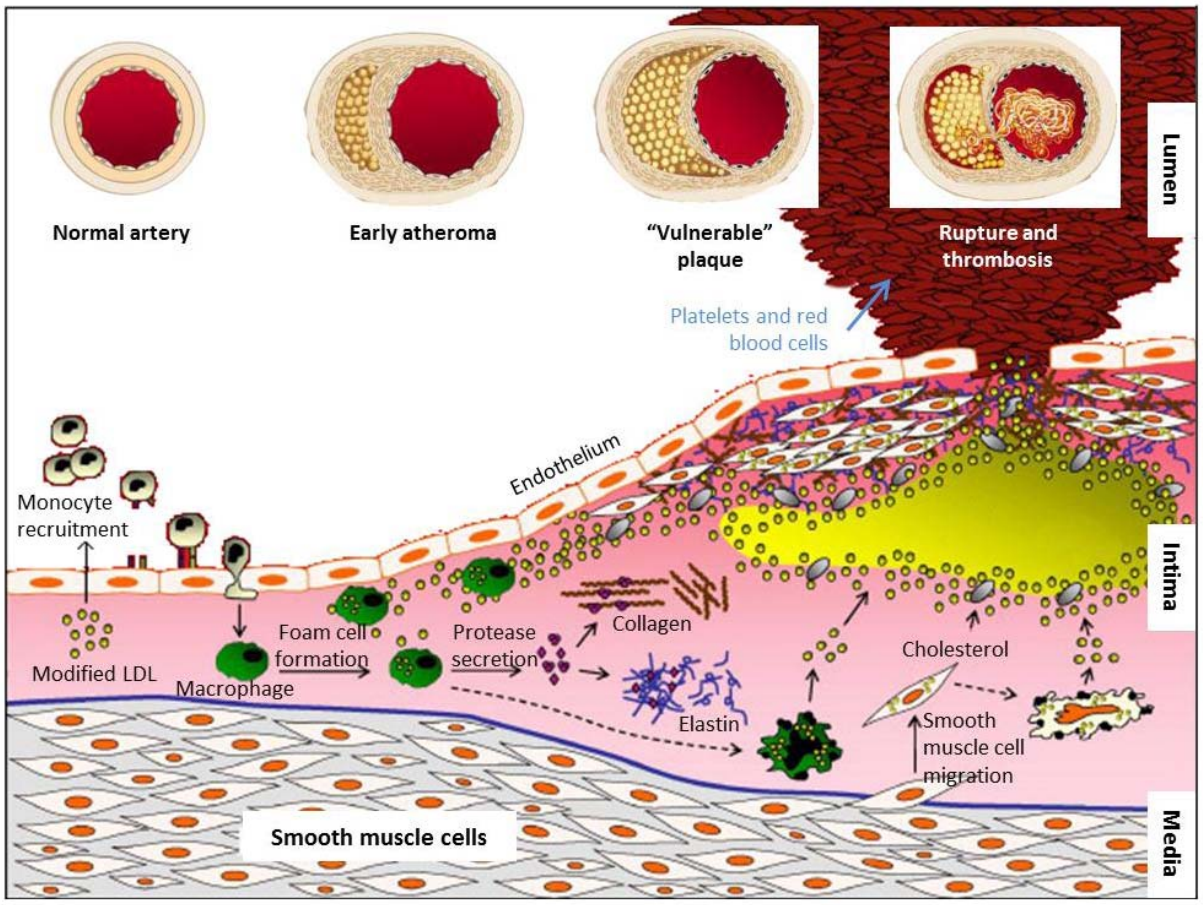


Fig.4 Formation of an atheroma. Upper boxes adapted from *Libby, Nature 2002* [101]. Rest of the picture modified after *McLaren et al, Progress in Lipid Research 2011* [102].

3.4 Role of inflammation in atherogenesis

High plasma concentrations of cholesterol accumulating within the artery, and particularly LDL cholesterol, have been considered for long time the principal risk factors for atherosclerosis. However, it is now known that atherosclerosis is not only due to hypercholesterolemia, but that a complexity of factors contributes to this process, chronic hyperglycemia and local and systemic inflammation playing a major role.

In endothelial dysfunction and formation of the atherosclerotic plaque are involved, besides elevated LDL, hypertension, diabetes mellitus, genetic alterations and free radicals caused by cigarette smoking. There has been also reported association between elevated plasma homocysteine concentrations, infectious microorganisms such as herpesviruses or *Chlamydia pneumoniae* and atherosclerosis [97].

These pathogenic causes of local and systemic inflammation have been underestimated for a long time, even though their significance is in our times important as well. Hence, the presence of systemic inflammation is evident even before the detection of myocardial infarction.

Patients with autoimmune diseases (as rheumatoid arthritis), untreated infections (like periodontal disease), chronic extravascular infections (eg, gingivitis, prostatitis, bronchitis) or even chlamydial infection have been shown to present elevated possibilities of atheroma formation[95,103,104].

3.5 Role of inflammation in atherothrombosis

It has been assumed that atherosclerosis is the consequence of the excessive inflammatory and prothrombotic response of the endothelium to activators like diet compounds, hypercholesterolemia, elevated free fatty acids, factors secreted from the obese adipose tissue and chronic hyperglycemia [105,106,107,108,109]. This causes endothelial and smooth muscle cells of the large arteries to synthesize proinflammatory proteins, including chemokines, CAMs and cytokines, growth factors and thrombogenic substances [95,110].

Development of atherothrombotic plaques in the bifurcation regions is facilitated by the turbulent blood flow, which reduces the activity of endothelial atheroprotective molecules such as the endothelium-produced nitric oxide (NO), and favours regional VCAM-1 expression under the presence of circulating inflammatory stimulus. Nitric oxide maintains the vasodilatory properties of the endothelium, and opposes the effects of vasoconstrictors like endothelin-1 (ET-1) and angiotensin II (AGT II). Reduced production of NO and increased synthesis and secretion of endothelin-1 enhances the vasoconstrictor tone and increases synthesis and release of proinflammatory cytokines maintaining endothelial activation [107,108]. On the other hand, diabetes- and obesity-associated elevated FFA levels decrease NO activity by activating innate inflammatory pathways.

The development of atheromatous plaques is a major health problem that is becoming an even greater issue when it comes to plaque rupture and thrombosis, triggering acute life-threatening cardiovascular events, as stroke or myocardial infarction. According to studies, an arterial narrowing of <50% is usually produced before the acute event, responsible for unstable angina and myocardial infarction [111]. Atherosclerotic lesions trigger acute life-threatening cardiovascular events such as myocardial infarction or stroke only when an occlusive thrombus (or clot) forms. The initial step for arterial thrombosis comes when the superior layer of the atherosclerotic plaque gets disrupted, and blood comes in contact with the lipid core of the thrombogenic subendothelium. Plaque rupture under the pressure of the blood flux would take place depending on plaque fragility, plaques with a lower content in lipids being more susceptible. The consequent events that lead to the arterial occlusion after plaque injury are: the contact of blood with the prothrombotic subendothelial components, the activation and aggregation of platelets and dynamic changes in cellular adhesion to the atheromatous plaques [95,97].

Plaque rupture is associated with local inflammation and degradation of the collagen and elastin in the extracellular matrix (ECM) by extracellular proteases present in the subendothelium. Endopeptidases, like MMPs, secreted by cytokine-activated macrophages and smooth muscle cells play an important role in plaque rupture. Besides ECM degradation, MMPs can promote macrophage invasion and angiogenesis [112].

The platelets are disc-shaped cell fragments that circulate in the blood as sentinels of vascular integrity and rapidly form a primary haemostatic plug at the site of vascular injury. When atherosclerotic plaque ruptures, platelets are rapidly recruited to the lesion site through the

interaction of specific platelet cell-surface receptors with collagen and VWF [113,114]. After adhesion to the vessel wall, platelets aggregate to one another, determining a rapid growth of the provisory thrombus. The formation of the final thrombus is induced by the activation of the coagulation cascade. The ultimate result is thrombus formation and the acute coronary or cerebral atherothrombotic event [100].

To conclude, inflammation initiates the atherosclerotic lesion and contributes to the formation of the complex plaque, weakens the fibrous cap (which causes easy plaque rupture) and enhances the thrombogenicity of the lipid core [115].

3.6 Endothelial activation and inflammation markers in atherothrombotic cardiovascular risk prognosis

Inflammation plays an essential role in the initiation and progression of atherosclerotic lesions, and plaque disruption. A good perception of the inflammatory mechanisms contributes to the prediction of potentially useful markers of CVD. Clinically, those markers have to complete a number of conditions in order to be able to be utilized as such. Those are [116]:

(1) the ability to standardize the assay and to control the variability of the measurement
(2) the independence from established risk factors
(3) their association with CVD clinical end points in observational studies and clinical trials
(4) the presence of population norms to guide interpretation of results
(5) the ability to improve the overall prediction beyond that of traditional risk factors
(6) the generalization of results to various population groups
(7) un acceptable cost of the assays.

A good sample of studies has examined the connection between inflammation and CVD through measurement of a variety of analytes [116].

Cellular Adhesion molecules (CAMs)

Cells interact collectively and this is mediated partially by different families of adhesion molecules. Patients with coronary artery disease possess higher serum levels of CAMs than lean subjects [108,117]. Plasma concentrations of soluble adhesion molecules may be higher in patients with atherosclerosis [118,119] and hyperlipidemia [120,120]. Out of those we are going to focus our analysis on two of them, which were also used in the methodology of this thesis.

Intercellular Adhesion Molecule 1 (ICAM-1), also known as CD54, is a membrane receptor of 80-114 kDa of size, structurally related to the immunoglobulins (Ig). It is expressed by various cell types, like epithelial cells and fibroblasts. But constitutively expressed it can only be found, at low levels, on vascular endothelial cells and on some lymphocytes and monocytes. Several cytokines, as the inflammatory interleukin (IL-1), tumor necrosis factor (TNF-α), and interferon gamma (IFN-γ) or lipopolysaccharides (LPS) can induce elevated ICAM-1 expression [121]. Its role has been found to be in inflammatory cells trafficking, in T-cell mediated host defense system [122] in microbial pathogenesis, and in signal transduction pathways [121]. Circulating ICAM-1 levels were found to be higher in patients with stable or unstable angina and acute myocardial infarction, compared with control patients [123]. In another study, CVD risk factors and BMI were associated

with systemic inflammation, determined by ICAM-1 levels. Thinking of the heritability of ICAM-1, it was suggested that systemic inflammation could have a genetic background [124].

Vascular Adhesion Molecule 1 (VCAM-1), alternatively CD106, weights 80-100 kDa [94]. It is a sialoglycoprotein whose expression is restricted to the endothelium of small and large vessels, and permits the binding of monocytes, lymphocytes, eosinophiles and basophiles to the activated endothelium. VCAM-1 is not constitutively expressed by endothelial cells, but is only synthesized by induction of inflammatory stimuli and its synthesis depends on the persistence of the deleterious input [125]. The promoter region of VCAM-1 gene presents binding sites for nuclear factor-κB (NF-κB)[126]. VCAM-1 plasma levels were increased in diabetic patients with microalbuminuria, giving to VCAM-1 the important role as a marker of ongoing vascular dysfunction and progressive vascular disease [127].

High sensitive C-reactive protein (hs-CRP)

CRP is a protein synthesized mainly in the liver, participating in innate host defense by recognising and further mediating elimination of pathogens and apoptotic cells. Thus, injured tissues are restored and their normal structure and function redeemed. IL-6 and TNF-α are inflammatory cytokines inducing the secretion of C-reactive protein in the liver [128]. CRP inter-relates with fibrinogen, a factor that has been found to double the risk of coronary attack and stroke in high-risk men [129]. Harmful effects of CRP have been found to be implicated in arteriosclerotic lesions [130,131] and in the tissue damage that accompanies acute myocardial infarction.

Abnormally high CRP levels can be caused by various reasons, since it is a protein involved in the mechanisms of the immune system. For that, it can not be considered as a specific prognostic indicator of systemic chronic inflammation in obesity and atherothrombotic disease. A better quantification of such conditions is achieved by measuring the high-sensitivity CRP (hs-CRP), which measures lower CRP levels with the help of laser nephelometry. The reliability considering hs-CRP a systemic marker of chronic low-grade inflammation present in atherothrombotic disease and obesity has been shown in various studies [132,133]. Many physicians consider hs-CRP levels as an add-on to overall cardiovascular risk prediction, and intensive research targeting vascular anti-inflammatory agents for the treatment and prevention of coronary disease is being conducted [133].

Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine relating to inflammation, host defense, and tissue injury. Many factors contribute in the production of IL-6, including infection, IL-1, interferon-γ, and TNF. IL-6 is a central mediator of the acute-phase response and a primary determinant of hepatic production of CRP. Moreover increases fibrinogen and plasminogen activator inhibitor-1 (PAI-1) concentrations.

IL-6 is being produced by vascular endothelial and smooth muscle cells, has been found expressed in human atherosclerotic lesions, and may have procoagulant effects. Healthy individuals with elevated levels of IL-6 are prone to developing coronary and cerebrovascular events [134].

Interleukin-1β (IL-1β)

IL-1β is a critical early mediator of inflammation. Its natural receptor antagonist (IL-1Ra) is also released during inflammation and may limit the potential deleterious effects of IL-1. Thus,

such balance may determine the progression of inflammation. Relative absence of IL-1Ra is suggested to play a role in the pathogenesis of some inflammatory disorders [135].

Monocyte chemoattractant protein (MCP-1)

Monocyte chemoattractant protein is a chemokine being given the role to recruit monocytes into the developing atheroma and may contribute to atherosclerotic disease development and progression. Plasma levels of MCP-1 are associated with prognosis in patients with acute coronary syndromes, but few population-based data are available from subjects in early atherosclerosis [136].

Tumor necrosis factor-alfa (TNF-α)

TNF-α is a multifunctional circulating cytokine, that enhances monocyte recruitment at atherosclerotic lesions, and is expressed by endothelial, smooth muscle cells and macrophages associated with coronary atheroma. TNF-α levels are noticeably elevated in proximal coronary events [137].

Interleukin-8 (IL-8)

IL-8 has been directly linked to the development of early atherothrombotic lesions since its production starts when phagocytes and mesenchymal cells get exposed to inflammatory stimuli, for example IL-1 or TNF-α. The activation of neutrophils with induction of chemotaxis, exocytosis and respiratory burst is triggered. In fact, IL-8 produces an immense neutrophil accumulation at the site of injection, believed to originate mainly by stimuli such as infection, inflammation, ischemia, trauma etc [138]. Moreover, it has been demonstrated that IL-8 is fundamental in monocyte capture [139].

Interleukin-10 (IL-10)

IL-10 is produced by T cells, B cells, monocytes, and macrophages, and inhibits a variety of immune parameters [140]. Besides that, it presents anti-inflammatory properties that inhibit the production of proinflammatory cytokines. Anti-inflammatory cytokines have been shown to have antiatherogenic properties [141]. In a study was demonstrated that IL-10 was able to downregulate ICAM-1 and VCAM-1 expression on HUVECs having been activated by IL-1 [142], and decrease IL-8 and IL-6 production in irradiated HUVECs [143].

Impediment of atherosclerosis development due to the protective effect of IL-10 could also be credited to inflammatory cell deactivation [144,145]. Expression of IL-10 in the atherosclerotic lesion is associated with low iNOS expression by macrophages and low levels of cell death [144].

Interleukin-12 (IL-12p70)

IL-12 is a cytokine consisting of two chains, one of 35 and another of 40 kDa, resulting in the biologically active 70 kDa (p70) form. IL-12 has been proven critical in several pathogeneses, whereas high IL-12 levels have been described in autoimmune diseases and chronic inflammatory reactions, such as osteoarthritis, rheumatoid arthritis and atherosclerosis [146].

Extracellular matrix (ECM) biomarkers

Being under normal conditions, the vascular endothelium produces a number of substances that contribute to hemostasis, fibrinolysis and regulation of vessel tone and permeability.

One such substance is the large glycoprotein **von Willebrand factor (VWF)**, whose production is undertaken mainly by endothelial cells and its presence can also be found in platelets. It is very important in the coagulation cascade, having the ability to enhance platelet adhesion and aggregation. Plasma levels of VWF are found elevated in different states of endothelial damage and are therefore candidates as useful markers of endothelial dysfunction. Stimulated release of VWF in humans is achieved by blockade of nitric oxide [147]. People with inherited deficiencies of von Willebrand factor may suffer a bleeding disorder, von Willebrand disease, while substantially increased levels are associated with thrombotic disorders.

Tissue factor (TF) is the key initiator of the coagulation cascade and thereby crucially involved in the maintenance of vascular hemostasis. As an ultimate step it can lead to fibrin and clot formation. TF is actively expressed and detectable in vascular smooth muscle cells and fibroblasts and its action can be provoked by various stimuli including cytokines. Apart from triggering coagulation extracellularly, TF also functions as a transmembrane receptor, regulating the expression of genes involved in cellular responses such as proliferation and migration. For that, it is apparently involved in the pathogenesis of neointima formation and tumor growth, and increased levels of TF have been detected in patients with cardiovascular risk factors or coronary artery disease in addition to those with cancer [148].

Elevated levels of TF are observed in patients that suffer under cardiovascular risk factors, i.e. hypertension, diabetes, dyslipidemia, and smoking, as well as in those with acute coronary syndromes, where TF may indeed promote thrombus formation besides migration and proliferation of vascular smooth muscle cells [149].

4. Factors contributing to the cardiovascular disease in obesity and type 2 diabetes mellitus

4.1 Classical and emerging cardiovascular risk factors in obesity and type 2 diabetes.

Traditional cardiovascular risk factors of atherothrombotic disease in obesity, as partially depicted in Chapter 1, are hypertesion, hyperglycemia, hyperinsulinemia and insulin resistance, elevated levels of circulating free fatty acids and abnormalities in the lipid profile.

Arterial hypertension

Hypertension is the cardiovascular pathology most attributed to obesity, and especially to central obesity. The percentage of hypertensive people among the obese is around 35% [150,151], with even more elevated levels in obese females. In the National Health and Examination Survey (NHANES) III, the prevalence of hypertension according to body mass index (BMI) and adjusted for age was about 14% in participants with a BMI less than 25 kg/m^2 *versus* about 40% in participants with a BMI greater than 30 kg/m^2 [152]. Hypertension appears up to five times more among obese people than among those of normal weight [52]. Increased blood pressure due to excess weight gain occurs due to the enhanced intravascular blood volume connected to greater body mass and a rise

in blood viscosity, an increased peripheral vascular resistance and adipose tissue release of angiotensinogen [153].

Lipid profile alterations

Obesity observed abnormalities in the lipid profile consist of an atherogenic lipid profile with increased triglycerides (TGs) [154], LDL-, VLDL cholesterol, decreased HDL cholesterol [155,156,157] and increase in the *circulating free fatty acids (FFAs).* These modifications result from various actions: increased production by the hepatocytes, enhanced release of FFAs from the AT [158], reduced clearance of TGs-rich lipoproteins due to a reduced activity of lipoproteinlipase (LPL) and, therefore, a decreased HDL synthesis, diminished expression of the LDL receptor followed by lower very low-density lipoprotein (VLDL) clearance [159,160], and therefore, weakened synthesis of HDL.

The levels of *FFAs* are elevated in obesity. Raised cellular levels of FFAs can produce insulin resistance in skeletal muscle and liver, and reduce beta-cell function, in what has been referred to as lipotoxicity [161]. Clinical evidence suggests that FFA levels in obese subjects may also accelerate atherosclerosis and predict cardiovascular mortality. FFAs may induce inflammatory processes, possibly through the activation of the Toll-like-receptor family. They may also induce endothelial apoptosis and impair endothelial-dependent vasodilatation [106].

Hyperinsulinemia and insulin resistance

Insulin resistance is known as the state in which a target cell or a whole organism act with reduced responsiveness to the insulin concentration to which they get exposed. Compensatory hyperinsulinemia contributes to the maintenance of normal glucose levels, not seldom for decades, until the beta cells become unable to overcome insulin resistance through hypersecretion, and chronic hyperglycemia, respectively diabetes, instaurates [162].

Obesity is most commonly linked to insulin resistance. It has been reported [163] that insulin resistance is a potent predictor of a wide range of serious illnesses, including stroke, type 2 diabetes, cardiovascular disease, hypertension and cancer. Regardless of whether the insulin resistance or the basal hyperinsulinemia came first, the hyperinsulinemia itself might perpetuate the insulin resistance. In other words, hyperinsulinemia is often both a result and a driver of insulin resistance[164].

Insulin sensitivity is quantified ideally by the euglycemic hyperinsulinemic clamp, a test that cannot be used in the clinical practice due to its length and complicated protocol. Alternative well-correlated methods are not substantially simpler than the clamp and unsuitable for clinical practice. The Homeostatic Model Assessment **(HOMA): HOMA-IR=**Insulin mU/l x Glycemia (mmol/l)/22.5 remains the most popular one among other calculation models based on glucose and insulin levels in the fasting state. HOMA data on insulin sensitivity correlate well with data obtained from the glucose clamp, and are able to predict future development of diabetes. However, it remains a surrogate marker, and has a poor reproducibility across laboratories and populations, cut-off values for HOMA insulin resistance (HOMA-IR) being dependent from this variables [165].

Hyperglycemia

Diabetes sets up after a period following a long phase of asymptomatic hyperglycemia. During this prediabetic state [166], which falls between normal glucose tolerance and diabetes [167],

fasting blood glucose can be maintained at an almost normal range for years, on the contrary to postprandial or postglucose levels that are gently elevated [166].

Hyperglycemia is responsible for the induction of numerous harmful effects that contribute to atherothrombosis. It is therefore linked to elevated oxidative stress [168], enhanced interaction between leukocytes and endothelial cells [169], and glycosylation of a plentiful of proteins such as lipoproteins, apolipoproteins, and clotting factors. Ultimately, a number of dehydration and oxidation reactions leads to the formation of advanced glycosylation end products (AGEs) [170] that promote atherothrombosis through their effect on endothelial cell function [171].

Inflammation

It is widely appreciated that adipose tissue has important endocrine functions under both physiological and pathophysiological conditions. The proteins recognized to be secreted from the obese adipose cells, both adipocytes and stromal cells, are known collectively as adipokines (or adipocytokines) and their main functions are to participate in various processes as inflammation, blood pressure control, lipid metabolism and immunity, appetite and energy balance, insulin sensitivity and angiogenesis [172]. The conversion from the lean to the obese state demands high triglyceride storage, so that the adipose tissue undergoes hyperplasia and hypertrophy. During and after this process, as consequence of the activation of stromal cells, infiltration with inflammatory immune cells and adipocytes hypertrophy, the obese adipose tissue produces an increased number of inflammatory adipokines and demonstrates an altered pattern of adipocyte secretion. Data have shown that initiation and integration of pathways of inflammation and insulin action in obesity and moreover in type 2 diabetes, depend critically on endoplasmic reticulum (ER) stress. The ER, as an organelle of protein modification and distribution, might be a sensor of the high metabolic stress induced by adipocyte hypertrophy and its conversion into signals of inflammatory response [173].

This results in a systemic maintained low-grade inflammation, endothelial activation and development of atherothrombotic disease [105,174]. The detailed role of local and systemic inflammation in the development of atherothrombosis has been detailed in chapter 3 Waist circumference correlates significantly with systemic inflammatory response [175].

Conceptually, inflammation is described as the principal response of the body attempting to deal with injuries and trying to integrate many complex signals. Whereas this reaction takes place as a **short-term** response, the long-term consequences of prolonged inflammation are often not beneficial, as seen in metabolic diseases, including obesity and diabetes. In the **chronic low-grade** inflammation associated with metabolic disease, few of the classic features of inflammation have been observed; the response is lower in intensity and more heterogeneous [173].

Low grade inflammation is characterized by activation of the immune system and increased levels of various sera cytokines. Despite this, due to the great variability, only the high-sensitivity C reactive protein (hs-CRP) [176], synthesized in the liver as response to the activation of the inflammatory cascade, and the increased count of leucocytes [177] have been validated as systemic markers for chronic inflammation. In atherothrombotic disease and obesity these markers are also utilized for the evaluation of the degree of systemic inflammation.

4.2 Obesity-associated atherosclerotic risk based on regional adipose tissue distribution

In mammals, the adipose organ consists of several subcutaneous and visceral depots, some areas of which are brown and correspond to brown adipose tissue (BAT), while many are white and correspond to white adipose tissue (WAT).

4.2.1 Brown and white adipose tissue and cardiometabolic risk

The Brown Adipose Tissue (BAT)

BAT is a thermoregulatory organ. Heat production in BAT is under central control from the hypothalamus, where the outgoing signal is transmitted via the sympathetic nervous system to the brown adipose tissue [178]. Being a heat regulator, it utilizes as a substrate FFAs in order to produce thermogenesis [179]. BAT is equipped with a great number of mitochondria that give to the tissue its distinctive dark color. Brown adipocytes express the uncoupling protein-1 (UCP-1), that generates heat at the expense of ATP [180].

Initially believed to be present only in young subjects/newborns, BAT is known nowadays to be active in various adult groups, as outdoor workers, northern populations, and less active in obese or overweight people, making it a suspect for obesity prevalence and a target for obesity treatment.

In humans, brown adipose tissue surrounds the heart and great vessels in infancy but tends to disappear over time. Metabolically active BAT is identified by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography scans (FDG-PET) combined with computed tomography (CT). Several studies have documented that the metabolically active areas detected on PET-CT actually are UCP-1-positive brown adipocytes [181,182,183] and have reached the conclusions that, irrespective of age and gender, metabolically active BAT is found in adult humans in the cervical–supraclavicular region and upper-chest regions [184].

The white adipose tissue (WAT) under normal and obesogenic conditions

On the contrary to BAT, WAT is active as an energy storage site. Dispersed all over the human body as part of the connective tissue and embedding muscle and nervous tissue, white adipocytes are the main storage depot for triacylglycerides (TAGs), leading to eventual release of FFAs.

Traditionally, WAT was attributed a role as a simple energy storage organ, that was accumulating triglycerides (TGs) and releasing FFAs and glycerol when energy intake was significantly lower than the fuel needed. However, nowadays it has proven to possess a complex metabolic role, which is to secrete and release a variety of proteins and biomolecules, therefore interacting with other tissues and organs [185,186].

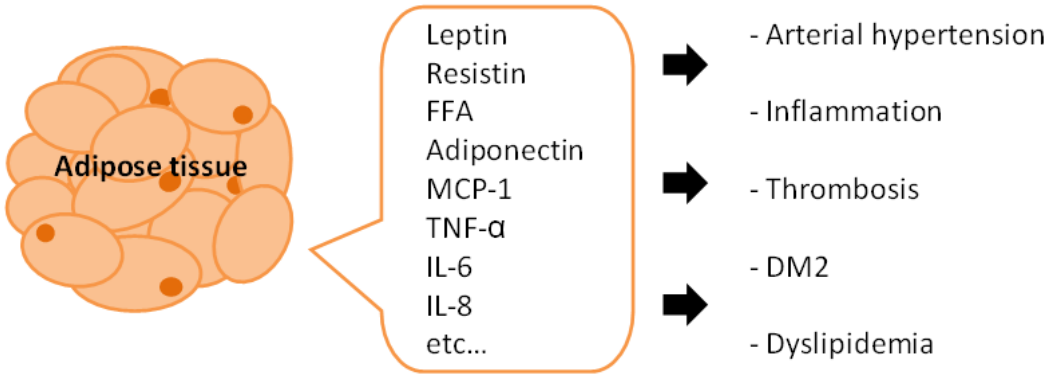


Fig.5 Adipose tissue as an important endocrine organ.

On its total, the adipose tissue (AT) is not composed of only adipocytes. Other types of cells to be found within this tissue are the stromo-vascular cells, formed by blood cell, leukocytes, macrophages, fibroblasts, preadipocytes, endothelial cells, holding one with another with collagen and elastic fibres [187,188,189,190]. WAT has significant endocrine functions and is well vascularized and innervated.

Human weight increase is due to the excessive accumulation of WAT. The white adipocyte is crucial for energy homeostasis and has significant endocrine functions, secreting hundreds of factors like enzymes, cytokines, hormones and growth factors [191,192] with autocrine, paracrine and endocrine actions. In obesity there is a misbalance in the secretion of these biomolecules that leads to a disrupted crosstalk between WAT and the other tissues inducing. WAT has the ability to grow and expand itself under positive energy balance and decline when energy expenditure is in excess of intake.

Obese AT as source of circulatory atherothrombotic cytokines. Cellular origin.

White adipocytes are nucleated cells comprising a lipid droplet which occupies most of the cell and a thin cytoplasm displaced to the periphery. Adipocytes derive from mesenchymal stem cells (MESCs) [193]. MESCs initially develop into preadipocytes, cells that are morphologically indistinct from MESC precursors but meant to convert into WAT or BAT [193]. Finally they differentiate into mature adipocytes under well known regulations.

The number and size of adipocytes generates the WAT mass, and cell death contributes to that together with the development and differentiation of progenitor cells, namely the preadipocytes. The growth of adipocyte mass occurs by elevated differentiation of the progenitors or by hypertrophy of the already existing adipocytes, precisely enhanced intracellular lipid deposition [188,194,195].

Studies performed during the last years demonstrated that adipokines and bioactive peptides released by the adipose tissue are not only secreted by the mature adipocytes, a fact that is true in the case of some proteins only, like leptin or adiponectin.

The theory about the inflammatory state of the obese adipose tissue emerged in 1993. Scientists were lead to this by the observation that TNF-α expression was upregulated in adipose tissue of obese mice [196]. Mice fed a high-fat diet had a weight gain allied to the activation of various inflammatory pathways, being 59% of the total adipose tissue mRNA linked to inflammatory genes[197]. Additionally, mRNA levels and protein secretion of IL-6, TNF-α, PAI-1, TF,

angiotensinogen (AGT), complement factor C3 and other inflammatory, angiogenic and prothrombotic cytokines have been found elevated in adipose explants from obese patients [107].

More recently [196,198], activation of a proinflammatory cell phenotype was suggested in the obese adipose tissue. According to evidence, adipose tissue dysfunction, resulting from fat accumulation in obesity, starts from the increase in both number and size of adipocytes, the associated hypoxia [199] and the defects in the accumulation and storage of lipids. Therefore, an imbalance of secreted cytokines and extracellular lipids is being generated, which further induces intracellular endoplasmic reticulum (ER) stress, produces an excess of reactive oxygen species (ROS) by the mitochondria and activates inflammatory signaling pathways [174,200]. Once the initial impulse has been generated by hypertrophic mature adipocytes, further cellular activation in the obese AT involves mostly other cell types. Most factors secreted by the adipose tissue of morbidly obese humans derive from the non-adipocyte stromal cell fraction [201].

Although macrophages embody only 5-10% of the adipose tissue's cell population, weight gain induced by diet causes a significant macrophage infiltration, resulting in macrophages representing up to 50% of all cells found in adipose tissue [202,203]. Studies performed on humans reported that insulin action is mostly connected to macrophage activation rather than the content[204]. For that, suggestions have been made linking inflammatory stimuli, generated by mature adipocyte-derived factors, to the polarization of the resident anti-inflammatory M2 macrophages to a proinflammatory M1 phenotype, secreting chemoattractant biomolecules [205]. This determines supplementary transmigration of circulatory monocytes and obese adipose tissue infiltration with proinflammatory macrophages, maintaining a vicious proinflammatory cycle [197,202,205].

In relation to this, our group has recently reported that the stromo-vascular cell fraction is responsible on majority for the secretion of cytoadipokines with endothelial injury potency [206]. Fair enough has been also proposed that the number of committed preadipocytes able to undergo adipogenesis is reduced in obesity due to the proinflammatory environment [200,207,208].

While less evidenced, worth mentioning are various other theories about inflammation onset in obesity. One of them [174] postulates that obesity itself is a stress condition comparable to that of an infection, explaining therefore the activation of similar pathways. Moreover, activation of inflammatory pathways could represent a catabolic defense response to the anabolic accumulation of adipose tissue.

All these occurrences may then interact in order to generate a nasty cycle, maintaining the obese adipose tissue inflammation and intensely disturbing the secretion of adipose tissue-derived cytoadipokines. Consequently, an amplified number of inflammatory and thrombogenic proteins/peptides enter the central circulation and induce systemic low-grade inflammation, endothelial activation and development of atherothrombotic disease [105,109].

4.2.2 Visceral *versus* subcutaneous adipose tissue in atherothrombotic disease

Evidently, changes in the adipose tissue's size and metabolism that define its pass from the lean to the obese state, but also changes within the very same obese condition (e.g. obese to morbid obese), determines an altered adipocytokine synthesis pattern, proinflammatory and prothrombotic ones getting dysregulated. Table 4 presents a summary of the till date most studied cytoadipokines emerged from the inflamed obese adipose tissue and their presumed actions.

Table 4. *Adipokines and their functions*

Adipokine	Metabolism	Inflammation (Adhesion, Chemotaxis)	Extracellular Matrix	Growth	Fibrinolysis , Hemostasis
Adiponectin (ADIPOQ)	√	√		√	
Adipocyte fatty acid binding protein (FABP4)	√	√			
Adipsin (CFD)	√	√			
Alpha 1 acid glycoprotein (ORM)		√			
Alpha 2 macroglobulin (A2M)			√		
Angiopoietin 1, 2				√	
Angiotensinogen (AGT); Angiotensin II		√	√		√
Apelin (Apln)	√	√			
Apolipoprotein E	√				
Cathepsin (CTSD)			√		
Chemokine ligands and chemokines: CXCL5,CXCL10, CCL4-5, CCL14,CCL23, CXCL3, XCL1,CXCL12 LIF, LYVE-1		√			
Collagen I, III, IV, VI			√		
Fibronectin (FN)			√		
Insulin-like growth factor 1 (IGF-1), IGF-binding proteins (IGFBP 1-7)	√			√	
Interleukines: IL-1β, IL-4, IL-6, IL-6sR, IL-7, IL-8 IL-10,IL-18	√	√		√	√
Leptin(Lep)	√	√		√	
Lysyl Oxidase			√		
Lipoprotein lipase (LPL)	√				
Macrophage chemoattractant proteins (MCP1, MCP2)		√			
Macrophage migration inhibitory factor (MIF)		√			
Macrophage colony stimulating factor (MCSF), Monocyte differentiation antigen (CD14)		√			
MMP1-3, 7, 9-12, 14,15, 19 TIMP 1- 4			√		
Omentin (ITLN)	√				
Oncostatin M (OSM)		√		√	
Resistin (RETN)	√	√			
Retinol binding protein (RBP4)	√				
Secreted frizzled-related protein 5 (Sfrp5)	√				
TNFα,sTNFαRI, sTNFαRII		√			
Visfatin (NAMPT)	√				
Vaspin (serpin)	√	√			

Serum amyloid A3 (SAA),C-reactive protein(CRP), Complement factors (C3-4, C7) , Haptoglobin (Hp)		√			
Angiogenin (ANG), Angiopoetin(ANGP), Fibroblast Growth Factor (FGF), Hepatic growth factor (HGF), Nerve growth factor (NGF),Stromal derived factor (SDF-1), Vascular endothelial growth factor (VEGF), Tumor growth factor (TGF-ß), Platelet derived growth factor (PDGF), Thrombopoietin (THPO)				√	
Tissue factor (TF), VCAM-1, ICAM-1, E-selectin		√		√	√
Plasminogen activator inhibitor (PAI-1), Antithrombin (SerpinC) Trombospondin (Tsp)	√				√

Both increased adiposity and reduced physical activity are strong and independent predictors of CHD [209] and death [210,211,212,213]. Depending on the regional distribution on the human body, excess WAT has been correlated to cardiometabolic complications (accelerated atherothrombosis and premature cardiovascular death [214,215]. There is widespread support in the literature for the conclusion that **central adiposity** (fat in the trunk and/or **abdomen**) confers more cardiovascular risk than **peripheral** adiposity (i.e., that present in the hips and thighs) [106]. To the first form of adipose tissue accumulation is also referred as **android** obesity, whereas to the subcutaneous deposition of fat at the hips and thighs as the **gynoid** form of obesity.

The waist-to-hip ratio reflects abdominal fat in predicting type 2 diabetes, stroke, myocardial infarction and cardiovascular mortality in middle-aged individuals. The Nurses' Health Study confirmed these findings using the waist circumference [216]. There is an independent curvilinear association between visceral adiposity and mortality [217], suggesting that a large amount of visceral fat is required for an increased risk of mortality.

In general, for each unit of BMI increment, the risk of CHD increases by 8% [209]. On the other hand, each 1 h-MET (metabolic equivalent) increase in activity score is associated with an 8% decrease in CHD risk. Most heart attacks can be predicted from easily measurable and modifiable factors, including abdominal adiposity and regular physical activity [214,217,218,219,220].

AT is not structured out of one component only and is separated to the visceral and subcutaneous one. Both store excess calories while visceral AT supplies the inner organs with energy. There is 3 to 4 times more subcutaneous than visceral AT [221], and it appears that the two tissue types can interact in a coordinated and compensatory manner. An increase in total body fat, particularly in the visceral deposit, takes place with age and is associated with insulin resistance, dyslipidemia and increased risk for diabetes and CVD [220,105].

Compared with the subcutaneous AT, visceral AT is more cellularized, vascularized and innervated, and contains a larger number of inflammatory and immune cells, a lesser preadipocyte differentiating capacity and a greater percentage of large adipocytes. Visceral AT cells are more metabolically active, more sensitive to lipolysis and more insulin resistant than subcutaneous adipocytes [187,222]. Several proteomic studies have revealed significant differences in many proteins/peptides secreted from the visceral *versus* subcutaneous fat pads in obese *versus* lean individuals [223,224]. Interestingly, a current comparative proteomic study in lean subjects suggests significant differences between the secretomes of visceral and subcutaneous stromal cell fraction, but not between the secretomes of adipocytes isolated from the same fat pads [225].

Thus, visceral AT has a greater capacity to generate free fatty acids and to take up glucose than subcutaneous AT, and is more sensitive to adrenergic stimulation. On the other hand, subcutaneous AT is more ardent in the absorption of circulating FFA and triglycerides. The subcutaneous fat is a more active storage depot, being more responsive for the anabolic actions of insulin and demonstrating a greater readiness for proliferation and expansion [222]. In this sense, AT inflammation and macrophage infiltration is higher in obese visceral AT than subcutaneous AT [197,202], which may be due to the reduced expandability of the former. Accordingly, data from various clinical *ex vivo* studies of different AT depots in humans indicate that visceral fat accumulation represents the highest risk factor for CVD, hypertension, insulin resistance and type 2 diabetes [105,220]. Visceral AT mass is also a more important predictor of myocardial infarction than the total body mass in women[226].

Therefore the increased secretion of deleterious adipokines in obesity has been attributed to visceral AT, suggesting that proinflammatory and prothrombotic protein secretion increases proportionally to the BMI [201,227]. However, subcutaneous AT from obese subjects with a BMI>40kg/m^2 also presented an altered cytokine secretion pattern [201,228]. Thus, recently there have been reports that increased circulating hs-CRP associates with enlarged visceral AT mass in patients with vascular disease, apparently as a consequence of the induction of CRP synthesis by IL-6 secreted by the visceral AT [229]. By that has been demonstrated the connection between the visceral AT secreted cytokine IL-6 and systemic inflammation. Circulating levels of IL-6 were associated with visceral adiposity, whereas TNF-α showed an association with overall obesity. These results support the hypothesis that IL-6 mediates the hyperinsulinemic state related to excess visceral fat while TNF-α contributes to the insulin resistance of overall obesity [230]. Overall adiposity has also been found to be responsible for plasma levels of CAMs, being ICAM-1 and E-selectin the most relevant ones[231,232].

In this sense, previous results from our group showed in a translational study model that in both lean and obese subjects the secretome of the visceral stromal cell fraction determines a higher proinflammatory and prothrombotic injury in macroendothelial cells, being highest the one induced by the visceral obese adipose tissue secretome [206].

On the other hand, pericardial or perivascular ectopic AT depots associated with visceral obesity, have also been associated with increased CV risk in a paracrine manner, although to a lesser extent than the intra-abdominal AT [220]. Nevertheless, the relationship between CVD and visceral AT is not straightforward, as racial and ethnic differences do exist. For example, African Americans demonstrate greater insulin resistance with less visceral fat as compared with Caucasians [233]. Moreover, earlier studies in humans showed a stronger association between subcutaneous abdominal fat and insulin resistance [234,235]. Thus, the role of anatomical location in mediating the deleterious effects of visceral AT, remains unresolved.

4.2.3 State of the art: Atherothrombotic disease in obesity and type 2 diabetes; the hyperinsulinemia- hyperglycemia- inflammation ensemble

Evidence demonstrates that increased adipose tissue mass contributes directly to an increase in ***systemic inflammation***. The earliest indications of this phenomenon were reported in 1985, when an article noted positive correlations between body mass and peripheral leukocyte count [236]. Neutrophil activation increases with the grade of obesity. As neutrophils have a short life span, this indicates that the chronic inflammatory condition associated with morbid obesity is characterized by a continuous activation of the innate immune system [177]. Further research in the '90s has evidenced, as previously mentioned, that obese adipose tissue is infiltrated with proinflammatory immune cells that activate local non-adipocyte AT cells inducing profound alteration in the secretory pattern of the AT [196].

Since then, a large number of studies have found that increased BMI correlates with increases in systemic circulating levels of inflammatory, prothrombotic proteins and markers of endothelial activation, such as hs-CRP, IL-6, PAI-1, VWF and vascular cell adhesion molecules like VCAM-1, ICAM-1, P-selectin, fibrinogen and AGT [106,107,201,237,238]. Moreover, endothelial activation and expression of ICAM-1 and E-selectin seems to be determined more by the obese AT grade of overall adiposity than insulin resistance [231]. Further investigation confirmed that the obese AT secreted cytokines, especially the visceral ones, reach systemic circulation and determine directly, or by inducing the hepatic synthesis of other proinflammatory proteins like hs-CRP, the modulation of the sera prothrombotic and proinflammatory biomolecules [196,198,229,239].

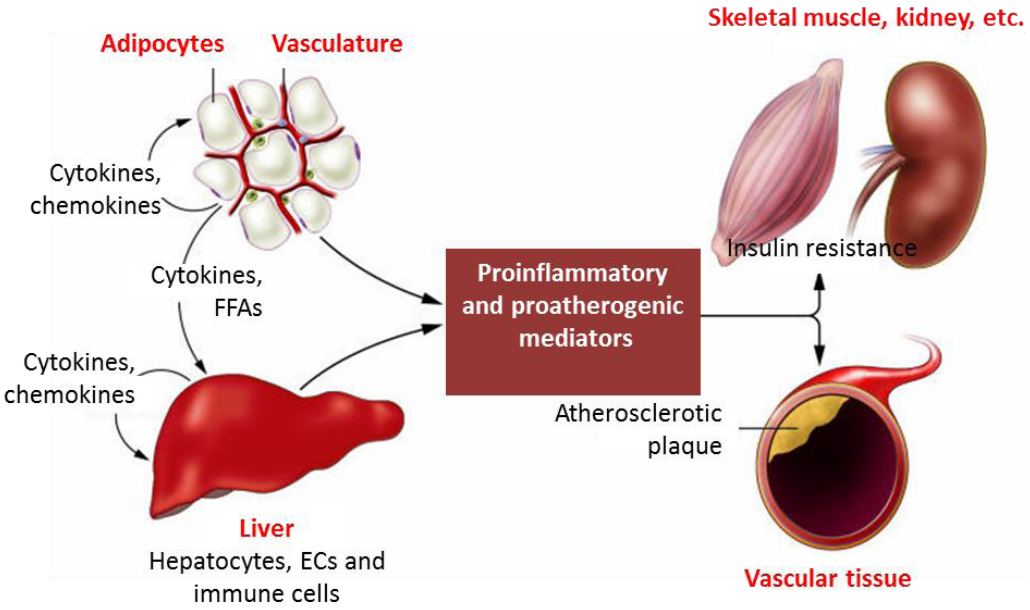


Fig.6 Local, portal, and systemic effects of inflammation in insulin resistance and atherogenesis.
Increasing adiposity activates inflammatory responses in fat and liver, with associated increases in the production of cytokines and chemokines. Portal delivery of abdominal fat–derived cytokines and lipids contributes to hepatic inflammation and insulin resistance. Proinflammatory and proatherogenic mediators are produced in the adipose tissue and liver and associated immune cells. This creates a systemic inflammatory diathesis that promotes insulin resistance in skeletal muscle and other tissues and atherogenesis in the vasculature. FFA= free fatty acids. Adapted from *Shoelson et al, J Clin Invest. 2006* [240].

The "chicken or the egg" question. As a result of the above, we can see that obesity is able to encourage both **chronic inflammation and insulin resistance** development. But there is still a blurred vision of whether insulin resistance leads to or results from systemic inflammation.

Considerable studies on this field have been made on animals. Therefore, insulin resistance can be provoked in lean animals by infusion with inflammatory cytokines or lipids. Mouse obesity models were protected against insulin resistance when inflammatory mediators or pathway components by genetic manipulation were removed [174]. Additionally, insulin resistance was caused in animals when infused with inflammatory cytokines or lipids and at the absence of obesity [174].

In humans, the facts are that subjects with chronic inflammation are predisposed to CVD and diabetes [174,237]. Insulin sensitivity is improved by salicylates [240], and obese patients with insulin sensitivity present a lower risk of cardiometabolic complications and a higher degree of adipose tissue inflammation [241,242]. The same studies suggest activation of insulin resistance associated with obesity depends on the activation of inflammatory cascades and that IL-6, IL-1b and NF-κB seem to be important effectors, mediating the inflammation effects promoting insulin resistance [242]. The inflammatory state can be reverted by weight loss, which is essentially a reduction in AT mass. Weight loss induces the constant decrease in CRP levels and other systemic inflammatory cytokines[107], with the exception of adiponectin, which rises.

Significant source of endothelial dysfunction can be considered insulin resistance and the effects proceeding hyperinsulinemia, even though evidence suggests that the roots of endothelial activation and at least the first crucial step of leukocyte adhesion to the endothelium are the consequences of the systemic inflammation associated with obesity [239].

Promising experimental data are suggesting that **insulin** has a beneficial effect on the endothelium [243]. Anyway debate persists as follows.

Anti-inflammatory effect of insulin. Another possible reason why obesity and type 2 diabetes are associated with inflammation is that the state of insulin resistance promotes inflammation. This is due to the fact that insulin exerts an anti-inflammatory effect at the cellular and molecular level *in vitro* and *in vivo*. A low dose infusion of insulin (2.5 IU h21) reduces ROS generation by mononuclear cells, suppresses NADPH oxidase expression and intranuclear NF-κB binding, induces I-κB expression and suppresses plasma intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) concentrations. It also suppresses plasma tissue factor, PAI-1 and MCP-1 concentrations [244,245]. An interruption of insulin signal transduction would prevent the anti-inflammatory effect of insulin from being exerted [246].

An important implication in the relationship between inflammation, obesity, insulin resistance and type 2 diabetes, is that atherosclerosis, which is responsible for the major cause of death (acute myocardial infarction) in this patient population, is itself an inflammatory process [97]. An increase in the plasma concentration of inflammatory mediators, such as CRP and IL-6, increases the risk of atherosclerotic complications, such as acute myocardial infarction. Thus, inflammation underlies both insulin resistance and atherosclerosis. One mechanism that might connect these features is the anti-inflammatory and potential anti-atherosclerotic effect of insulin, which in the presence of resistance will lead to a proinflammatory state. It is interesting in this light that insulin sensitizers as thiazolidinediones are also anti-inflammatory and potentially antiatherogenic [247,248,249]. Thus, insulin and thiazolidinediones (TZDs) exert a suppressive effect on ROS generation, NADPH oxidase expression and intranuclear NF-κB binding, and a stimulatory

effect on I-κB expression along with a reduction in plasma concentrations of ICAM-1, MCP-1 and PAI-1. TZDs also suppress the plasma concentrations of TNF-α and CRP [246].

Proinflammatory and other proatherogenic effects of insulin. Intriguingly, results from *in vitro* studies in the HUVEC-established macroendothelial model, mostly from the group of Madonna *et al*, reported that prolonged pathologic concentrations of insulin induce the endothelial expression of adhesion molecules VCAM-1, ICAM-1 and activation of NF-κB pathways in endothelial cells through stimulation of the p38 MAP kinase [250,251,252]. Moreover, recent researches regarding insulin growth factor potential have reported that high concentrations of insulin stimulate angiogenesis in the atherosclerotic plaque, making it more vulnerable and prone to an acute rupture [253].

Hyperglycemia, glucose variability and inflammation *in the atherothrombotic disease related to type 2 diabetes-associated obesity.*

Earlier we referred to hyperglycemia as causative of cardiovascular complications which constitute the major source of morbidity and mortality in patients with diabetes.

These have been linked to hyperglycemia-induced oxidative stress and to the formation of advanced glycosylation end products (AGEs), that promote atherothrombosis through their effect on endothelial cell function [254,255,256].

However, there is a considerable debate about the range of glucose adjustment. It is a fact that glucose control in DM treatment undergoes strict lines, since intensive glycemic control prevents the development and progression of microvascular complications in patients with type 1 or type 2 diabetes. Nonetheless, recent studies demonstrated that intensive glycemic control to near normoglycemia also can result unworthy, having no deriving benefit on cardiovascular outcomes. Apart from this, prejudicious intensive glucose-lowering therapy was reported to have an effect on mortality [257].

An interesting theory suggests that glucose variability is more important than hyper- or hypoglycemia *per se*. Exposure of human umbilical vein endothelial cells (HUVECs) to oscillating glucose revealed its damaging effects compared to constant high glucose, inducing a metabolic memory even after glucose normalisation, most probable due to hyperactivation of p53 during glucose oscillation [258]. With the term “metabolic memory” has been described the ability of hyperglycemia to leave a mark in the cells of the vasculature and therefore supporting forthcoming complications even at moments of good glycemic control [259]. Hence, a very early aggressive treatment of hyperglycemia has been suggested as mandatory [255].

Data remains unchanged when comparing effects of high glucose, hypoglycemia and glucose variability on the acute and chronic CV disease. Both high values of glycemia and hypoglycemia and high oscillation seem to predict endothelial apoptosis and prothrombotic short term complications[260].

Moreover it has been observed that systemic inflammation increases in obesity once diabetes is restored [261]. The effect of high glucose concentrations (varied doses and time of exposure) on the activation of inflammatory endothelial pathways has been studied in various *in vitro* models of endothelial injury with contradictory results [262,263,264,265,266,267,268]. Most evidence indicates that chronic hyperglycemia is not sufficient to directly promote endothelial inflammatory activation. At clinical level, inflammatory activation of endothelial cells in acute myocardial infarction is related to glucose impairment in diabetic subjects [269], but as mentioned above, no relation has been established in the evolution of chronic CVD. These findings are highlighting the

pivotal role of a proinflammatory environment in type 2 diabetes associated to obesity, suggesting it as a critical factor conditioning the early pro-atherosclerotic actions of hyperglycemia.

Till date no study was performed on the direct concomitant effect of glucose and adipose tissue secreted molecules on the endothelium. Our group has previously demonstrated in an *in vitro* model the capacity of cytokines secreted from the adipose tissue to directly activate endothelial injury mechanisms. How glucose, insulin and type 2 DM change the effect of the adipokines secreted from the obese adipose tissue has therefore been one of the three goals of this thesis.

4.3 Metabolism products as emerging cardiovascular- and diabetes-predictor factors in obesity and diabetes mellitus

4.3.1 Metabolomics, the concept

Since the 1990s, there has been a revolution in the techniques and approaches used in molecular biology culminating with the development of system biology and of the -omic sciences[270]. Among "-omics" sciences, metabolomics is of the youngest, but its use has been rapidly increased, accounting for its great impact. It shows a great potential in identifying possible biomarkers for drug discovery, involving the bio-chemical profiling of all the metabolites in a cell, tissue, or organism.

In reality it depicts the comprehensive and simultaneous systematic profiling of multiple metabolite levels and their systematic and temporal changes caused by factors such as diet, lifestyle, environment, genetic effects and pharmaceutical effects, both beneficial and adverse, in complex systems and whole organisms. Actually metabolomics focuses on the measurement of the physiological state of an organism's metabolites through the combination of data-rich analytical techniques and multivariate data analysis [271,270].

The objective of systems biology is to obtain an integrative model of systems and their interactions. Genomics', transcriptomics', metabolomics' and proteomics' data are integrated for construction and validation of models. The value of metabolomics is justified by the straight correlation between the metabolome and the phenotype, since the metabolites reflect the activities of the cell at the phenotypic level. It is worth to note that a large variety of metabolites change far faster than nucleic acids or proteins, reflecting more adequate changes in cell functions. Differently from genomics and transcriptomics, metabolomics is sensitive to both the genetic and the modifying environmental influences that determine the metabolic pattern of an organism. Metabolic analysis observes the biochemical effects in a system being close to the biological end-point.

Metabolomics applies techniques suitable for a high throughput analysis of metabolites in fluids (mainly blood and urine) and tissues. The product is the metabolic profile, whose comparison with others, from different phenotypes, is used to recognize precise metabolic changes. The latter leads to understanding of biochemical pathways, complex biomarker combinations, toxic effects and disease progression. Metabolomics is mainly functional in

mammalian systems in phenotyping, discovery of biomarkers, diagnosis of diseases and therapeutic efficacy, toxicology (pre-clinical drug candidate safety assessment), etc.

4.3.2 Detection techniques

There are two basic different approaches to metabolomics: targeted and untargeted. **Targeted** metabolomics refers to the identification and quantification of defined sets of structurally known and biochemically annotated metabolites. It has the chief advantage to generally provide quantitative data (the concentrations of the involved metabolites) and facilitate the instantaneous understanding of deviations from normal. Interpretation of the data is straightforward, regardless of the cohort investigated, for example, healthy vs. diseased or treated vs. untreated. A further advantage of targeted metabolomics is that it is well suited for high-throughput and routine applications [272]. Another major benefit of the targeted method is speed, since minimal effort and resources are required to profile the specific metabolites over a large number of samples [273].

On the other hand, **untargeted** metabolomics involves comparison of total relative quantities of the metabolome in samples without previous identification. The objectives of untargeted metabolomics are to determine metabolomic profiles in relation to complex sets of genomic and proteomic changes. This method is a potent technique for identifying and quantifying relative changes in complex samples, since identification of the peaks found to be dysregulated in the untargeted data still remains a challenging determination [273]. In contrast to targeted metabolomics, where ions from known metabolites are being measured, untargeted metabolomics records all ions within a certain mass range, including those of structurally novel metabolites [274].

The main analytical techniques that are employed for metabolomic studies are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), thanks to their sensitivity and resolution capabilities. The latter technique requires a connection to another technique for a pre-separation of the metabolic components, for instance gas chromatography (GC) after chemical derivatization, or liquid chromatography (LC), with the newer method of ultra-high-pressure LC (UPLC) gaining more and more use. The use of capillary electrophoresis (CE) coupled to MS has also shown to be promising. Other more specialised techniques as Fourier transform infra-red (FTIR) spectroscopy and arrayed electrochemical detection have been used with less extent [270].

NMR spectroscopy

NMR spectroscopy is a non-destructive technique that supplies detailed information on the molecular structure of pure compounds and complex mixtures, as well as on absolute or relative concentrations. The NMR spectroscopic methods are employed in the survey of metabolite molecular dynamics and mobility in addition to substance concentrations through the interpretation of NMR spin relaxation times and by the determination of molecular diffusion coefficients [270].

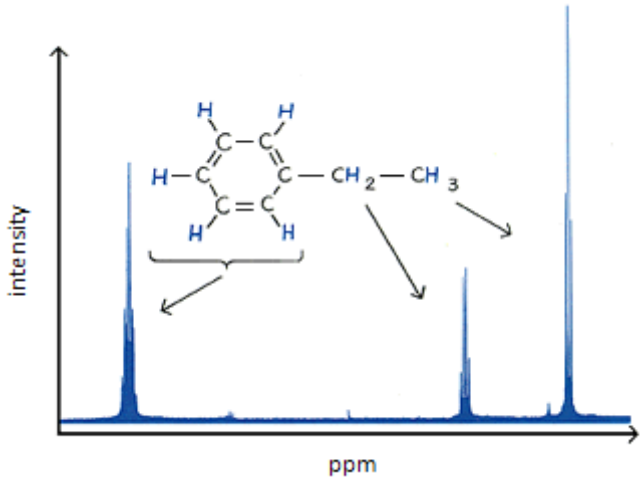


Fig.7 A proton NMR spectrum of a solution containing a simple organic compound, ethyl- benzene. Each group of signals corresponds to protons in a different part of the molecule. Adapted from *http://www.nobelprize.org.*

The benefits and weaknesses of the technique, with respect to its use in metabolomics, are depicted in Table 5. In the pros are included the high throughput, the minimum sample preparation, the non-destructive nature of the technique and the possibility for *in vivo* application on whole live subjects, among others. The cons are allied to a relatively poor resolution as far as complex samples are concerned, a reduced sensitivity compared to MS, and the need for a fairly high sample volume, as being the most important ones.

Table 5. *Advantages and disadvantages of metabolomics-applied NMR*

Advantages	Disadvantages
○ Rapid and simple sample preparation required.	• Poor sensitivity and selectivity.
○ Does not require chromatographic separation.	• Relatively poor resolution in complex samples.
○ High reproducibility.	• Difficult automatization despite robotized systems.
○ Relatively high throughput in 1DNMR experiments.	• Very low throughput in 2D-NMR experiments.
○ Useful for identification purposes.	• Difficult to unequivocally assign signals.
○ Versatility. Possibility of direct analysis of solids and *in-vivo* studies.	• Less suitable for targeted analyses.
○ Non-destructive.	• Large sample volume (0.5 ml).

Adapted from *http://www.tdx.cat*.

Targeting NMR analysis is gaining attention owing to the wide range of applications that has emerged lately. Interestingly enough there have already been studied and published biomarkers related to diseases or human metabolism, including schizophrenia [275], multiple sclerosis [276], diabetes [277], organ rejection [278], and rheumatoid arthritis [279]. Moreover, NMR-based serum-lipoprotein quantification is now an ordinary clinical diagnostic tool, since *in vivo* identification and quantification is currently well practiced.

Mass spectrometry (MS)

Being currently the most reliable platform for identification, MS is a detection technique based on the differential displacement of ionized molecules through vacuum by application of an electrical field. The ions are separated according to their mass-to-charge ratio (*m/z*) and are this way detectable. A general prior separation of the complex mixture sample is required, being mass spectrometers usually joined to a separation unit such as a chromatograph or capillary electrophoresis equipment [271]. For example, connection to a UPLC (UPLC-MS) provides better chromatographic peak resolution and increased speed and sensitivity in complex mixture separation [270].

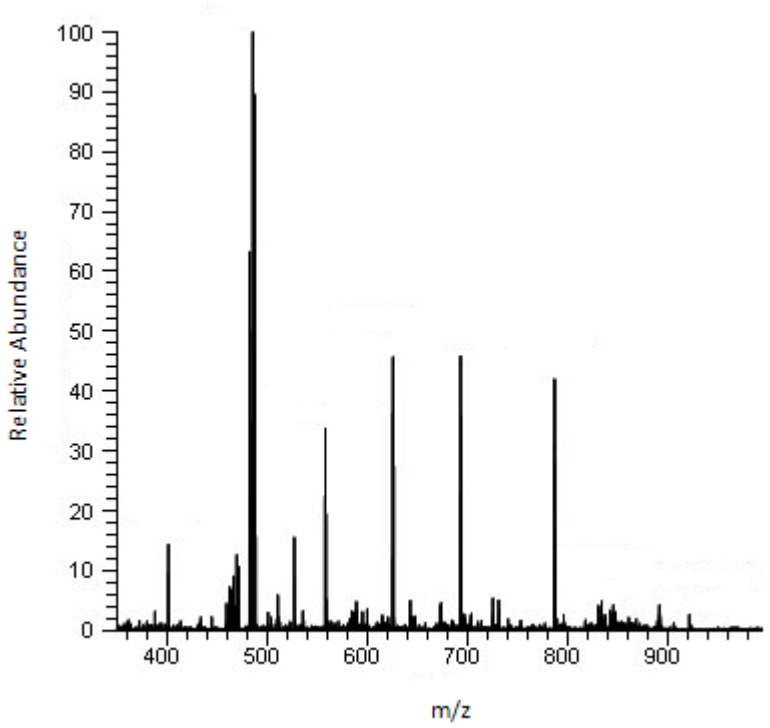


Fig.8 Example of a mass spectrum. Each peak shows a component of unique m/z in the sample, and heights of the peaks indicate the relative abundance of the various components in the sample. Adapted from *http://www.broadinstitute.org*.

The use of MS is extensive in metabolic fingerprinting and metabolite, as well as in the identification and quantitation of drug metabolites, since it is considerably more sensitive than NMR spectroscopy [270]. In fact, MS-based metabolomics offers quantitative analyses with high

selectivity and sensitivity and the potential to identify metabolites with high mass accuracy. On the contrary, quantification requires suitable internal standards, MS measurements can vary in time requirement, and sample preparation might cause metabolite losses. For more information see Table 6.

Table 6. *Advantages and disadvantages of metabolomics-applied MS*

Advantages	Disadvantages
○ Highly sensitive in its different modes.	• Detection depends on ionization efficiency.
○ Accurate *m/z* measurements ideal for identification of metabolites.	• Limited to liquid samples or extracts isolated from solid samples.
○ Suitable for quantitative target analysis.	• Destructive, particularly complicated for analysis of valuable samples.
○ Very selective.	• More efficient when coupled to separation techniques.
○ Structural information throughout fragmentation patterns.	• Quantification requires chemically-related internal standard.
○ Excellent resolution (improved by separation).	• Difficult sample preparation protocols.

Adapted from *http://www.tdx.cat*.

Mass spectrometry is the preferred technique for targeted analysis in several areas, including pharmaceutics, clinics, forensics, food, agriculture, etc., being a great tool for molecular identification. The recently introduced highly accurate mass spectrometers (Orbitrap and TOF analyzers) have enabled the use of MS for global profiling with a relatively high throughput. Mass-spectrometric metabolic profiling has been used broadly in the diagnosis of metabolic disorders by sampling diverse biofluids; as an example, the urinary metabolome that has been evaluated by Kuhara [280] in the diagnostics of metabolic disorders using GC–MS. Additionally, MS has become valuable in assessing disorders of amino acids metabolism, fatty acids, organic acids biosynthesis, etc[271].

To summarize, NMR and MS approaches are highly complementary, and mutual use is frequently necessary for global molecular characterisation. The lower detection limits of MS constitute it more sensitive, provided the substance of interest can be ionised, but NMR spectroscopy is particularly functional for distinguishing isomers, for the obtention of molecular conformation information and for studies of molecular dynamics and compartmentation [270].

4.3.3 Applications on obesity and type 2 diabetes mellitus

Nowadays' scientists are confronting themselves with the discovery of treatment or prevention of diseases. Development of obesity-associated metabolic disease, cardiometabolic complications and diabetes are among them [271].

Alterations of carbohydrate and lipid homeostasis in obesity have been widely recognized [281] but the role of specific metabolites remains under study, with controversy surrounding the question of whether protein metabolism is affected in human obesity. Metabolomic sera studies performed till now in obese humans have provided important evidence about the role of obese adipose tissue emerged metabolites in the systemic metabolic disease have been performed till date various study in animal models and in obese humans. Adipose tissue-secreted metabolites are not only indicative of the obesity-induced psychopathological state but may also act as initiators of pathological cascades and play endocrine roles as regulatory signals [282].

Metabolic markers for insulin resistance, development of the cardiometabolic disease and diabetes have been a constant motive of investigation.

As far as animal models are concerned, the Zucker obese rat has been widely used for metabolomic studies. Considering male and female Zucker rats as models for diabetes, metabolomic analysis has been applied on urine samples of those animals, with the help of capillary HPLC [283]. Chromatographic separation using capillary HPLC-MS provided higher sensitivity than simple HPLC-MS, as well as a greater peak count (ca. 3000 ions compared to 1500 for HPLC) for the same analysis time. From the data resulted also markers of diurnal variation, for both male and female rats.

Moreover, the relationship between visceral adiposity and intramyocellular lipid levels associated with the development of type 2 diabetes was surveyed in animal models of insulin resistance, induced by diet or glucocorticoids [284], applying metabolomic measurements as well as *in vivo* and plasma NMR spectroscopy.

In humans, the first metabolomic studies applied NMR spectroscopy in the urine and plasma of diabetic patients [285]. Insulin removal lifted plasma levels of ketone bodies and glucose, whose levels, together with the ones of lactate, valine and alanine, were in agreement as when measured by standard clinical chemistry methods, confirming NMR accuracy. This, in addition to some later studies, demonstrated the usefulness of NMR spectroscopy in the diagnosis of diabetes, the monitoring of the development of the disease and the monitoring of patient therapy in a fast and non-destructive way.

It was not until recently that capillary gas chromatography was applied in the diagnosis of type-2 diabetes mellitus based on serum lipid metabolites, suggesting that serum fatty acid profiles might distinguish type 2 diabetic patients from healthy subjects [286].

Kim *et al* identified three lyso-phosphatidylcholine derivates as potential plasma markers and confirmed eight known metabolites for overweight/obesity men. Overweight/obese subjects showed higher levels of lysoPC C14:0 and lysoPC C18:0 and lower levels of lysoPC C18:1 than lean subjects. Moreover they present an abnormal metabolism of two branched-chain amino acids (BCAA), two aromatic amino acids, and fatty acid synthesis and oxidation [287].

In a functional study in obese humans, Huffman *et al* observed that elevated concentrations of large, neutral amino acids were independently associated with insulin resistance and fatty acids were inversely related to the pancreatic response to glucose, suggesting that these

metabolites might play a role in the progression of type 2 diabetes by inducing insulin resistance and pancreatic injury [288].

Hypercaloric high fat and BCAA containing diets (mainly leucine) contribute to development of obesity-associated insulin resistance by chronic phosphorylation of mTOR (mammalian target of rapamycin), JNK (c-Jun N-terminal kinase), and IRS1Ser307 (insulin receptor substrate 1), and by accumulation of multiple acylcarnitines in muscle [289]. Plasma long-chain AcylCN species are increased in obesity and type 2 DM, suggesting that more fatty acids can enter the mitochondria. In type 2 DM, many shorter species accumulate, suggesting an extended oxidation defect [290].

Moreover, butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms [291].

Other small study on serum from young obese, nondiabetic women reports that, serum acetate was negatively associated with visceral adipose tissue and insulin levels [292].

Interestingly, other scientists proposed that a high glutamine to glutamate ratio would exert a preventive role on the cardiometabolic status [293].

Moreover, recent clinical evidence from the Framingham offspring study indicated increased levels of a group of five BCAA and aromatic amino acids (namely isoleucine, leucine, valine, phenylalanine and tyrosine) as candidate metabolic risk markers for diabetes in obesity, predating the clinical onset of diabetes by years [294].

In summary, up to date have been reported notable differences in the metabolic sera signatures of obese, mild insulin resistant but nondiabetic and lean individuals, while a decrease in certain metabolites has been found to predict improvement in insulin resistance (HOMA-IR) independent of the weight loss. Moreover, specific plasmatic metabolites are emerging as strong predictors of insulin resistance. What is more important, very recently have been described predictive sera metabolites of the onset of diabetes in obesity.

Despite all this extensive sera basal and functional metabolite study, until nowadays the contribution of the metabolites produced by the different obese adipose tissue depots to the systemic altered metabolite pool has not yet been defined. Hence, as in the present thesis one of our aims was to study the particularity of regional adipose tissue depots, we comparatively analyzed the secretome pattern of subcutaneous and visceral obese and nonobese adipose tissue using an untargeted metabolomics approach. Our goal was to decipher how obesity affects the metabolic profile of adipose tissue secretomes.

5. Metformin role in the treatment of type 2 diabetes

5.1 History

Back in the 18th century, the British botanist Sir John Hill, briefly illustrated in his work a plant named Goat's Rue (*Galegus officinalis*), a description that still stands until today: 'This is a perennial, native of Spain, and Italy; of Greece and Africa. A specious plant, of a yard high, that

flowers in August. The stalk is juicy, and green: the leaves are of a fine fresh green: the flowers are purple; sometimes white [295].

Goat's Rue, also known as French Lilac or Italian Fitch, is the natural source of guanidine, the active component of galega. Guanidine was popular in the 1920s for the synthesis of several antidiabetic compounds, out of which in the 1950s were introduced metformin and phenformin, two main biguanides [296].

Whereas phenformin presented a connection with lactic acidosis and was withdrawn from clinical use in many countries in the 1970s, metformin got not related with such associations and it is still used in more than 90 countries as the most widely prescribed oral antidiabetic agent. Metformin has an efficacy of glycemic control similar to that of the sulfonylureas, even if with a different mode of action. It is administered as first line therapy in patients with type 2 diabetes, with a special indication in obese patients. Metformin acts as an insulin sensitizer lowering blood glucose concentrations, improving insulin sensitivity and thus decreasing insulin resistance [296].

5.2 Metformin as antidiabetic drug: mechanisms of action

Fig.9 shows the structure of metformin and in Table 7 are delineated its pharmacokinetics. The main mode of action of metformin relies on the hepatic suppression of **gluconeogenesis**, fatty acids and cholesterol biosynthesis and on the reduction of the glucagon-stimulated gluconeogenesis[296]. This reduces the levels of circulating glucose, increases hepatic insulin sensitivity, and reduces hyperinsulinemia associated with insulin resistance. Metformin performs effectively only in the presence of insulin[296]. Most type 2 diabetic patients have three times the normal rate of gluconeogenesis and metformin treatment reduces this by over one third.

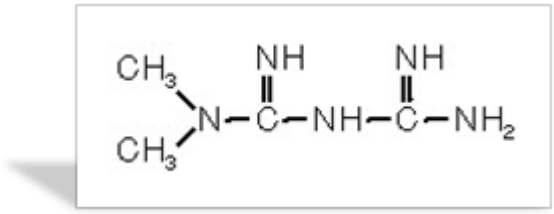


Fig.9 Structure of Metformin.
Adapted from *Bailey et al, NEJM 1996* [296].

Several other actions may contribute to the positive actions of metformin, such as **increased intestinal use of glucose** and **reduced fatty acid oxidation.**

Peripheral tissues, for instance skeletal muscle, increase insulin-mediated glucose uptake due to metformin, predominantly after meals. Metformin activates the adipose tissue AMPK [297]. Therefore it increases the activity of anaerobic glucose metabolic pathways with the formation of lactate and the suppression of lipolysis. This sinks the availability of substrates for gluconeogenesis[296], a process linked to its antilipolytic effect by lowering serum free fatty acids. Metformin **increases therefore the uptake and oxidation of glucose by the adipose tissue and the lipogenesis [298].** Consequently, glycemic control gets enhanced and serum insulin levels diminish.

However, the actions of metformin on peripheral tissues *in vitro* require high concentrations and are slow in onset. **Tissues acutely insensitive to insulin,** like the brain, the renal medulla and the skin, show ineffectiveness to metformin [296].

Table 7. *Pharmacokinetic Aspects of Metformin*

About…	Observations
Bioavailability	- 50-60% - absorbed mainly from the small intestine - estimated absorption half-life 0.9-2.6 hours
Plasma concentration	- 1-2 μg/ml 1-2 hours after an oral dose of 500-1000mg - negligible binding to plasma proteins
Plasma half-life	- 1.5-4.9 hours
Metabolism	- not measurably metabolized
Elimination	- ~ 90% is eliminated in urine in 12 hours - multiexponential pattern involving glomerular filtration and tubular secretion
Tissue distribution	- distributed in most tissues at concentrations similar to those in peripheral plasma - higher concentrations in liver and kidney - highest concentrations in salivary glands and intestinal wall

Table adapted from *Bailey et al, NEJM 1996* [296].

As much as is known, the molecular mechanism by which metformin acts is to inhibit liver cell glucose production by activation of AMP-activated protein kinase (AMPK), a liver enzyme important in insulin signalling, whole body energy balance and metabolism of glucose and fats. Metformin's regulation of AMPK works through liver kinase B1 (LKB1), a tumor suppressor, and activation of AMPK through LKB1 may play a role in inhibiting cell growth. Hence, metformin seems to exert its cell-growth inhibitory effects through two distinct mechanisms: a direct mechanism that inhibits the mTOR pathway and an indirect mechanism depending on insulin levels. For that, metformin use has been observed to decrease cancer incidence [296].

Interestingly, besides pathways dependent from the activation of AMPK, it has been stipulated that metformin increases insulin sensitivity by increasing the affinity of insulin to its receptors and the phosphorylation and tyrosine kinase activity of these receptors *in vivo*, but the actions have been irreproducible *in vitro*. Moreover, metformin increases the cellular translocation of GLUT-1 and GLUT-4 isoforms of **glucose transporters**. *In vitro*, cultured hepatocytes and adipocytes exposed for long periods to high insulin concentrations are prevented from developing insulin resistance by metformin treatment [296].

The decrease in the **fatty acid oxidation** induced by metformin rises up to 10-20% [296], resulting in a following reduction in plasma glucose concentrations by means of the glucose–fatty acid cycle. Metformin affects overall faintly the oxidative metabolism, since it causes a small decline in fatty-acid oxidation and a small augmentation in glucose oxidation, implicating glycogen formation as an important part of the increased glucose disposal.

Another characteristic of metformin is that it **increases basal and postprandial blood lactate** levels at small, but within normal, degrees. The explanation of these ascends needs to be searched between obesity and diabetes, which have the ability to slightly raise blood lactate concentrations. The latter effect is probably due to metformin-induced conversion of glucose to lactate by the **intestinal** mucosa. Continuously, the lactate enters the portal circulation and gets degraded by the liver, where it serves as a gluconeogenic substrate. After a meal, when liver gets immersed in loads of fuel, greater quantities of lactate enter into the systemic circulation. In animals, metformin administration contributes to the conversion “glucose–lactate–glucose” and the overall increase in glucose turnover [296].

5.3 Safety and tolerability

Metformin is being used widely in Europe, Latin America, Canada and the United States for its antidiabetic action, as well as in the rest of the world. Even so, historically its use has been through adverse prohibitions, due to an affected safety profile compared to other biguanides. Being lactic acidosis the most severe reaction to biguanides, as it leads to fatal results in 50% of the cases, phenformin, a member of the family, had to be withdrawn from the market due to its high incidence of lactic acidosis, making the use of metformin considerable of approval in the U.S. [299].

Lactic acidosis is defined as the accumulation of lactate and lowering of the pH in body tissues and blood, occurring when the body regenerates ATP without oxygen (tissue hypoxia) [300,301]. It is rare but a serious effect in metformin therapy, with an incidence of 9.7 episodes per 100,000 patient-years among patients with type 2 diabetes, translated to 3 cases per 100,000 patient-years with metformin therapy [299]. In the vast majority of the cases it occurs because one or more contraindications were overlooked, precipitating the reaction. Moreover, causal consideration of metformin to lactic acidosis has been present in patients with renal insufficiency, and thus at risk for metformin accumulation, or in patients going through hypoxic states, as hypotension with low tissue perfusion, liver disease, alcohol abuse, heart failure and surgical procedures, which increase the risk for developing lactic acidosis [296,299]. The risk of death from lactic acidosis in metformin-treated patients is similar to that of hypoglycemia in sulfonylurea-

treated patients[296]. Metformin-attributable lactic acidosis can be confronted by removal of the drug with hemodialysis [296].

More commonly, adverse effects occurring during metformin therapy are of gastrointestinal nature and are dose dependent. In a lesser extent, around 7% of patients under metformin treatment present reductions in serum vitamin B_{12} and folic acid, probably in relation with impaired gastric absorption of these vitamins. Other events include hypoglycemia episodes, but occur at lower rates similar to those of other antidiabetic drugs [299].

5.4 Metformin and the cardiometabolic risk factors associated with type 2 diabetes and obesity

As suggested by data from clinical trials, metformin, compared with any other oral diabetes agents or placebo, dicreases the cardiovascular mortality and also the cardiovascular morbidity due to its glucose lowering effect [302,303]. Sustained evidence from animal and *in vitro* studies suggests also a beneficial, but glucose-lowering, independent effect of metformin on the CV outcome in type 2 diabetes. This effect has been stipulated to rely initially basically on its insulin sensitizer proprieties.

Nowadays there has been provided evidence about pleiotropic actions of metformin in the reduction of the CV risk. The United Kingdom Prospective Diabetes Study (UKPDS) reported that metformin was associated with a 32% reduction in any diabetes related endpoint ($p<0.002$), a 39% reduction in myocardial infarction ($p<0.01$) and a non-significant 29% fall in microvascular complications [304].

Interestingly, metformin therapy has been also observed to decrease **platelet sensitivity** to aggregating agents [305], most probably due to reduced blood glucose levels. Moreover, there has been an association with increased fibrinolytic activity and small reductions in plasma concentrations of the fibrinolytic inhibitor PAI-1 [296].

Moreover metformin seems to modulate, although discrete, the lipid profile, by decreasing fatty acid oxidation. Patients receiving long-term metformin treatment show a moderate reduction in plasma triglyceride concentrations (10-20%) because of decreased hepatic synthesis of very low density lipoprotein [296].

Very recent reports from *in vitro* experiments suggest that metformin suppresses the metabolic memory of high glucose in diabetic retina by targeting Sirtuin 1 (SIRT1). This fact, although focusing on a microarterial complication in diabetes, raises questions about the importance of this mechanism in macrovascular disease, as SIRT1 exerts an important antiatherogenic role [306,307].

Interestingly, also *in vitro* has been provided sustained evidence on its capacity to improve nitric oxide endothelial dysfunction and prevent nitrosative and oxidative stress under basal conditions, as well as alter exposure of EC to high glucose and lipids [308,309,310,311,312,313]. Metformin also ameliorates proliferation and apoptosis of human coronary artery endothelial cells [314].

From other *in vitro* and animal model experiments, we have evidence that metformin independently reduces carotid arterial wall thickness and improves arterial dilatation in type 2 diabetes and other insulin resistant models [315,316].

Recently has been questioned the role of metformin on the chronic systemic inflammation, an important CV risk factor associated to obesity and type 2 diabetes. In *in vitro*

experiments on an established macroendothelial study model (HUVEC), metformin has been observed to inhibit cytokine-induced NF-κB activation via PI3K-dependent AMPK activation in vascular endothelial cells [317,318,319]. In this sense, some sera studies on the hs-CRP inflammatory marker report the decrease of systemic inflammation in metformin-treated patients [320,321,322]. Contrarily, a recent study in obese children with normal glucose tolerance and the PIOCOMB study on type 2 diabetic patients report that metformin does not improve inflammatory parameters [323,324].

In vivo controversy persists on the beneficial role of metformin on CV outcomes beyond glucose control. In this sense, one recent meta-analysis concludes that treatment with metformin is not associated besides the glucose lowering effects with significant harm or benefit on cardiovascular events [325]. Moreover, other recent meta-analysis of 26 trials on metformin and insulin *versus* insulin alone, reported that there was no evidence ore even a trend towards improved all-cause mortality or cardiovascular mortality in the metformin treated groups [326]. These data strongly indicate that CV disease prognosis is not definitively determined by the amelioration of insulin resistance in the metformin-treated patients and moreover raise the question if really metformin acts or not on the systemic inflammation.

Concerning the inflammation-related CV risk, in obesity and associated type 2 diabetes the driver of the low systemic inflammation is considered the crosstalk between the obese adipose tissue-emerged cytoadipokines and the hepatic response to the complex fatty acid and adipokine aggression [206]. In humans with type 2 diabetes, metformin has been shown to activate AMPK in the adipose tissue and to enhance lipogenesis [297]. *In vitro*, metformin decreases in human adipose tissue, adipocytes and stromal cells the expression of MCP-1 [327]. Recent studies on sera samples reported mostly that metformin does not influence adiponectin levels neither in humans [328] nor in db/db mice[329]. An animal study reported a decrease in chemerin expression (an adipokine known to influence adipocyte differentiation) and alleviation of the ER stress in the visceral adipose tissue of high fat diet-fed rats, when treated with metformin [330], proposing its use in obese, insulin resistant states. Nevertheless, in humans the effect of metformin on the deleterious inflammatory cytoadipokine secretion in obesity-associated type 2 diabetes has not been studied yet.

In order to understand the effect of metformin on the obese adipose source of inflammatory cytokines one aim of the present thesis was to investigate the effect of metformin on the endothelial proinflammatory and prothrombotic response induced by the secretomes of visceral adipose tissue fat pads obtained from obese subjects with established type 2 diabetes under treatment with metformin.

II. Aims

As depicted in the introduction, obesity is a strong predictor for the development of cardiometabolic disease. Type 2 diabetes and atherothrombotic disease are the milestones of the evolution of obesity.

New paradigms are under debate nowadays. On the one hand there is still under debate which is the importance of chronic inflammation associated to obesity in the interplay of hyperinsulinemia and hyperglycemia in atherothrombosis. Moreover, the impact of metformin, the worldwide first-line treatment agent in type 2 diabetes associated to obesity, on the endothelial atherothrombotic dysfunction induced by the obese adipose tissue-secreted cytokines has not been yet investigated.

One the other hand, sustained by the evolution of recent investigation tools, beside cytokines, metabolites are emerging as new endocrine key players in the genesis and evolution of diseases. The changes in the obese adipose tissue metabolism have not been studied yet.

Previous studies in our group[206] have reported that cytokines present in the secretome of simple obese subjects without other cardiometabolic risk than their BMI determine the direct proinflammatory and prothrombotic activation of endothelial cells. On the contrary, secretomes of normo-weight subjects do not injure the endothelium.

Parting from this previously acquired knowledge, in order to advance in the understanding of pathophysiological processes involved in atherothrombosis and type 2 diabetes in obesity, we have proposed the following aims:

1. To investigate the effects of physiological and pathophysiological concentrations of insulin and glucose on the proinflammatory and prothrombotic endothelial injury induced by the cytokines secreted from the visceral obese adipose tissue.

2. To investigate the potential protective effect of metformin on the endothelial inflammatory and prothrombotic injury induced by cytokines originating from the visceral adipose tissue of obese subjects with established type 2 diabetes.

3. To study the impact of obesity on the metabolism of the regional adipose tissue depots, namely visceral and subcutaneous ones, by comparing the metabolite profile of obese visceral and subcutaneous adipose tissue secretomes with similar ones isolated from lean subjects.

NOTE:

Obese patients with established diabetes have been submitted to bariatric surgery only after the initiation of the treatment of diabetes, respectively after the initiation of metformin. For this reason, although it would have been an important goal of our investigations, the study of the secretomes of visceral adipose tissue from obese patients with established diabetes without any treatment was for ethical reasons not possible.

III. Materials and Methods

Experimental model for aims 1 and 2: Justification of choice

In order to accomplish our aims, we employed an established model of macroendothelial injury, respectively the HUVEC (human umbilical vein endothelial cell) model. For that we used primary human endothelial cells deriving from umbilical cords of various donors. This cell type has been utilized extensively in physiological and pharmacological investigations for the study of the regulation of endothelial cell function and the role of the endothelium in the response of the blood vessel wall to various stimuli, between them inflammatory activation pathways of endothelial cells being one of the most used.

1. Cell handling and experimental techniques

Umbilical cords from healthy neonates were maintained in physiological serum at 4°C until use within a maximum of 48 h after birth for EC isolation according to the protocol described in 1.1. HUVECs are in general adherent and grow in a monolayer. They were cultured with appropriate culture medium in a humidified incubator under standard conditions (37°C and 5% CO_2), expanded by tripsinization and media was changed every 48 hours, until reaching confluence at passage 2. Subsequently they were passed to culture plates (TPP) or glass coverslips, depending on the subsequent technique to use after stimulation: respectively for RNA extraction-targeted stimulation, and for immunohistochemical labeling and extracellular matrix reactivity experiments.

RNA was further retrotranscribed and processed for/through real time analysis.

Culture media preparation

MEM 199 supplemented

To obtain the culture medium for the HUVECs, MEM 199 (Medium 199 EARLE, Gibco BRL, Life Technologies) was supplemented with pooled human serum at 20% (previously filtered, 0.2µm), penicillin and streptomycin 5% (P/S, HyClone), mixed softly, and left at 37°C for immediate, or 4°C for future use.

The preparation was all set under sterile conditions.

PBS Dulbeco's and *Hanks' balanced salt solution* (HBSS) were obtained from Gibco.

Additional solutions

The following solutions were prepared under sterile conditions and moreover used filtered through a 0.2µm filter (Millipore).

Gelatin

The gelatin (Type B: from bovine skin, Sigma) is in a concentration of 2% and we diluted it

down to 0.1% in PBS for our experiments.

Collagenase

The collagenase (Collagenase A from *Clostridium histolyticum*, Roche) arrives lyophilized. For use, we dissolved 0.5mg per ml (HBSS, MEM 199 or PBS). Each cord needs 15ml of collagenase solution.

Trypsin-EDTA

The trypsin solution arrives concentrated (10x, Gibco) and was therefore diluted at 1x in HBSS for use.

Glutaraldehyde

The glutaraldehyde solution (Merck) arrives in a concentration of 25%, and we diluted it in PBS at 0.5%.

Preparation of culture flasks with gelatin

1. Prepare gelatin solution (see above).
2. Filter it.
3. Add 5ml per small flask, or 8ml in case of a medium flask.
4. Keep flasks at 37°C for 30min until 1h.
5. Aspirate the remaining gelatin.

1.1 Isolation of Human Umbilical Vein Endothelial Cells

Following procedure was performed in order to separate the ECs from the umbilical cord:

1. Place the umbilical cord on the silver paper previously sprayed with alcohol.
2. Spray the cord with alcohol and clean away the blood with a paper.
3. Cut off one extreme of the cord with a scalpel.
4. Dilate the cordon's vein with the help of the forceps.
5. Bind the cannula and the cord with a thread, and check that the cannula is well fixed.
6. Clean the interior of the vein with 20ml of PBS (Gibco), using a syringe.
7. Cut off the other extreme of the cord and bind the cannula.
8. Introduce in this extreme of the vein 15ml of collagenase with a syringe, leaving the interior of the vein filled with the collagenase solution.
9. Wrap the cord with a silver paper and incubate it at 37°C during 15min.
10. Perform a soft massage to the cord for 2min.
11. Remove the whole solution from the vein's interior with the help of a syringe and pass it to a sterile falcon tube. Add 20ml PBS with a new syringe.
12. Pass the solution from one syringe to another through the cord, various times.
13. Collect the solution in a sterile falcon.
14. Centrifuge 5min at 1,100 rpm.
15. Aspirate the overflow leaving only a few microliters of it on the pellet.

16. Resuspend the cellular pellet in 4ml supplemented culture medium.
17. Transfer the suspension to a small flask, previously treated with gelatin.
18. Wash the perfusion tube with 3ml of medium and transfer it to the small flasks.
19. Leave the flasks in the CO_2 incubator for culture growth, with the lid slightly open for gas exchange.
20. Carry on changing the culture medium until the cells reach confluence. Keep them for maximum 7 days in the flask. If by the end of this time they haven't reached 80% confluence, discard the cells. Slow growth indicates that something is wrong with them and we'd better not use them.

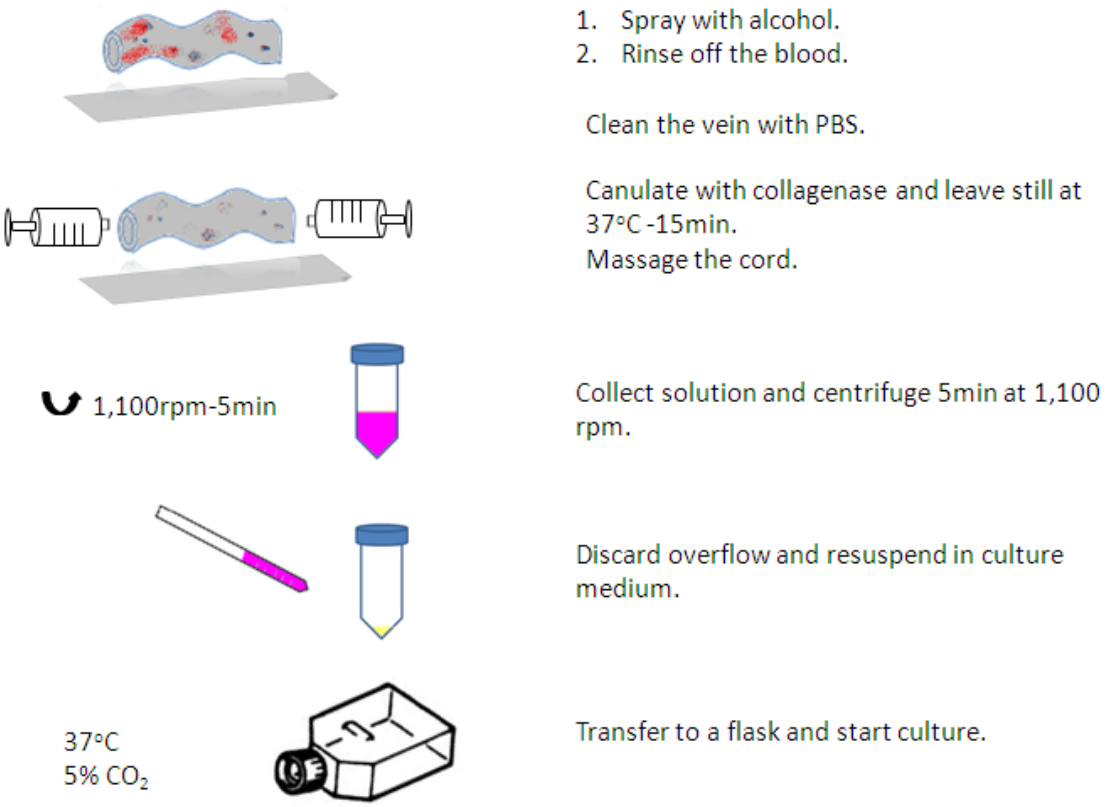


Fig.10 Isolation of endothelial cells from an umbilical cord. Gross procedure.

Tripsinization

This method was performed maximum 7 days after having proceeded the umbilical cord, depending on each culture, and under sterile conditions. After transition of the first 48h in culture, cells were observed in the microscope for reaching confluence and in positive case tripsinized as in the next protocol:

1. Aspirate medium (overflow).
2. Add 4ml HBSS (Gibco), mix softly and aspirate.

3. Add 1.5ml Trypsin (Trypsin-EDTA 10x, Gibco).
4. Leave small flask for 1-2min at 37°C (lid completely closed).
5. Shake gently the flask in order to detach the monolayer and observe the cells in the microscope.
6. Add to the cell suspension with the trypsin 4.5ml of culture medium and mix for breaking the cell aggregates.
7. Transfer the cell suspension to a medium flask previously treated with gelatin for 1h and subsequently removed from the flask (add cell solution against the wall of the flask).
8. Wash the small flask with 6ml culture medium and transfer them to the medium flask.
9. Leave the flasks in the CO_2 incubator for additional 48 hours.
10. Keep changing the culture medium until the cells reach confluence.

1.2 Planting and Culture of the Human Umbilical Vein Endothelial Cells

1.2.1 Planting in 6-well plates

Plates Preparation

1. Add 1ml of gelatin 0.1% to each well and keep it for an hour.
2. Aspirate and add 2.5ml culture medium per well.

Procedure

1. Aspirate the culture medium from the medium flask.
2. Wash with 5ml HBSS, mix softly and aspirate.
3. Add 2ml trypsin and leave 1-2min at 37°C.
4. Hit gently the flask on the sides in order to detach the cell monolayer.
5. Add on the cell suspension 4ml of culture medium and mix.
6. Add 6ml culture medium and mix the total 12ml.
7. Transfer 0.5ml cell suspension to the sterile culture plates containing 2.5ml of culture medium per well.
8. Leave plates in the incubator.
9. Maintain the cells changing the medium (3ml) every two days.

When cells obtained from one umbilical cord were not enough to cover all our conditions, we used 12-well plates for the fulfillment of the experiment, and during cell planting was utilized the same volume of cell suspension, but half the volume of culture medium per well. That results in reaching quicker the desired confluence.

The use of those plates was destined only for RNA extraction.

1.2.2 Planting in 6-well plates with coverslips

1. Place a coverslip in each well and add alcohol 70% during 15min.

2. Aspirate the alcohol and wash 3 times with PBS.
3. Incubate the coverslips with 1ml gelatin **1%** for 30min.
4. Aspirate the gelatin and wash one time with PBS (try not to add it directly on the coverslip).
5. Incubate coverslips with 1ml glutaraldehyde for 30min.
6. Aspirate the glutaraldehyde and wash 3 times with PBS.
7. In new sterile plates add 2.5ml culture medium/well.
8. Transfer the coverslips to the new plates, with the treated surface looking up.

2. Secretomes of the human adipose tissue

2.1 Study participants

For reasons of simplification we describe once all included study groups of this thesis, independently of the aim inclusion.

To respond to aim 1 and 2 we performed a comparative analysis between patients of group 1 (Ob) and 2 (DMO).

Participants were recruited in the surgery unit of our hospital. Visceral adipose tissue samples in order to obtain the secretomes were obtained during laparoscopic surgery. Therefore, a total of n=16 morbid obese subjects (Ob and DMO, BMI=42-46 kg/m^2) from the Obesity Unit of the Hospital Clínic about to undergo gastric bypass surgery were included in the experiments. Six nonobese age- and sex-matched subjects (NOb, BMI=24-26 kg/m^2) undergoing elective procedures of digestive surgery to correct benign conditions (vesicular lithiasis and eventrations) served as controls. The study was approved by the hospital's Ethics Committee. Written informed consent was obtained from all participants.

<u>The study groups included:</u>

1. Obese participants (Ob) without other cardiometabolic risk factors than their own BMI (n=8); diabetes was excluded after negative-resulting standard oral glucose tolerance test (OGTT) subsequent to oral administration of 75 g glucose [55].
2. Obese subjects with established type 2 diabetes (DMO) on treatment with oral antidiabetic agents, namely metformin 850mg x 2 times/day for at least 1 year and maximum 3 years, at maximum 5 years of diagnosis of diabetes (n=8) [55].
3. Nonobese subjects (NOb, n=6) served as controls.

<u>Exclusion criteria:</u>

1. Subjects receiving hormones (i.e. glucocorticoids, oral contraceptives, or other substitutive treatment), non-steroid anti-inflammatory drugs, immunosuppressive drugs, medications known to affect fat mass metabolism (i.e. thiazolidinediones, modulators of adrenergic receptors) were excluded.
2. Insulin treated patients and patients with history of hypoglycemias were excluded

from the type 2 DM group.

3. Subjects with major chronic illnesses or recent antecedents of neoplasias were also excluded.

Biochemical phenotype of the study participants:

Main biochemical parameters were measured in serum after an overnight fast in the hormonal and core unit of the central laboratory of our hospital, employing standardized methods for each parameter. Insulin resistance was estimated by calculating HOMA-IR index, with a threshold of 2.9, the reference value for the control population from our hospital.

Clinical data from the study participants are resumed in Table 8.

Table 8. *Clinical and metabolic parameters of the studied subjects*

	1) Obese (Ob) Subjects n=8	**2) DM Obese (DMO) Subjects n= 8**	**3) Nonobese (NOb) Subjects n=6**
Age (years)	41.4 ± 10	45.0 ± 4.2	44.4 ± 14
Sex (men/women)	2/6	1/7	2/4
BMI (kg/m^2)	44.1 ± 2.2	47.8 ± 2.6	24.6 ± 2.3
Waist circumference (cm)	126.3 ± 4.02	132 ± 3.01	82.9 ± 2.1
SBP/DBP (mmHg)	126.1/75.4 ± 2.7/1.8	135.2/75 ± 1.2/1.4	116.1/70.6 ± 1.5/1.3
Fasting Glucose (mmol/l)	5.5 ± 0.2	6.85 ± 2.65	4.8 ± 0.5
2h–OGTT plasma glucose (mmol/l)	7.5 ± 1.5	NA	-
Total Cholesterol (mmol/l)	5.0 ± 1.1	4.8 ± 0.8	4.3 ± 1.3
HDL/LDL (mmol/l)	1.3/3.3 ± 0.1/0.7		1.5/3 ± 0.1/0.5
Triglycerides (mmol/l)	1.5 ± 0.2	1.7 ± 0.3	1 ± 0.3
Leukocytes (x 10^9/l)	8.2 ± 1.7	9.01 ± 1.42	6.3 ± 0.8
High Sensitive-CRP (mg/l)	13.2 ± 4.5	16.2 ± 7.5	(<5)*

HbA1c (NGPS/DCC)	5.2 ± 0.3	7.5 ± 0.05	(4.0 - 5.5)*
Insulin (pmol/l)	201.9 ± 77.2	156.67 ± 57.6	(15.3 - 98.6) *
HOMA-IR	5.7 ± 1.9	NA	(<2.93)*
Time from diagnosis of diabetes	NA	1-3 years	NA
Time of treatment with metformin	NA	≥1year	NA
Dyslipidemia on statin/fibrates treatment	1/8	6/8	-
Dyslipidemia without treatment	1	1	
Hypertension on diuretic treatment	2/8	4/8	-

Data are presented as mean ± standard error of the mean (SEM) in international units; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: C-reactive protein; OGTT: oral 75g glucose tolerance test; HOMA-IR: homa insulin resistance, NA: not applicable. *Laboratory age- and sex-matched normal parameter values.

Note: Previous comparative study on HUVECs regarding inflammatory endothelial activation between the simple obese and the nonobese group has been recently published from our group.
In the present thesis in order to respond to the proposed aims we comparatively investigated:

a) The inflammatory activation of endothelial cells between the visceral obese adipose tissue isolated from participants of group 1 and 2.
b) The metabolite compounds of groups 1 and 3.

Further comparison of metabolites between groups 1 and 2 remains the goal for future investigation.

2.2 Adipose tissue samples

Visceral (VIS, omental) adipose tissue biopsies (fat pads, FP) from all obese and nonobese study participants mentioned above and subcutaneous (SC, periumbilical) adipose tissue biopsies (AT) from the morbid obese and nonobese individuals were obtained during the laparoscopic surgery in the surgical unit of our hospital. The samples were collected in non-fetal medium provided by us (DMEM, Sigma) and placed on ice, immediately transported to the laboratory and further processed.

2.3 Preparation of the secretome

The following procedures were performed under sterile conditions provided by laminar airflow, according to the protocol of Fain and Rodbell [227,331] with minor modifications.

The medium used for the incubation of the fat pads in order to obtain the conditioned medium, the secretome, was the MEM 199 with a preparation as described underneath.

To note, the incubation media do not include fetal serum or other type of growth factors.

Medium for adipose tissue incubation

MEM 199 (Sigma) arrives in powder and therefore has to be dissolved and adjusted for pH before use. Supplements can be added prior to filtration or introduced aseptically to the sterile medium; we preferred to follow the second option.

Preparation Instructions (http://www.sigmaaldrich.com):

1. Measure out 90% of final required volume (=1l) of distilled water. Water temperature should be around 15-20^{o}C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved without heating it up.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 2.2g sodium bicarbonate or 29.3 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH 7.4 since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize by filtration using a membrane filter of 0.2µm porosity.
8. Aseptically dispense medium into sterile container.

The above medium was stored for longer periods at -20^{o}C and at 4^{o}C for shorter ones. It was supplemented with 1% P/S and 1% gentamycin (G, Sigma, 50 mg/mL in deionized water), and kept at 37^{o}C before addition to the fat pads.

Acquisition of the secretome

Each experimental replication included tissue samples from distinct individuals. Once in the laboratory and under the laminar airflow, the visceral adipose tissue was washed from the blood with PBS, and the burnt parts from the laser operation as well as the coagulations were carefully removed with scissors and forceps, previously sterilized. Next, it was placed in a Petri dish and brought to the balance in order to record its weight. Back to the laboratory hood, the tissue was cut into small pieces (around 10mg) so as to have as much surface as possible in contact with the medium. The non-fetal medium (P/S+G) was added to the tissue in an analogy 5ml medium per 1g of FP. The dish was subsequently positioned in the incubator, at 37^{o}C and 5% CO_2, for 2h. After transition of this time, the medium was cautiously separated from the FP pieces by aspiration, discarded and replaced by the same quantity. Incubation carried on for further 24h under the same conditions, in order to obtain the visceral obese (Ob-VIS FP) and nonobese FP (Nob-VIS FP)

secretomes.

At the end of the 24h, the secretome was collected in a syringe, sterile filtered through a filter membrane of 0.2µm and stored at -80°C.

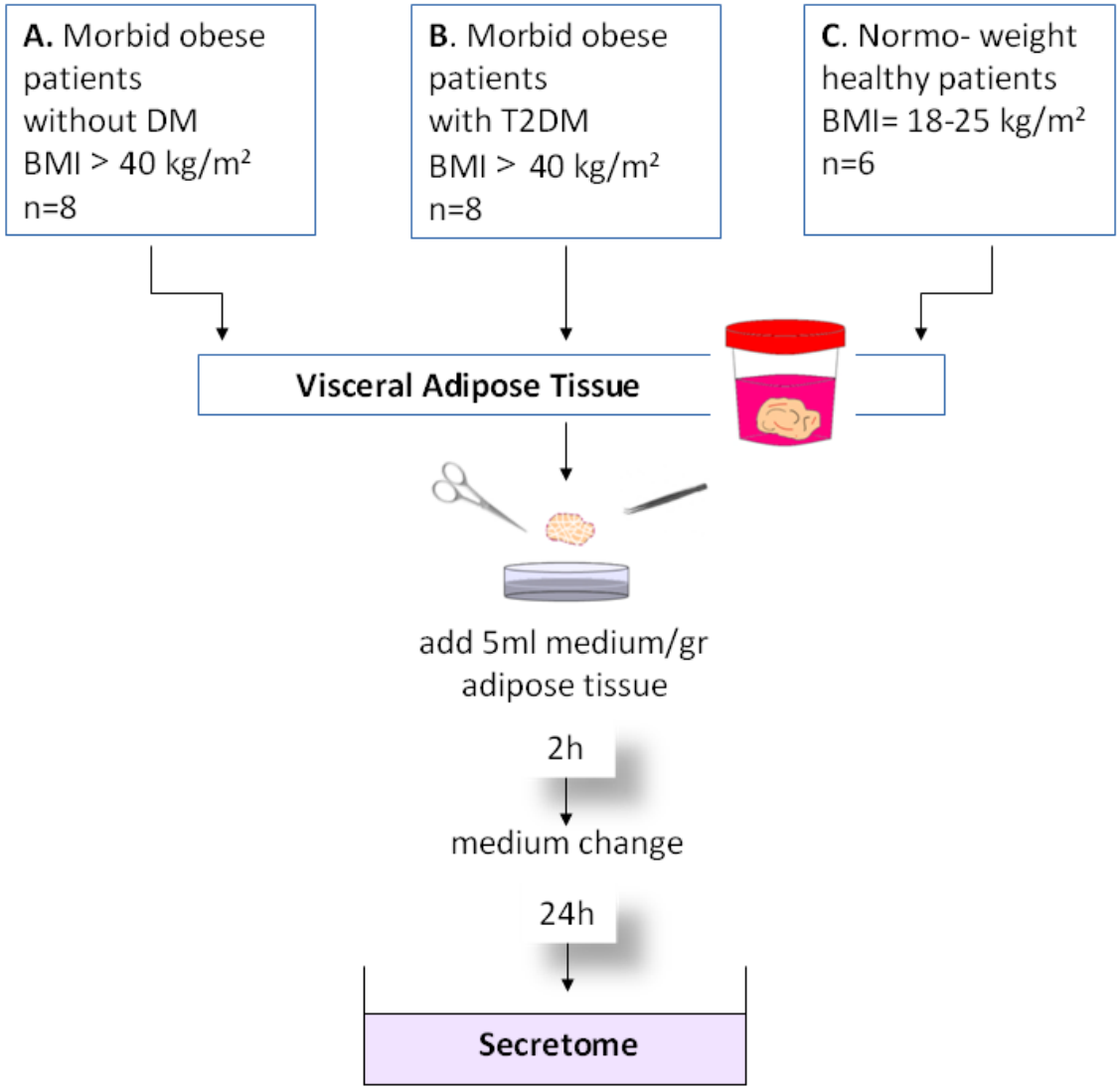


Fig.11 Obtaining the secretome. Gross procedure.

3. Experimental design for the *in vitro* studies

HUVECs, obtained and cultured as described above, were exposed to different secretomes on one hand, and control medium on the other. As control medium (Ctr) is referred to the standard culture medium for HUVECS, respectively the MEM 199 supplemented at 20% with human serum obtained from a pool of healthy donors.

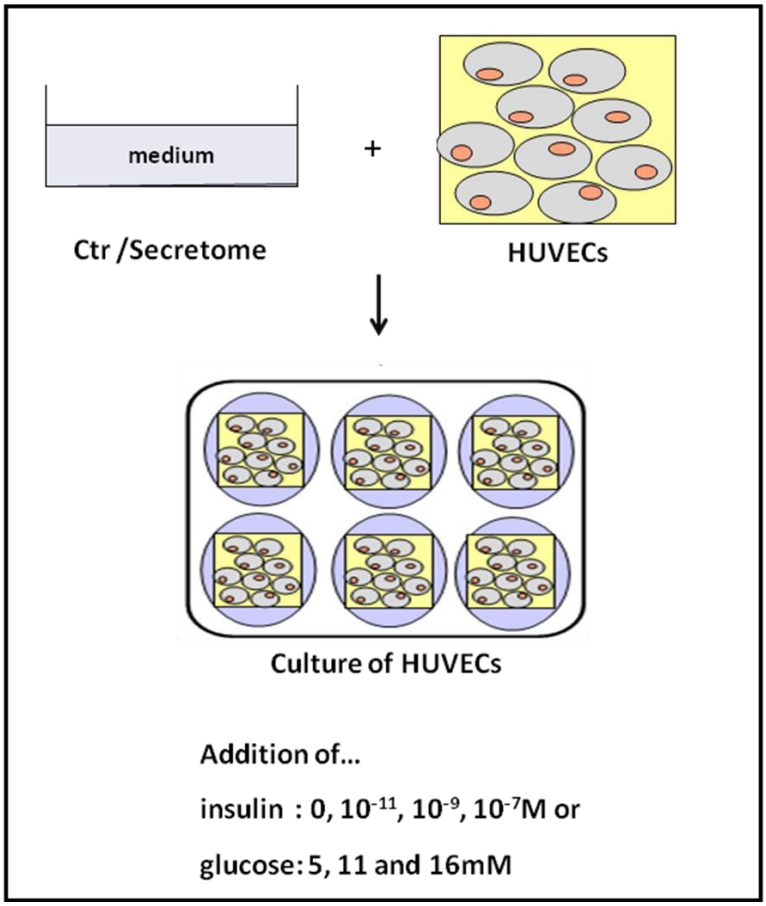


Fig.12 Basic experimental design.

To the secretomes and the control medium were furthermore added progressively increasing concentrations of D-glucose: 5mM, 11mM and 16mM respectively (Merck) or insulin: 0, 10^{-11}, 10^{-9} and 10^{-7}M respectively (Sigma), as illustrated in Fig.12.

Genetic changes and receptor expression were assessed after 24h stimulation, whereas protein expression on the extracellular matrix and its reactivity required a coculture of endothelial cells with the secretomes for 7 days.

The stimulations were made in order to assess changes in the expression and synthesis of (a) the cellular adhesion receptors VCAM-1 and ICAM-1 in endothelial cells, (b) the von Willebrand factor (VWF) and tissue factor (TF) expression in the endothelial cells and synthesis in the subendothelial extracellular matrix (ECM), (c) the expression of the transcription factor NF-κB and (d) the platelet adhesion to the ECM mentioned above.

Genetic changes and expression of receptors VCAM-1, ICAM-1 and VWF, TF and NF-κB were assessed after 24h stimulation, whereas adhesion of platelets to the ECM matrix by perfusion studies and protein synthesis of VWF and TF on ECM required an exposure to the experimental conditions of 7 days.

For the gene expression level we employed Real-Time PCR and for the quantification of the protein synthesis immunocytochemistry studies. The reactivity of the ECM was investigated through perfusion studies. Methods will be detailed in the following paragraphs.

Insulin facts and dilutions

Kinetics of the experiments and insulin concentration studies have been selected with regard to data previously presented in the literature. Insulin concentrations ranging from 10^{-11} to 10^{-7}M are spanning from 1.7mU/l to $1.7x10^{4}$mU/l, covering pharmacological to pathophysiological levels of insulinemia. Moreover, those refer to far overwhelming insulin concentrations in fasting and postprandial states of obese hyperinsulinemia in the presence of insulin resistance [332].

Evidence of endothelial cell insulin receptors has been recognized long before by Lee *et al*[333] and Bar *et al* [334].

Human insulin solution was obtained from Sigma, at 1.7mM. The desired concentrations for our experiments were 10^{-11}, 10^{-9} and 10^{-7}M.

Starting from the given 1.7mM= 1.7 x 10-^{3}M, a dilution of 17 times (1/17) was made in PBS and filtered under the hood through a filter membrane (0.2μm), in order to reach the concentration 10^{-4}M. That was subsequently diluted 100x, arriving to 10^{-6}M, and further 100x, reaching 10^{-8}M.

Those three dilutions served as stock solutions from which was obtained the planned insulin concentration, by picking out the corresponding 1/1000 of the medium volume added to the cells. Since for each stimulation was enough 1ml of secretome per well of a 6-well plate, 1μl from every insulin stock was added to the regarding wells.

Scheme:
from 10^{-4}M → 10^{-7}M
from 10^{-6}M → 10^{-9}M
from 10^{-8}M → 10^{-11}M

Glucose stock preparation and addition

Glucose (Merck) was dissolved in as much PBS as necessary in order to prepare a stock solution of 1M, and sterile filtered through a filter (0.2μm).

It was to expect that even if the medium destined for secretome acquisition had a glucose concentration of 5mM, the adipocytes, after 24h of incubation, would consume glucose from it. Therefore, in order to perform the experiments under the same starting glucose concentration, the latter was measured before addition to the cells with a glucometer (both glucometer and stripes from Arkray Factory, Inc.) and the equation below was used for the calculation of the necessary glucose to be added:

$$\mathbf{C_1 \times V_1 = C_2 \times V_2}$$

C_1 = final glucose concentration in the secretome/control medium
V_1 = final secretome volume in each well (1ml)
C_2 = glucose stock concentration
V_2 = glucose stock volume to calculate in order to be added

Starting from the fact that C_1 had to be from our experimental conditions 5mM, 11mM or 16mM (corresponding to 90, 198 and 288 mg/dl respectively), and continuing with the consumption of glucose from the adipocytes, C_1 was calculated by deduction of the measured

glucose from the desired concentration, i.e. C_1 = (16mM – obtained measurement).

The glucometer gives values in a scale between 10 and 600mg/dl (both included). Our samples were not out of this range, but to ease our work they had to be calculated in the mM scale by division of the glucometer's value by 18 (number obtained after simplification of the necessary calculations and taking on account the molecular weight of glucose).

4. RNA

RNA purification and manipulation requires certain precautions to be taken on account. The principal reason of RNA degradation is contamination from ribonucleases (RNases), enzymes very active and stable, which can generate important problems in RNA integrity. For that, all objects used such as gloves, pipettes, surface and corresponding material, has to be previously cleaned with RNaseZAP (Sigma) in order to ensure an RNase free environment. Moreover, the use of gloves is very important so as to avoid direct contact with skin, the main contamination source.

4.1 RNA extraction

Various methods have been developed for the extraction of RNA; the one that was used in this thesis was with TRIzol® (Invitrogen). The method consists in cell disruption and components dissolves, before separation into aqueous and organic phase, acquisition of the RNA in the aqueous phase and subsequent precipitation.

Protocol

Homogenization

1. Aspirate culture medium from the plates and wash cells once with PBS.
2. Add appropriate TRIzol volume to the plate. The volume does not depend on the number of cells, but on the area of culture (1ml per 10cm^2).
3. Mix with the pipette various times.

Phase separation

4. Incubate samples for 5 min at room temperature and transfer them into eppendorfs.
5. Add 0.2ml of chloroform per 1ml of TRIzol in each eppendorf.
6. Shake tubes vigorously by hand for a few seconds; then leave them still at room temperature for 2-3min.
7. Centrifuge at 12,000g for 15min at 4^{o}C.

RNA precipitation

8. Collect the aqueous phase to a fresh tube.
9. Add 0.5ml of isopropyl alcohol per 1ml of TRIzol used initially.
10. Incubate samples at room temperature for 10min.
11. Centrifuge at 12,000g for 10min at 4^{o}C.

RNA wash

12. Remove the supernatant.
13. Wash the RNA pellet once with 75% ethanol, adding at least 1ml of 75% ethanol per 1ml of TRIzol. Vortex.
14. Centrifuge at 7,500g for 5min at 4°C.

Redissolve the RNA

15. Remove the supernatant.
16. Dry the RNA pellet (air-dry).
17. Dissolve RNA in RNase-free water, pipetting few times.

In those experiments the RNA was dissolved in 8µl of RNase-free water.

4.2 RNA quantification

RNA concentration was determined by Nanodrop (Thermo Scientific), analyzing the RNA absorbance of 1µl of sample at 260nm. The purity of the sample was observed through the ratio obtained by the sample's absorbances at 260 and 280nm (Abs_{260}/Abs_{280}). Ratios greater than 1.8 indicate optimal RNA quality.

4.3 Retrotranscription (RT)

The obtained RNA was used to synthesize first-strand DNA (cDNA), using the enzyme SuperScript™ III (SS III, Invitrogen).

Protocol

- To a PCR-reaction tube add:
  Random primers (3µg/µl)……..0.5µl
  RNA……0.15-1µg
  H_2O (RNase free)……..up to 10µl
- Incubate at 70°C for 10min.
- Chill on ice.
- Add to each sample:
  H_2O (RNase free)……..1.5µl
  First strand buffer (5x).....4µl
  DTT (0.1M)……2µl
  dNTP mix (10mM)……1µl
  RNase OUT (40U/µl)……0.5µl
  SS III (200U/µl)……1µl
- Leave at room temperature for 10min.
- Incubate in a thermal cycler:
  42 °C ……. 15min
  50 °C ……. 90min

55 °C ……. 15min
70 °C ……. 15min
4 °C ……. ∞

- Remove samples from the thermal cycler and store at -20 °C until further use.

4.4 Primers design and validation

The primers used in Real-Time PCR analysis (Table 9) were designed with the program Primer3 Input (v. 0.4.0), having previously obtained the related mRNA sequence in the webpage of NCBI (*http://www.ncbi.nlm.nih.gov*).

Table 9. *Primer sequences used*

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
VCAM-1	CAA ACA AAG GCA GAG TAC GCA A	GCT GAC CAA GAC GGT TGT ATC TC
ICAM-1	AGC CAG GAG ACA CTG CAG ACA	TGG CTT CGT CAG AAT CAC GTT
VWF	ACA TAA CAG CAA GAC AGT CCG GA	GCT GCG GCT ATC TCC AAG G
TF	GCC AGG AGA AAG GGG AAT	CAG TGC AAT ATA GCA TTT GCA CTA GC
NF-κB	TCC ACA AGG CAG CAA ATA GAC GAG	AAG CTG AGT TTG CGG AAG GAT GT
Actin	CCC CCA TGC CAT CCT GCG TCT G	CTC GGC CGT GGT GGT GAA GC

After reception, they were validated with known amplificated samples on an agarose gel 1.5%, by comparing the resulting band with the expected amplicon size, and by ensuring that negative cDNA samples don't present bands. Additionally, primers were tested for the presence of single-peaked-melting curves (see paragraph 4.5 for more details).

4.5 Gene expression analysis by Real-Time PCR

Whereas in a conventional PCR can be detected the number of copies generated during the reaction, a Real-Time PCR, or quantitative PCR, enables data collection throughout the amplification cycles, rather than at the end of the reaction, with the use of fluorochromes.

In this thesis we have used the dye SYBR Green, which emits fluorescence when intercalated in the DNA helix. As the PCR reaction increases in amplicons, so does the fluorescence, and the PCR products are quantified after each completed PCR cycle. The cycle at which the fluorescence exceeds a detection threshold, the Ct (threshold cycle) correlates to the number of target cDNA molecules present in the added cDNA. Therefore, by comparison to a

calibration curve, it is possible to quantify in absolute amounts the number of target molecules in added cDNA samples.

The MESA GREEN qPCR MasterMix applied (Eurogentec) combines the fluorochrome SYBR® Green I with stabilizers, the DNA polymerase Meteor *Taq*, dNTPs including dUTP, $MgCl_2$ and an appropriate reaction buffer. Real-Time PCRs were performed on Roche's Light Cycler® 480 II.

Protocol

- Prepare appropriate cDNA dilutions, so as to have a final quantity of 15ng/sample.
- To each well of a 96-well plate, add:
  SYBR Green Mix……..10µl
  Primers mix (10µM).....1µl
  cDNA……1-3µl
  dH_2O……..up to 20µl
- Centrifuge plate briefly so as for the content to descend.
- Place plate into the thermal cycler under following conditions:

Preincubation	95 °C ……. 5min
Amplification	95 °C ……. 15s
	60 °C ……. 1min
Melting curve	95 °C ……. 5s
	65 °C ……. 1min
	97 °C ……. instantly
Cooling	40 °C …….30s

At the end of each amplification cycle is created a melting curve for every sample, proving that the amplified product was unique. If this is true, the entire DNA from a sample gets separated at the same temperature and we obtain only one peak in the melting curve. The temperature where the two DNA strands are separated is detectable because SYBR Green does not emit fluorescence any more.

Quantification of results was performed by comparing the expression of our genes of interest with the expression of a control gene, that is, a gene being expressed alike in the variety of samples. Normalization of the results was achieved by calculating the ratio of the value of the studied gene in respect to the value of the control gene (ΔΔCt). Here, β-actin, the gene that codifies for actin, a multi-function protein present in all eukaryotic cells, was playing the role of the house-keeping gene (Act).

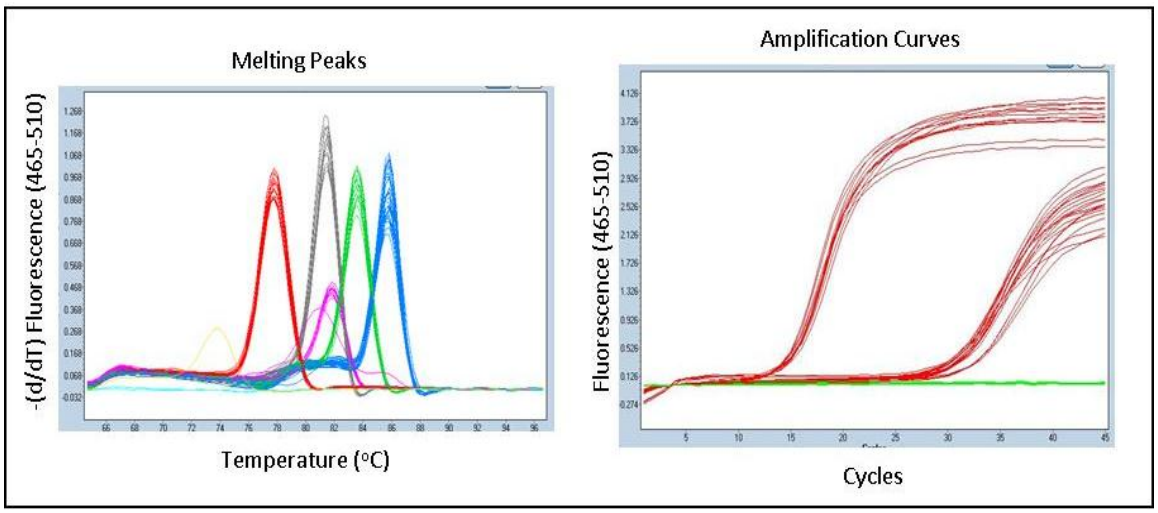


Fig.13 Melting curves (left) and amplification plots (right) of a Real-Time PCR.

5. Generation of the extracellular matrix

In some experiments we evaluated the composition and reactivity of the ECMs. For this, EC monolayers grown on glass coverslips were treated with a 3% solution of ethyleneglycoltetraacetic acid (EGTA) to extract the cells and maintain the ECM on the coverslip as followed:

1. Prepare the EGTA solution, 3% in Hank's balanced salt solution (HBSS). Adjust the pH to 7.4.
2. Wash the monolayers of cells with HBSS.
3. Add 3ml EGTA solution to each well, and apply mechanic action with a Pasteur pipette every 15min for 1 hour, leaving the plates at 37°C.
4. Change the EGTA after the hour and continue the same way for another half an hour. Be careful not to go too far with the pressure and detach the matrix besides the cells, too.
5. Aspirate the EGTA and leave the samples in HBSS until use in immunofluorescence or perfusion, the very same day.

6. Immunohistochemistry

6.1 Immunogold Labeling (IG) and Silver Enhancement

To evaluate the protein synthesis we performed the below described immunohistochemistry techniques. Glass coverslips coated with ECs and prepared as described in paragraph 1.2.2 were incubated with control medium, or secretomes only, or secretomes plus the highest insulin concentration, or secretomes plus the highest glucose concentration, for 24h. In continuation they were fixed to detect VCAM-1 and ICAM-1 adhesion receptor expression on ECs.

a) Cell fixation

This step was performed with paraformaldehyde (PFA) at 4%, diluted in PBS and filtered (membrane 0.2µm), so as to avoid the contact of possible formed crystals with the coverslips and thereby experimental background. Our PFA (Merck) stock solution is prepared at 8% and stored at -20°C.

Procedure

1. Remove the culture medium from the cells and add MEM 199, previously tempered at 37°C. Mix softly.
2. Remove the medium and add a tempered PFA solution 2%, prepared in MEM 199. Leave the plate for 10min at 4°C.
3. Remove the solution and add PFA at 4%, diluted in PBS. Place the plate at 4°C for at least 10min and maximum for one week.
4. With the aim of neutralizing the aldehyde groups of the PFA, add glycine (BioRad) at 1% (diluted in PBS and filtered 0.2µm), and leave it for 10min with soft agitation.

b) Cell wash

Wash the wells with PBS, 3 times of 5min each, and continuous agitation.

c) Antibody incubation

1. To avoid unspecific binding in the regions of our interest, after the last PBS wash incubate in continuous agitation for 15min with albumin (Sigma) solution at 1% in distilled water, filtered before addition.
2. In 30ml of PBS add some drops (around 10) of the albumin solution and mix well. From this new solution is going to be prepared the antibody's dilution.
3. Perform the antibody incubation for 1 hour. Monoclonal antibodies against VCAM-1 (Chemicon International) and ICAM-1 (Millipore) were diluted 1/100 in the solution prepared in step 2. Incubation in a humid environment is necessary, for that we prepared a petri dish of greater diameter (150mm) wrapped up in silver paper, in which we have placed humid filter paper and on top parafilm. On the parafilm we pipette 100µl of antibody solution and place the coverslips, the cell coating in contact with the drop. We close the petri dish and leave it still for the required time.
4. Remove the excess of antibody by washing 3 times with PBS-BSA for 5min, coverslips being placed back to the plate wells and coated surface looking up.

d) Incubation with colloid gold (Amersham)

1. Incubate with colloid gold-conjugated goat anti-mouse IgG (dilution 1/200 in PBS-BSA) for 1 hour, in the same system mentioned in paragraph c). At this point take out the silver staining from the fridge, in order for it to reach room temperature.
2. Wash 3 times with PBS in constant agitation, each 5min, to eliminate gold particles not bound to the primary antibody.

e) Intensification with silver staining

1. Wash 3 times with distilled water for 5min in constant agitation.
2. Prepare the intense silver enhancement reagent (Amersham), mixing 1:1 the intensifier

and the initiator. Treat samples in the same manner as for the primary antibody and the gold. Leave the incubation for 5 to 20min, checking out regularly for the intensity reached. The solution should not get a very dark color, since this results in increased background. Silver enhancement helps to visualize gold particles bound to the antigen.

3. Wash 3 times with distilled water for 5min in constant agitation.

f) May-Gruenewald-Giemsa staining

1. Discard the water from the wells.
2. Add 2ml May-Gruenewald staining, filtered with the help of a humid filter paper, for 1.5min.
3. Wash one time with tap water.
4. Add 2ml Giemsa for 7-10min, diluted 1/5 in distilled water and filtered through a humid filter paper.
5. Rinse off with tap water until color fades.

g) Mounting

1. Dry the coverslips overnight, coated part facing up.
2. Stick them on an objective holder, coated surface facing down and in touch with the glue (DPX, Surgipath).
3. Let them dry overnight at room temperature.
4. Samples can be stored at room temperature.

6.2 Immunofluorescence (IF)

Glass coverslips coated with ECs and prepared as described in paragraph 1.2.2 were stimulated with usual culture media for HUVEC (20% human serum), or culture medium (MEM 199) containing 10% pooled human sera and 10% secretomes, alone or with highest insulin or glucose concentrations, for 7 days.

The protocol of the IF is similar to that of the IG. Since this technique was used to evaluate protein expression in the ECM, here the coated coverslips were processed without cells. Nevertheless, the ECM has to be fixed as well, following basically the same protocol described in 6.1. After PFA 4%, cells can be stored in PBS at 4^{o}C. Subsequent steps including washes, inactivation of aldehyde groups with glycine and BSA blocking at 1% and incubations with antibodies continue identical, as well as the regarding times.

The primary antibodies used here were the human Tissue Factor (American Diagnostics) and the human von Willebrand Factor (Dako), in dilutions of 1/100 and 1/1000, respectively. The secondary antibodies were correspondingly anti-mouse (Alexa 555) and goat anti-rabbit (Alexa 488), both from Invitrogen, in the concentration of 2µg/µl.

Attention has to be paid to the manipulation of the samples once the secondary, fluorescence emitting antibody is in use. For maximum performance, samples have to be protected from light and kept covered.

Cell staining is not necessary, because theoretically the cells have been detached and what remains is only the ECM. Nevertheless, nuclei staining is achieved with DAPI (300nM) 1-5min under darkness.

Coverslips have to be left drying before their adhesion (ProLong®, Invitrogen) on objective holders and afterwards being stored at 4°C.

See Fig.14 for a general overview of the technique.

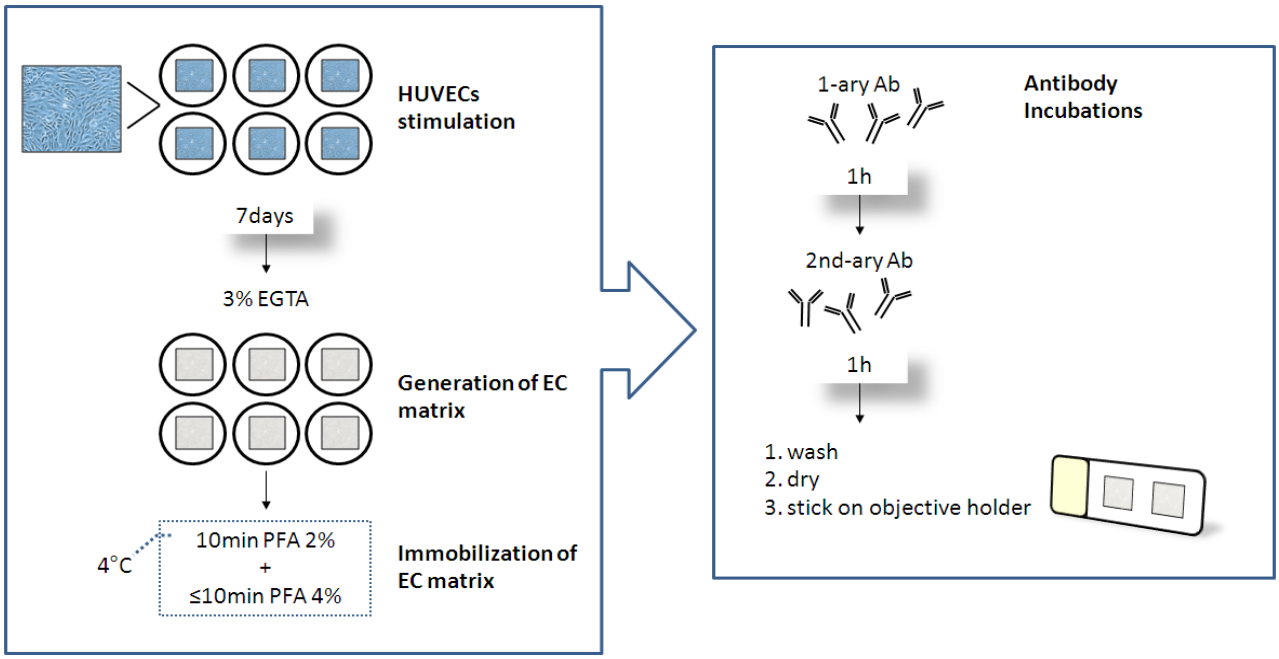


Fig.14 Immunofluorescence steps. Gross procedure. Ab=antibody, 1-ary=primary, 2nd-ary=secondary. For the rest of the abbreviations, see text. HUVEC picture by PromoCell.

7. Platelet adhesion on the ECM: perfusion studies with whole blood

Glass coverslips coated with ECs were prepared as described in paragraph 1.2.2 and cultured as in the experiments of IF, for 7 days. The ECM was acquired with EGTA 3% as in paragraph 6, but was not fixed yet, since the objective was to evaluate platelet adhesion on ECMs.

In the meanwhile, 100ml of blood were extracted from healthy, normoweight donors in the Haemotherapy - Haemostasis unit of our hospital.

Perfusion studies were carried out in a parallel-plate perfusion chamber at a shear rate of $800s^{-1}$ for 5 minutes. For each perfusion, two coverslips were inserted in separate receptacles of a parallel-plate perfusion chamber and perfused with 25ml-aliquots of whole blood anticoagulated with citrate (100mM sodium citrate, 16mM citric acid, 18mM sodium hydrogen phosphate, and 130mM dextrose; final citrate concentration, 19mM) from a single healthy donor, and left until use at 37°C.

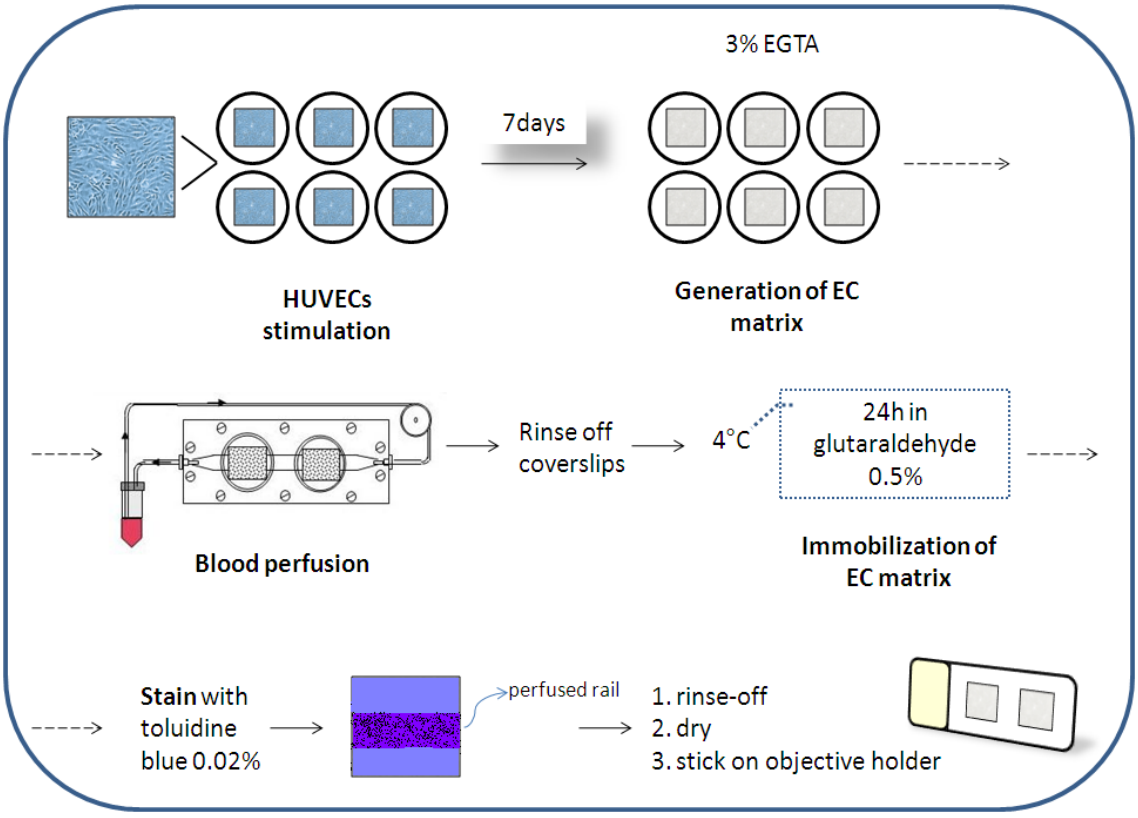


Fig.15 Platelet adhesion on the ECM. Gross procedure. For abbreviations, see text. HUVEC picture by PromoCell.

Protocol

1. Wash cell monolayers with HBSS.
2. Obtain the ECM as described, EGTA 3%.
3. Aspirate EGTA and leave the matrices in HBSS until perfusion.
4. Apply blood perfusion 5 minutes, 800s^{-1}, volume 25ml (blood kept in citrate phosphate dextrose solution, CPD).
5. Between samples, wash the pump system with tap water, distilled water and saline buffer, respectively.
6. Rinse off coverslips 4 times in falcons with PBS (1x).
7. Leave coverslips in glutaraldehyde 0.5% in PBS, at 4°C for 24 hours to fix the matrices.
8. Wash 4 times with distilled water.
9. Stain with filtered toluidine blue (Merck), 0.02% in distilled water for 10min. It is very important to have the matrices well stained and distinguish the perfusion rail, that's why staining concentration and time may vary.
10. Rinse off with tap water, until color fades.
11. Leave dry 24h at room temperature.
12. Fix the coverslip on an objective holder with DPX (glue), coated surface facing down and in touch with the glue.
13. Leave dry for 24h at room temperature.
14. Samples can be stored at room temperature.

8. Microscopy

Regarding the IG technique, the density of gold particles bound was morphometrically analyzed in a light microscope (Polyvar) equipped with epipolarizing filters. Observation of the cells in the microscope can be performed without time limitation, immersing the objective/lens in oil.

Immunofluorescent images were acquired by means of an immunofluorescence microscope (Leica, Germany), within one week after performance of the technique since fluorescence is prone to fading away with time.

Platelet adhesion was detected on the optical microscopy lens of the same Leica microscope. Neither here did we have time limitation for obtaining the images.

9. Measuring cytokines levels by flow cytometry

Pooled (n=7) secretome samples of each simple obese and diabetic obese patients were measured twice for cytokine analysis, using the Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD). The kit can be used to quantitatively measure interleukin-8 (IL-8), IL-1β, IL-6, IL-10, TNF and IL-12 protein levels in a single sample.

Principle of CBA assays: CBA assays provide a method of capturing one or more soluble analytes bound to beads, making their detection possible by recognizing the known size and fluorescence of the beads using flow cytometry. The capture beads in the kit have been previously conjugated with a specific antibody. The detection reagent in the kit contains a mixture of phycoerythrin (PE)-conjugated antibodies, which provide a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead+analyte+detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

Principle of this assay: Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1β, IL-6, and IL-10, TNF-α, and IL-12 proteins. The six bead populations are mixed together to form the bead array that is resolved in a red channel (FL3 or FL4) of a flow cytometer (see Fig.16).

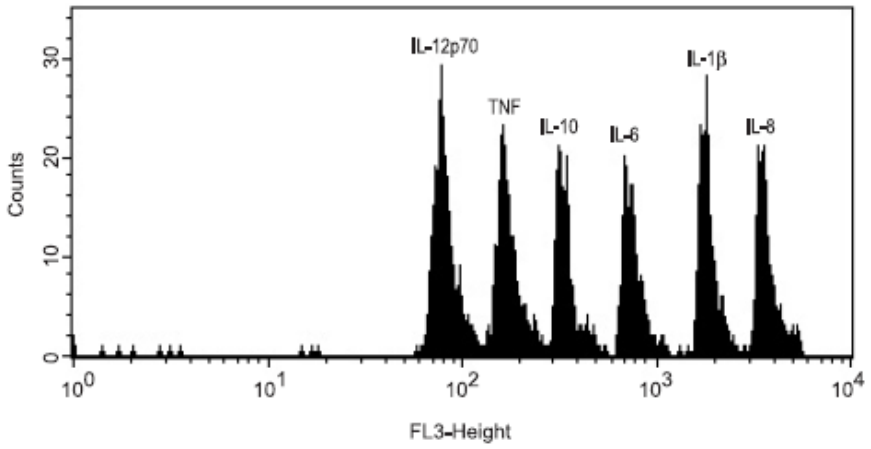


Fig.16 Red channel resolvation of a flow cytometer, including six bead populations.
Picture from the original protocol.

During the assay procedure are mixed the inflammatory cytokine capture beads with the recombinant standards or unknown samples and incubated with the PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine. After acquiring samples on a flow cytometer, FCAP Array™ software is used to generate results in graphical and tabular format.

Protocol

The steps to follow are the ones being provided by the BD assay kit. The reagents and solutions referred to are as they appear in the original protocol.

Preparation of the standards

1. Reconstitute the lyophilized standard spheres with 2ml of Assay Diluent (this is the "Top Standard").
2. Allow the reconstituted standard to equilibrate for at least 15min at room temperature.
3. Mix the reconstituted protein by gentle pipetting.
4. Pipette 300µl of Assay Diluent in each of eight 12 × 75mm tubes that are going to be used as dilution tubes (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256).
5. Perform a serial dilution by transferring 300µl from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipetting.
6. Continue performing serial dilutions by transferring 300µl from the 1:2 tube to the 1:4 marked tube and so on until the tube 1:256.
7. Prepare one tube containing only Assay Diluent as the negative control 0 pg/ml.

Mixing the capture beads

8. Vigorously vortex each Capture Bead vial for 3 to 5 seconds before mixing.
9. Add 10µl of each Capture Bead per assay tube to be analyzed into a single tube.
10. Vortex the bead mixture thoroughly.

Samples' dilution

11. Did not apply in our case.

Performing the assay

12. Vortex the mixed Capture Beads prepared in step 9 and add 50µl to all assay tubes.
13. Add 50µl of the standard dilutions to the control tubes, starting from the highest dilution tube. List them from the highest to smallest dilution (small to high concentration).
14. Add 50µl of each unknown sample to the appropriately labelled sample tubes.
15. Add 50µl of the PE Detection Reagent to all assay tubes.
16. Incubate the assay tubes for 3 hours at room temperature, protected from light. During this time, the cytometer setup can be performed.
17. Add 1ml of Wash Buffer to each assay tube and centrifuge at 200*g* for 5min.
18. Aspirate and discard the supernatant.
19. Resuspend the bead pellet by adding 300µl of Wash Buffer to each assay tube.
20. Acquire the samples on the flow cytometer.

Table 10. *Concentrations correspondence to standard dilutions in the BD Cytokine Kit**

Standard Dilution	Concentration (pg/ml)
no dilution/assay diluent	0 (negative control)
1:256	20
1:128	40
1:64	80
1:32	156
1:16	312.5
1:8	625
1:4	1,250
1:2	2,500
Top Standard	5,000

*Adapted from the original protocol.

Setup of the cytometer can be performed during realization of the protocol and according to the manufacturer's instructions.

Cytometry measurements were acquired on the BD LSRFortessa™ cell analyzer and analyzed on the BD FCAP Array™ v1.0.1 software.

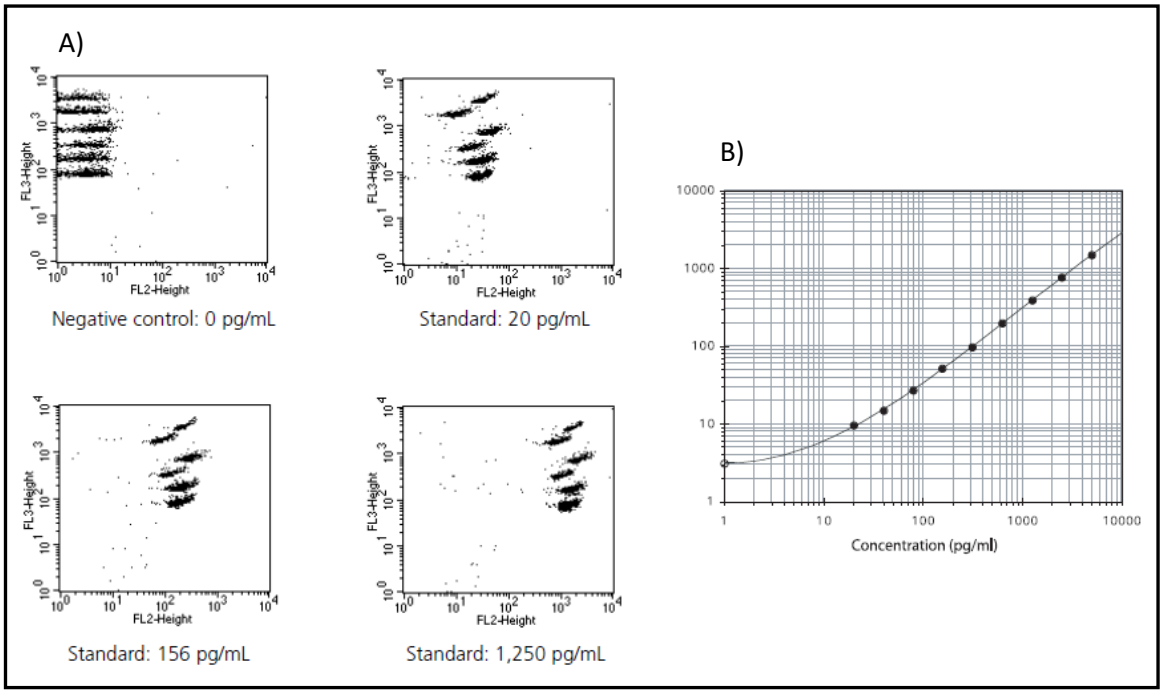


Fig.17 A) Standards and positive control detectors; flow cytometric data. **B)** Characteristic standard curve from the BD CBA Human Inflammatory Cytokine Standards. Adapted from the original protocol.

10. Enzyme-linked immunosorbent assay (ELISA)

Insulin concentration in the medium was measured applying the ELISA method, using Mercodia's Ultrasensitive Insulin ELISA kit.

This is about a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, sample insulin reacts with peroxidase-conjugated anti-insulin antibodies which are bound to microplate wells. Unbound enzyme labelled antibody is being removed with a simple washing step whereas the bound conjugate is detected by reaction with 3,3', 5,5'-tetramethylbenzidine (TMB). The reaction is stopped by addition of acid to give a colorimetric endpoint that is read spectrophotometrically.

Test procedure

The protocol followed is according to the kit's instructions. The reagents and solutions referred to are as they appear in the original.

All reagents and samples must be brought to room temperature before use and a calibrator curve is prepared for each assay run.

Before to start, prepare the Enzyme Conjugate 1X Solution and the Wash Buffer 1X Solution by diluting the reagents in Enzyme Conjugate Buffer (10 times) and distilled water (20 times), respectively. After making some tries, the samples were in general diluted 0 until 1/10 with calibrator 0.

1. Pipette 25 μ l each of Calibrators and samples into sufficient wells provided by the kit.
2. Add 100 μ l of Enzyme Conjugate 1X Solution to each well.
3. Incubate on a plate shaker (700-900rpm) for 1 hour at room temperature (18‑25°C).
4. Discard the reaction volume by inverting the microplate over a sink. Add 350 μ l Wash Buffer 1X Solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid and repeat 5 times. Avoid prolonged soaking during washing.
5. Add 200 μ l Substrate TMB into each well.
6. Incubate for 30 minutes at room temperature (18‑25°C).
7. Add 50 μ l Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
8. Read optical density within 30 minutes at 450nm and calculate results.

Table 11. *Concentrations correspondence to Calibrators**

Calibrator	Concentration (mU/l)
0	0
1	0.15
2	1
3	3
4	10
5	20

* According to the ELISA kit.

Computerized calculation of results

The concentration of insulin is obtained by computerized data reduction of the absorbance for the calibrators, except for calibrator 0, *versus* the concentration, using cubic spline regression (Fig.18).

The detection limit is 0.07mU/l calculated as two standard deviations above the calibrator 0.

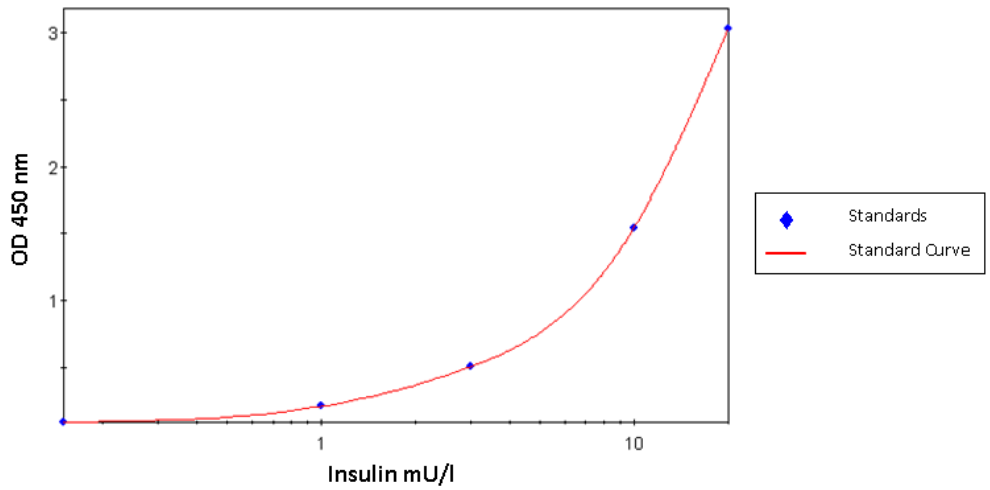


Fig.18 A typical calibrator curve.

In our experiments, when the results obtained were under the detection limit of the lowest Calibrator, we considered in our calculations as a valid value the minimum of 0.07mU/l.

Experimental model for aim 3: Justification of choice

Methods applied for the metabolomic analysis of the adipose tissue secretomes:

In order to respond to **aim 3** we were in the need to choose between the two available analysis methods present in our institution: magnetic resonance and gas chromatography- mass spectrometry. As magnetic resonance permits only a general landscape description of the samples, we preferred a more detailed approach utilizing the gas chromatography-mass spectrometry analysis.

1. Subjects inclusion

Participants included in aim 3 are described in detail above (2.1). To make a first metabolomic analysis, due to the novelty of the approach, we have first analyzed the metabolites between lean and obese subjects. The metabolomic analysis of secretomes among diabetic obese and obese patients represents a further study, under work for the moment.

2. Sample conditioning and gas chromatography-mass spectrometry analysis (GC-MS)

Samples

Visceral (VIS, omental) and subcutaneous (SC, periumbilical) adipose tissue biopsies (AT)

from the morbid obese (Ob, n=8) and nonobese (NOb, n=6) individuals were obtained and processed as explained earlier. Samples were transported on dry carbonic ice to the metabolomic platform of our institution**.** When ready to use, samples were thawed at 4°C.

Sample conditioning/treatment

The whole GC-MS analysis was performed according to Agilent's specifications [335] as followed:

1. Spike 100µl-aliquots of each sample with 20µl internal standard solution (1µg/µl succinic-d4 acid; Sigma-Aldrich).
2. Add 900µl of cold methanol/water (8:1 v/v) in order to precipitate the metabolites.
3. Ultrasonicate for 4 minutes and vortex 10 seconds.
4. Centrifuge at 19,000*g* for 10min at 4°C.
5. Prepare three technical replicates of each sample, transferring 200µl supernatant to a GC autosampler vial.
6. Spike samples with 20µl myristic acid-d27 (Sigma-Aldrich), used as the internal standard for retention time lock (RTL system provided in Agilent's ChemStation Software).
7. Lyophilize overnight (Lyotrap freeze dryer).
8. Derivatize metabolites in order to make them volatile and less polar and, by that, suitable to GC-MS analysis, in two different steps:
    a) Perform methoximation in order to prevent ring formation and stabilize carbonyl moieties. Thus, incubate lyophilized secretome residues with 50µl methoxyamine in pyridine (0.3µg/µl) for 16h at room temperature.
    b) Increase the volatility of the compounds. Silylate samples using 30µl N-methyl-N-trimethylsilyltrifluoroacetamide with 1%trimethylchlorosilane (MSTFA + 1% TMCS, Sigma) for 1 h at room temperature.

GC-MS analysis

Derivatized secretome samples were analyzed with a gas chromatography-mass spectrometry (GC-MS) system. All system components originated from Agilent Technologies (Santa Clara, USA) including the HP 6890 gas chromatograph coupled to a quadrupole HP 5973 mass spectrometric detector. The latter had a split inlet (split ratio 5:1) equipped with a J&W Scientific DB 5-MS+DG stationary phase column (30m × 0.25mm i.d 0.1µm film). Steps to follow were:

1. Inject 1 µl of each derivatized sample in a gas chromatograph system.
2. Set injector temperature at 250°C and keep the helium carrier flow rate constant at 1.1 ml/min.
3. Hold the column temperature at 60°C for 1min, then increase to 325°C at a rate of 10°C/min and hold at this temperature for 10min.
4. Maintain detector operation in the electron impact ionization mode (70 eV), and record mass spectra after a solvent delay of 4min with 2.46 scans/second (mass scanning range of mass-charge ratio: m/z, 50–600; threshold abundance value of 50 counts).
5. Whilst, keep source temperature and quadrupole temperature at 230 and 150°C, respectively.

To reduce systematic error associated with instrumental drift, samples were entirely randomized.

Statistical approaches

a) Gene analysis

Real-Time PCR results were expressed in relation to the control condition (having always the value 1) as folds mean ± standard error of the mean (SEM), including results from a minimum of n=3 different experiments, and analyzed using two-tailed Student's *t*-test for paired samples. Results were considered as significant when $p<0.05$.

b) Protein analysis of membrane antigens

Immunogold and immunofluorescence images were captured by a video camera (ProgRes MF) and transferred to a personal computer with an automated image analysis system (Image J software, version 1.43m) for the evaluation of the density of gold particles (expressed as gold particles on covered surface), with the help of the threshold tool.

c) Protein analysis regarding ECM activation

c.1) **Fluorescence** micrographs were taken by a video camera (Leica, Germany) and densitometrically analyzed with the help of the Image J software, which is used to analyze/process all sorts of images in biology-related research. This software automatically analyzes the gray density of each pixel in a scale ranging from 0 (black) to 255 (white). The mean gray value in the control pictures was considered as one hundred per cent. Results are expressed as the percentage of the mean grey intensity increase of the covered surface over the control samples.

c.2) Blood **perfusion** images were captured by a video camera (Leica, Germany) and transferred to a personal computer with Image J for evaluation of the degree of platelet deposition on the perfused surface and evaluated *en face* by means of an automated method. Platelet adhesion on the ECM was selected from the background with the threshold tool, and the intensity was measured only in the selected area. The surface covered by platelets was expressed as a percentage with respect to the total area of the coverslip screened (%SC).

Data from experiments a) to c) are expressed as mean ± standard error of the mean (SEM), and analyzed using two-tailed Student's *t*-test for paired samples. Results were considered as significant when $p<0.05$.

d) Cytometry & ELISA

Flow cytometry and ELISA results were expressed as mean ± standard error of the mean (SEM), and analyzed using two-tailed Student's *t*-test for paired samples. Results were considered as significant when $p<0.05$.

e) GC-MS data analysis

Due to the high difficulty of the metabolomic study analysis we performed the whole bioinformatics analysis with the help of the bioinformatics specialist from the metabolomic unit of our institution. The author of this thesis has participated to the analysis although not having

performed autonomous the procedure.

Raw GC/MS files were exported into the platform-independent netCDF (*.cdf) and loaded into XCMS software (version 1.6.1) based on R-program version 2.4.0, where peak finding, integration and alignment in the time domain were performed. XCMS data was exported to Matlab (version 6.5.1), where normalization to internal standard succinic acid-d4 was performed and the averaged integrated intensities for the three analytical replicates of each biological specimen were computed.

Secretome samples were compared based on either 6/21 multivariate or systematic multiple univariate statistical test performed 183 on XCMS-derived dataset. The false discovery rate (FDR) procedure described by Storey *et al* [336] was used to account for multiple testing. The AMDIS program (Automated Mass Spectral Deconvolution and Identification System) was run for peak deconvolution, and both the Fiehn GC/MS Metabolomics RTL Library and NIST mass spectral databases were used for identification. Further data processing, analysis, and statistical calculations were performed using Matlab.

IV. Results

1. Genetic expression in EC

Gene expression analysis was conducted in order to assess the proinflammatory and prothrombotic changes introduced by the visceral adipose tissue's secretome of morbid obese or morbid obese type 2 diabetic subjects treated with metformin on the EC. The related genes whose expression was analyzed in this thesis were VCAM-1, ICAM-1, von Willebrand Factor (VWF), tissue factor (TF) and the transcription nuclear factor-κB (NF-κB).

The expression of the adhesion molecules VCAM-1 (or CD106) and ICAM-1 (or CD54) is characteristic of the endothelium in that it indicates activation and the beginning and progression of inflammation, due to their participation in leukocyte migration on the endothelium and final diapedesis. NF-κB, implicated in various cell cycle processes, forms also part of the inflammatory cascade. The proteins VWF and TF are two major prothrombotic factors synthesized by endothelial cells and released to the subendothelial matrix in response to activation, playing a major role in atherothrombotic thrombus formation.

For the significant involvement of the above mentioned molecules in vascular damage, our study was preferentially focused on these very genes.

The duration of stimulation to evaluate gene expression was considered appropriate to last 24h after analyzing various studies from the literature with sera on HUVECs and performing time course studies. Results were obtained by Real-Time PCR gene expression analysis which (expression) was compared with a standard condition, that is, cDNA from cells having been stimulated in parallel to our conditions with regular growth medium, which we named control medium (CM).

1.1 Endothelial response to the stimulation with visceral simple obese (Ob) secretomes vs. secretomes of obese diabetic metformin-treated (DMO) patients

1.1.1 Proinflammatory endothelial activation

Endothelial cells exposed to the simple obese (Ob) or the diabetic obese (DMO) subjects presented a significant (between 3- to 8-fold) increase in the expression of VCAM-1, ICAM-1 and NF-κB as compared to the control medium (CM).

The response of the ECs to the obese diabetic secretome (DMO) resulted in a high expression of VCAM-1. This effect was notably lower when stimulating the cells with the simple obese (Ob) secretome. As can be seen on Fig.19A, there was a statistically significant (p=0.03) difference in the gene expression between the two types of secretomes. ICAM-1, which is constitutively expressed in the endothelium under resting conditions, increased three and two folds in response to the Ob and DMO secretomes, respectively, in comparison to VCAM-1. Nevertheless, ICAM-1 was expressed without significance between the two types of secretomes, but with a higher tendency at stimulation with the Ob secretome (Fig.19B).

NF-κB expression was more similar to the levels of VCAM-1. Even more alike, the DMO secretome had a greater effect than the Ob on the ECs, inducing vascular damage as expressed by the more noticeable presence of NF-κB, with a p equal to 0.03 (Fig.19C).

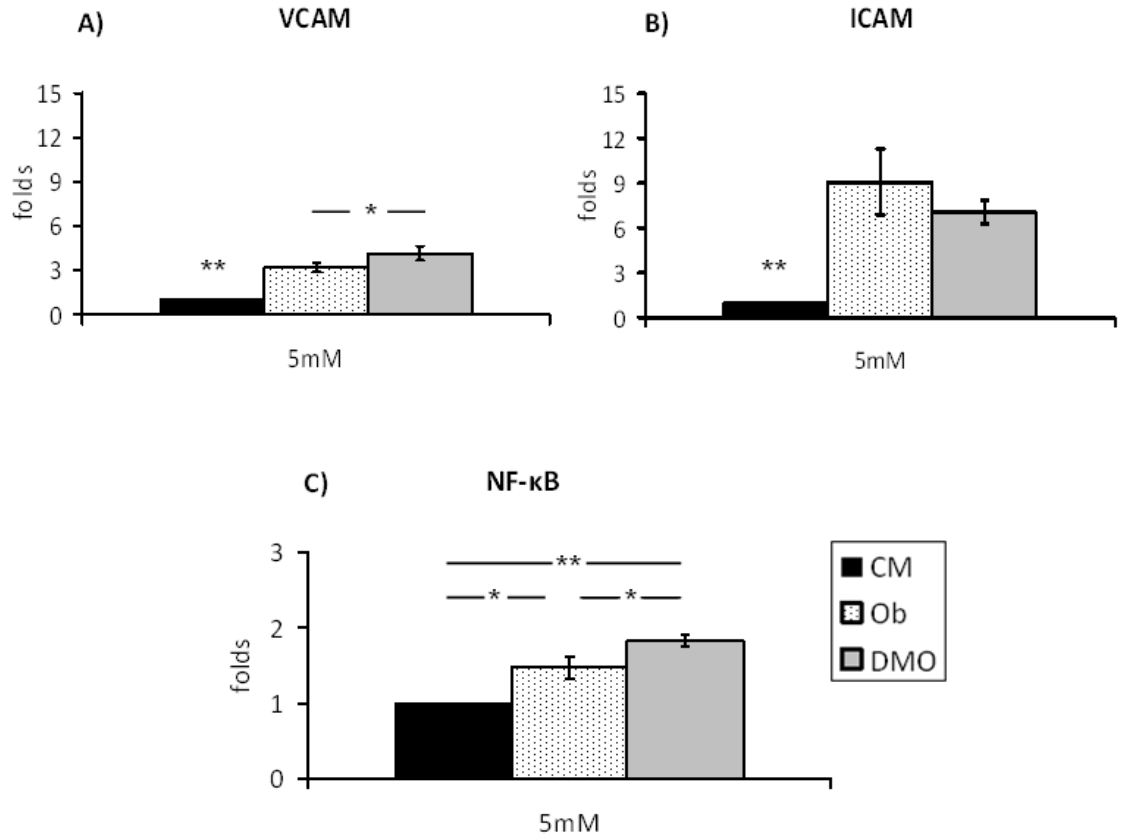


Fig.19 Effects of the secretomes on proinflammatory genes on ECs. Expression of **(A)** VCAM-1, **(B)** ICAM-1 and **(C)** NF-κB genes in cells stimulated 24h with regular growth medium (control medium, CM), simple obese (Ob) and obese diabetic secretomes (DMO). Results obtained by Real-time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± standard error of the mean (SEM). Ob n=6-9, DMO n=6. *$p < 0.05$, and ** $p < 0.005$ in CM vs. Ob and DMO, otherwise indicated.

1.1.2 Prothrombotic response

Ob and DMO induced a statistically significant increase in the expression of the genes of the prothrombotic factors VWF and TF. Endothelial cells did not respond in a diverse manner to stimulation with Ob and DMO secretomes, as far as the thrombosis markers were concerned. Therefore, although VWF and TF appeared at similar levels, VWF showed a tendency towards higher expression in the presence of the DMO secretomes, whereas we observed an opposite tendency for TF (Fig.20). Nevertheless, there was no statistical significance between the two conditions.

1.1.1 - 1.1.2: Summary/Conclusions

Both secretomes of visceral Ob and DMO induce activation of the inflammatory and prothrombotic pathways, as demonstrated by comparison with the control condition (CM). In our experiments we observed that the secretomes from the adipose tissue of obese diabetic subjects seemed to activate the inflammatory cascade in a slightly greater degree than the secretomes

obtained by simple obese adipose tissues as marked by the higher expression of VCAM-1, NF-κB. VWF seems to increase slightly without reaching statistical significance. Although results of ICAM-1 and TF are of opposite tendency, both are quite similar in both secretome groups. However, these results have to be regarded with precaution due to the high variability in the obese group. Overall we can conclude that the DMO secretome induces a slightly increased expression of the proinflammatory and prothrombotic genes studied, while both secretomes produce an important activation of endothelial cells with respect to the CM.

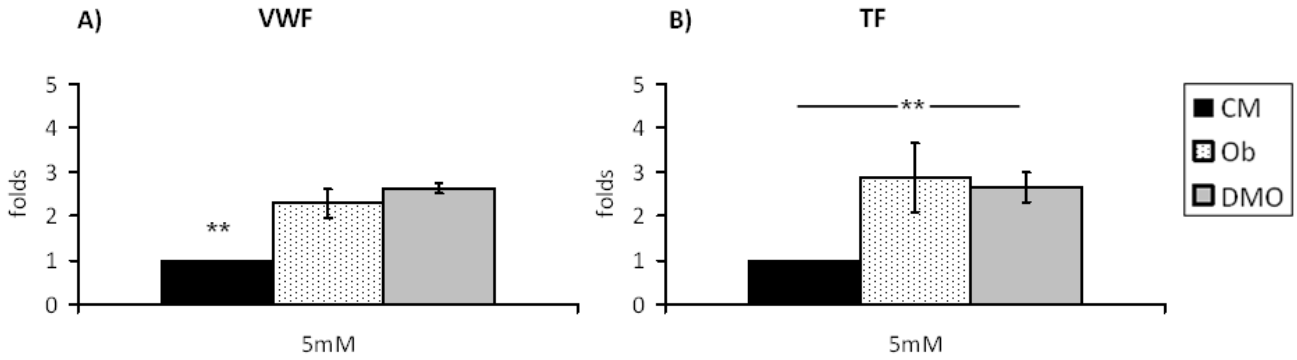


Fig.20 Effects of the secretomes on prothrombotic genes on ECs. Expression of **(A)** VWF and **(B)** TF genes in cells stimulated 24h with regular growth medium (control, CM), simple obese (Ob) and diabetic obese secretomes (DMO). Results obtained by Real-time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. Ob n=7, DMO n=6. ***p* < 0.005 in CM vs. Ob and DMO, otherwise indicated.

1.2 Stimulation with simple Ob secretomes supplemented with insulin

Hyperinsulinemia is a common feature of type 2 diabetes mellitus associated to obesity and there persists still debate about its effects on the endothelium. The prothrombotic and proinflammatory potency of visceral obese adipose tissue secretomes on endothelial cells has been previously reported and published (previous results from our group). Therefore we decided to evaluate in our *in vitro* experimental model the complementary effect on endothelial cells of pathophysiological concentrations of insulin to the one induced by the Ob secretomes.

1.2.1 Proinflammatory endothelial activation

VCAM-1 and ICAM-1 expression remained unchanged at the presence or not of insulin at physiological to pathophysiological concentrations in the secretomes (0M, 10^{-11} M, 10^{-9} M), being around 3- and 15-fold higher than the control condition, respectively. The pathological 10^{-7} M insulin level induced an increased VCAM-1 expression as compared to the 10^{-9} M insulin condition (p=0.05). Expression of ICAM-1 under the same 10^{-7} insulin condition presented the same tendency, although not reaching statistical significance. Obviously, the highest constant effect observed equally in all conditions was due to the obese secretomes themselves (Fig.21A and B).

Regarding NF-κB, insulin addition to the obese visceral secretome at 10^{-11} M and 10^{-9} M did not result in any significant fold change of NF-κB between the Ob conditions, whereas insulin at 10^{-7} M showed a capacity to increase NF-κB expression in comparison to the lower insulin

concentrations (p<0.05). All secretome-related expressions were around 2-fold elevated in respect to the control condition (Fig.21C).

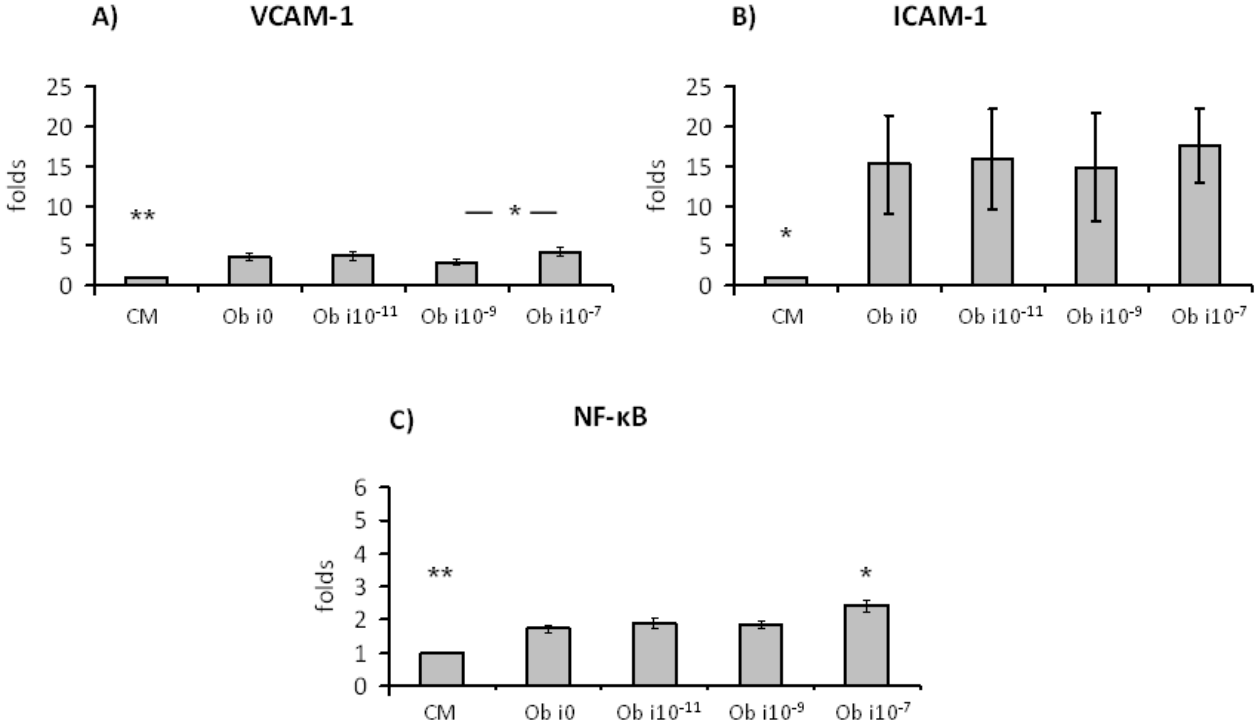


Fig.21 Effects of insulin on proinflammatory genes on ECs. Expression of **(A)** VCAM-1, **(B)** ICAM-1 and **(C)** NF-κB genes in cells stimulated 24h with regular growth medium (control, CM) and simple obese secretome (Ob), alone (Ob i0) or supplemented with insulin at 10^{-11} M, 10^{-9} M and 10^{-7}M (correspondingly symbolized as Ob i10^{-11}, Ob i10^{-9}, Ob i10^{-7}). Results obtained by Real-Time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=4-5 for VCAM-1, n=5-6 for ICAM-1, n=4 for NF-κB. In ICAM-1 *p < 0.05 where indicated, and, in ICAM-1 regarding the rest of the conditions, and in NF-κB regarding the rest of the Ob conditions. ** p < 0.005 with respect to the rest of the conditions.

1.2.2 Prothrombotic endothelial response

Results referring to thrombogenicity (Fig.22) revealed the ability of insulin to tend towards downregulating the gene expression of the VWF gradually in parallel with the increase in insulin concentration, until to reach statistical significance at the highest one (10^{-7} M), compared to the simple secretome stimulation (p=0.02). The same pattern was observed for TF although not reaching statistical significance probably due to the variability between samples. (Fig.22B).

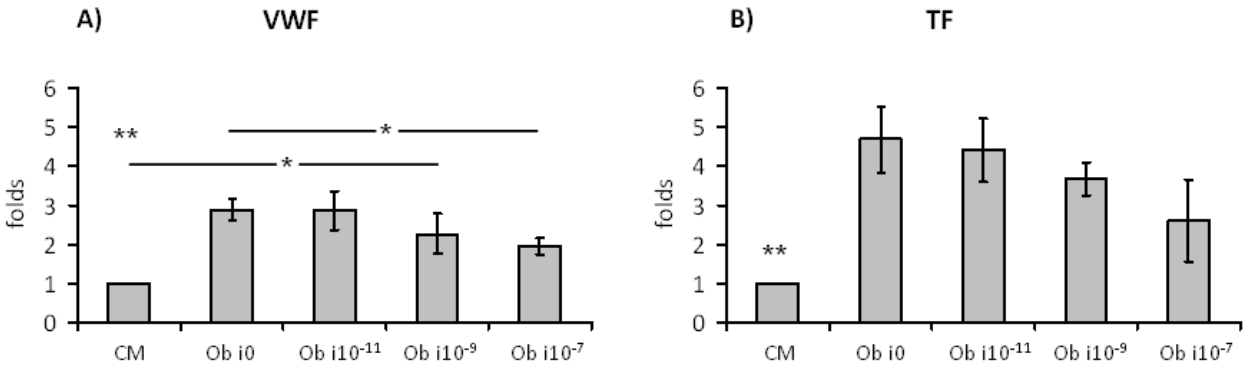


Fig.22 Effects of insulin on prothrombotic genes on ECs. Expression of **(A)** VWF and **(B)** TF genes in cells stimulated 24h with regular growth medium (control, CM) and simple obese secretome (Ob), alone (Ob i0) or supplemented with insulin at 10^{-11} M, 10^{-9} M and 10^{-7}M (respectively symbolized as Ob i10^{-11}, Ob i10^{-9}, Ob i10^{-7}). Results obtained by Real-Time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=3-5. n=4-5 for VWF, n=4 for TF. *$p < 0.05$, and ** $p < 0.005$ in CM vs. Ob conditions, except from comparison with Ob i10^{-7} in the TF.

1.2.1 - 1.2.2: Summary/Conclusions

Here we observed that simultaneous stimulation of ECs with insulin and secretome for 24h induced only at the very high level of 10^{-7} M an increased expression of the adhesion molecules VCAM-1, ICAM-1 and of the transcription factor NF-κB. Curiously, these changes were not paralleled by the changes in the expression of the genes of the prothrombotic factors VWF and TF, which may suggest to have a protective antithrombotic effect.

1.3 Stimulation with simple Ob secretomes supplemented with glucose

Next, in order to perform an overall evaluation of a glucose additive effect on the endothelium to the one induced by the obese secretomes, we concomitantly added glucose in increasing concentration to the endothelial cells while a 24h-stimulation with the secretome was performed.

To fulfill this purpose, we employed in our experiments the normal, basal glucose state of HUVECs culture, namely 5mM, which was the one generally present in the simple visceral obese secretomes, and two pathophysiological conditions of glucose: 11mM and 16mM.

1.3.1 Proinflammatory endothelial activation

Supplementing the secretomes with the glucose concentrations of 11mM and 16mM for 24h did not provoke changes in the gene expression. No statistically significant differences were observed in the fold change of the proinflammatory genes VCAM-1, ICAM-1 and NF-κB between the glucose conditions.

Interestingly, glucose at its highest concentration (16mM) presented a tendency to diminish the gene's expression of VCAM-1 and ICAM-1 in comparison with the lower ones. (Fig.23A, B) while the expression of NF-κB was quite equal between conditions (Fig.23C).

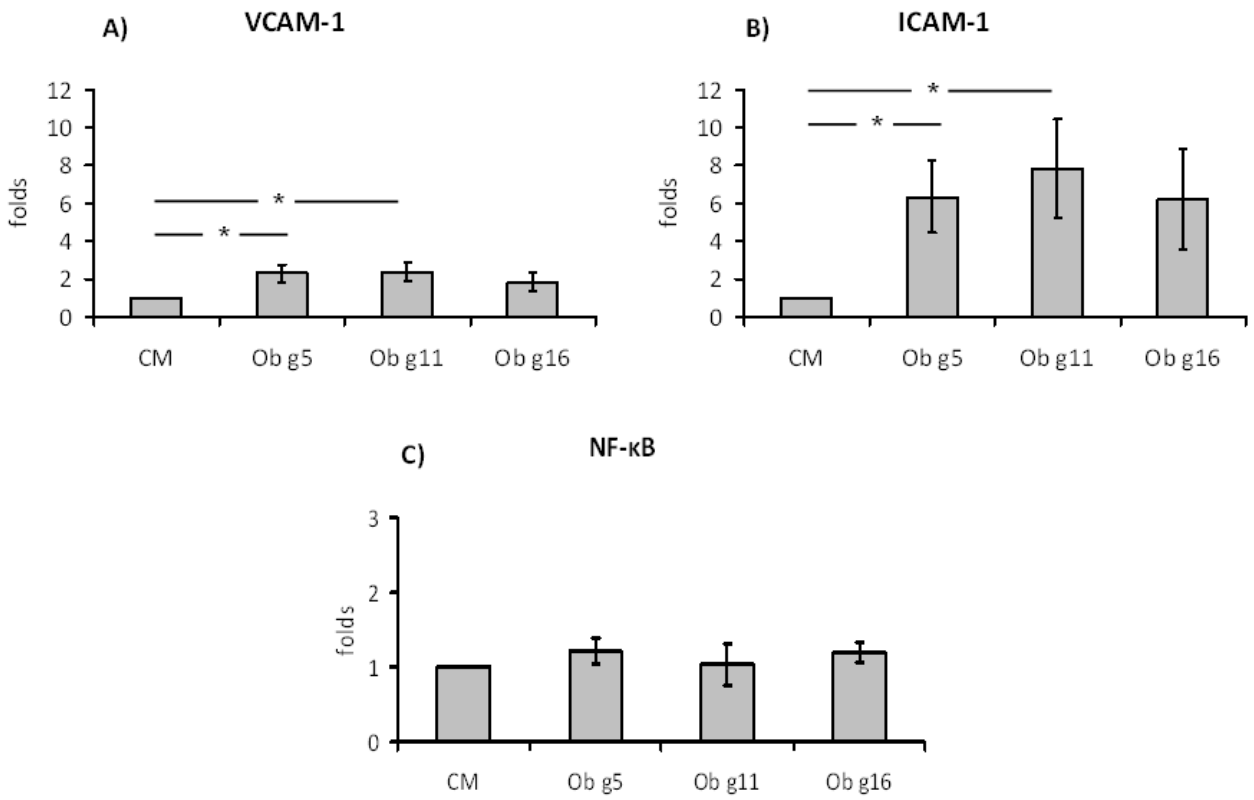


Fig.23 Effects of glucose on proinflammatory genes on ECs. Expression of (A) VCAM-1, (B) ICAM-1 and (C) NF-κB genes in cells stimulated 24h with regular growth medium (control, CM) and the obese simple secretome which contained glucose 5mM (Ob g5) or supplemented with glucose at 11mM and 16mM (respectively symbolized as Ob g11, Ob g16). Results obtained by Real-Time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=3-4. *$p < 0.05$ where indicated.

1.3.2 Prothrombotic endothelial response

Maintaining the same pattern as for the proinflammatory genes, the prothrombotic gene expression was not modified with the increasing glucose (Fig.24). To note that interpretation of the results regarding the expression of TF was difficult to be done, due to the high variability between samples.

1.3.1 - 1.3.2: Summary/Conclusions

Increasing concentration of glucose does not seem to result in any proinflammatory or prothrombotic additional damage to that already induced by the obese visceral secretome. However, results are difficult to evaluate due to the great variability. There was a tendency to peak for glucose at 11mM for ICAM-1 and TF genes, thereafter descending to the values of simple obese secretomes. Further increase in the number of experiments could lead to more relevant results.

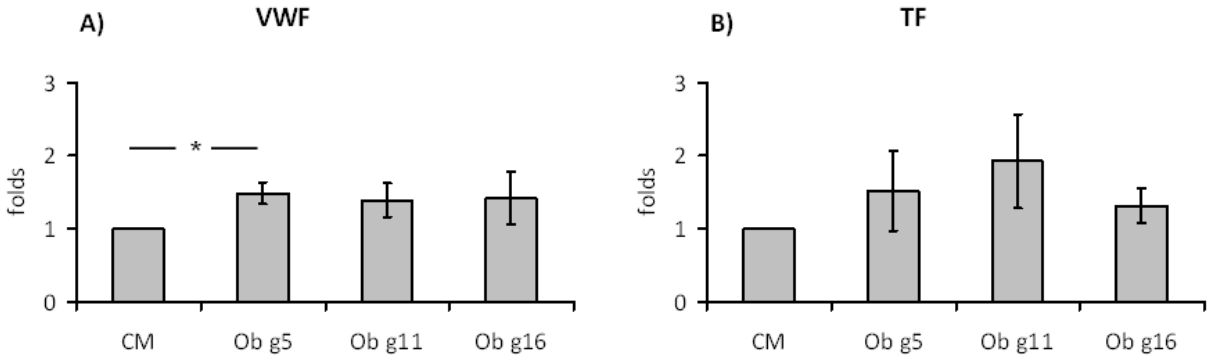


Fig.24 Effects of glucose on prothrombotic genes on ECs. Expression of **(A)** VWF and **(B)** TF genes in cells stimulated 24h with regular growth medium (control, CM) and simple obese secretome (Ob g5) supplemented with glucose at 11mM and 16mM (respectively symbolized as Ob g11, Ob g16). Results obtained by Real-Time PCR analysis. Bars representing fold change with respect to the control condition. Data are expressed as mean fold ± SEM. n=4. *$p < 0.05$ where indicated.

1.4 Stimulation with control medium (CM) supplemented with insulin

In order to investigate the 24h-effect of insulin on ECs, we stimulated for the same period of time ECs growing in regular medium supplemented with the corresponding insulin concentrations.

1.4.1 Proinflammatory response

Inflammation-related genes were not induced by insulin, with the only exceptions being ICAM-1 at the 10^{-9} M condition, and NF-κB at the lowest insulin condition. On the contrary, there seemed to be a slight protective effect of the hormone, causing a decrease in the gene expression of all proinflammatory genes quantified, although reaching statistical significance only for the gene expression of ICAM-1 for insulin at 10^{-9} M, and for the transcription factor NF-κB for insulin at 10^{-11} M (Fig.25).

1.4.2 Prothrombotic response

Results regarding the expression of the prothrombotic factors VWF and TF were heterogeneous. Generally insulin at 10^{-11} M and 10^{-9} M seemed to induce again a certain protection, while at the pathological concentration of 10^{-7} M to increase the expression of VWF (Fig.26).

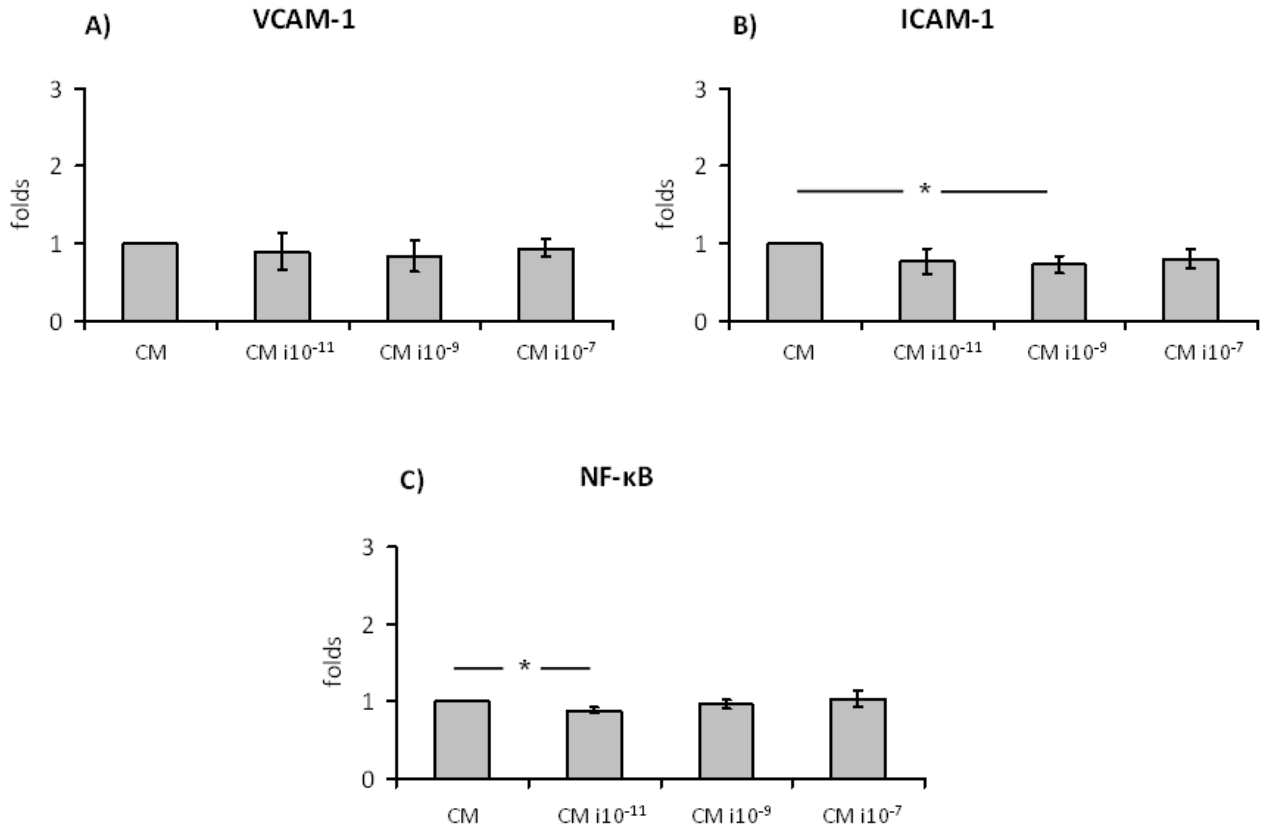


Fig.25 Effects of insulin on proinflammatory genes on ECs. Expression of **(A)** VCAM-1, **(B)** ICAM-1 and **(C)** NF-κB genes in cells stimulated 24h with regular growth medium (control, CM) alone, or supplemented with insulin at 10^{-11} M, 10^{-9} M and 10^{-7}M (respectively symbolized as CM i10^{-11}, CM i10^{-9}, CM i10^{-7}). Results obtained by Real-time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=3. *$p < 0.05$ where indicated.

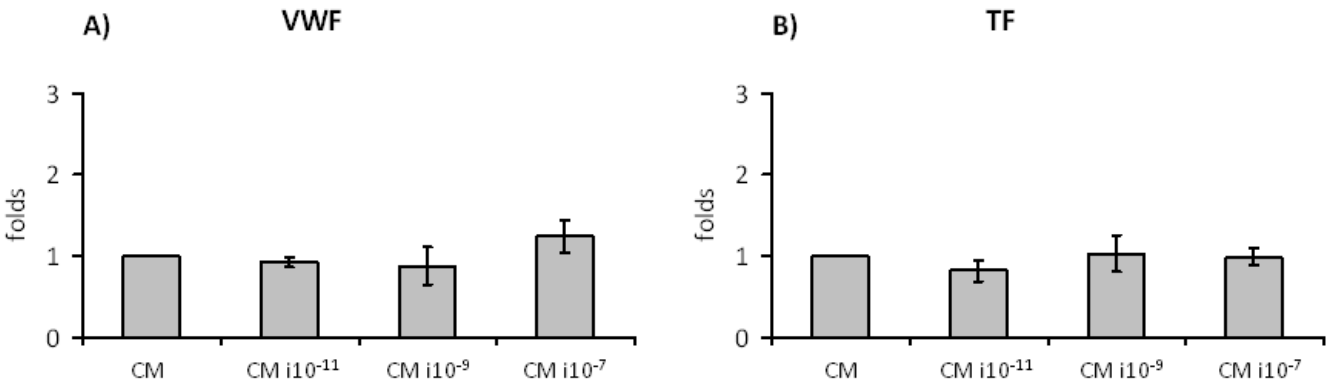


Fig.26 Effects of insulin on prothrombotic genes on ECs. **(A)** Expression of VWF and **(B)** TF genes in cells stimulated 24h with regular growth medium (control medium, CM) alone, or supplemented with insulin (concentrations 10^{-11} M, 10^{-9} M and 10^{-7}M). Results obtained by Real-time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=4.

1.4.1 - 1.4.2: Summary/Conclusions

Insulin has a heterogeneous action on the expression of proinflammatory and prothrombotic factors with a slight decrease for the low concentration and a tendency to increase for high insulin concentrations, presenting the highest effect at 10^{-7} M in the VWF.

1.5 Stimulation with control medium (CM) supplemented with glucose

Verification of the effect of glucose on endothelial cells was applied also in this case, keeping the same stimulation time and conditions.

1.5.1 Proinflammatory response

Glucose did not affect at all the gene expression of VCAM-1, ICAM-1 and NF-κB (Fig.27).

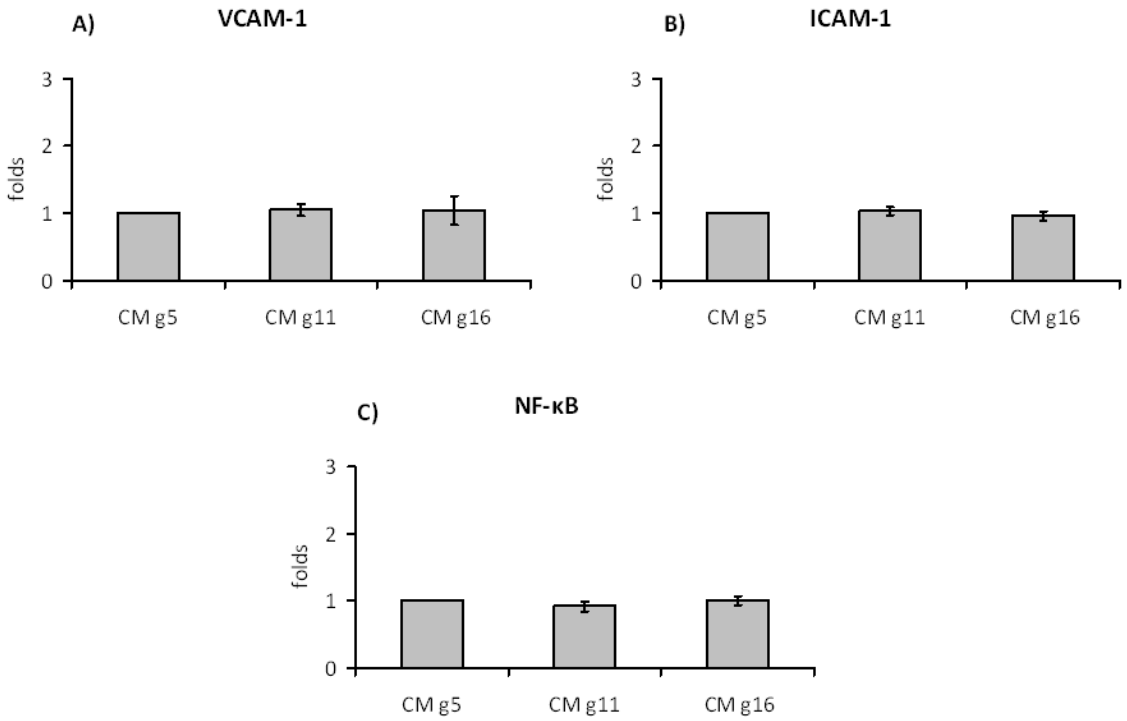


Fig.27 Effects of glucose on proinflammatory genes on ECs. Expression of **(A)** VCAM-1, **(B)** ICAM-1 and **(C)** NF-κB genes in cells stimulated 24h with regular growth medium (control, CM g5) or supplemented with glucose at 11mM and 16mM (respectively symbolized as CM g11, CM g16). Results obtained by Real-time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=3.

1.5.2 Prothrombotic response

Results regarding glucose influence on a possible prothrombotic reaction were not encouraging, either (Fig.28).

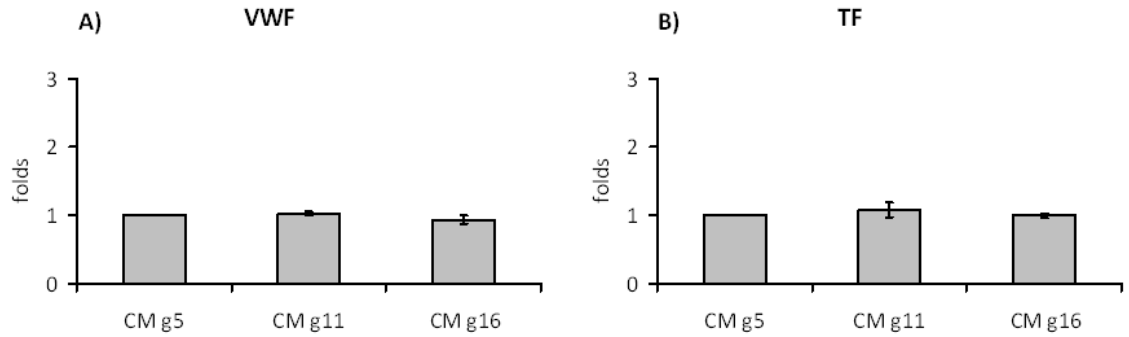


Fig.28 Effects of glucose on prothrombotic genes on ECs. Expression of **(A)** VWF and **(B)** TF genes in cells stimulated 24h with regular growth medium (control, CM g5) or supplemented with glucose at 11mM and 16mM (respectively symbolized as CM g11, CM g16). Results obtained by Real-time PCR analysis. Bars representing fold change in respect to the control condition. Data are expressed as mean fold ± SEM. n=3.

1.5.1 – 1.5.2: Summary/Conclusions

Glucose was unable to provoke cell damage when added to regular growth medium, in normal to very high glucose concentrations, confirming our first results.

2. Protein expression in EC

Changes in gene expression were necessary to be confirmed at the protein level. In previous pages was made reference on VCAM-1 and ICAM-1 as molecules present on the surface of the endothelium, participating in the adhesion of the leukocytes to the vasculature and signalling among others the initiation of the inflammatory mechanism. Precisely, they are adhesion receptors on the endothelial cell membrane.

On the other hand, the genes for VWF and TF codify for proteins that leave the interior of the cell and are released to the extracellular matrix. From there they can take up action and carry out the thrombosis cascade.

Therefore, it was necessary to apply the appropriate technique in order to detect each protein. It was decided to perform immunogold labelling (IG) for the receptor detection, and carry on with immunofluorescence (IF) for the detection of the proteins of the extracellular matrix.

Receptor's expression was quantified after 24h of stimulation, whereas the expression of the ECM proteins was quantified after 7 days of coculture, time requested for their expression on the subendothelium. Due to the complexity of the technique, endothelial cells were exposed to the extreme conditions of our experimental design, respectively to basal and maximal concentrations of insulin and glucose.

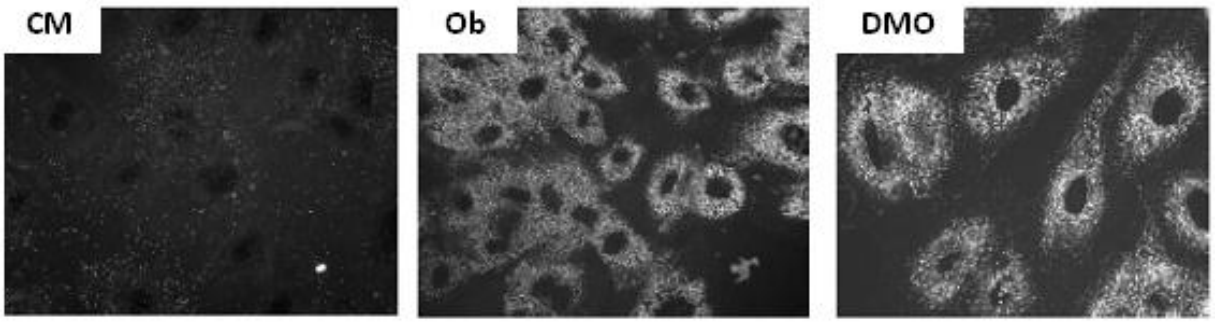
2.1 Visceral simple obese (Ob) secretomes vs. the secretomes of diabetic obese (DMO) subjects treated with metformin

2.1.1 Adhesion receptor expression on EC: Ob secretomes vs. DMO

Stimulation of the cells with DMO secretomes for 24 hours did present a discrete decrease in the expression of the adhesion receptors VCAM-1 and ICAM-1 as compared to the ones exposed to the Ob secretomes. Both types of secretomes induced on ECs a significantly increased expression of VCAM-1 and ICAM-1 in comparison to the control condition (Fig.29).

It will be noticed in- and referred to- upcoming pages that the expression of the VCAM-1 protein was not studied any more. This was due to the fact that the present experiment was performed in an early time point compared to the rest; meanwhile the commercial company retired the antibody and thereby new immunocytochemistry methods are at the time evaluated for the quantification of this antibody in our laboratory.

A)



B)

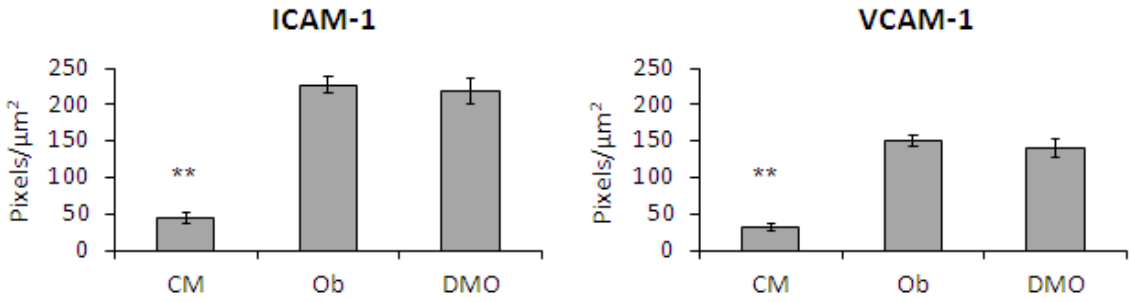
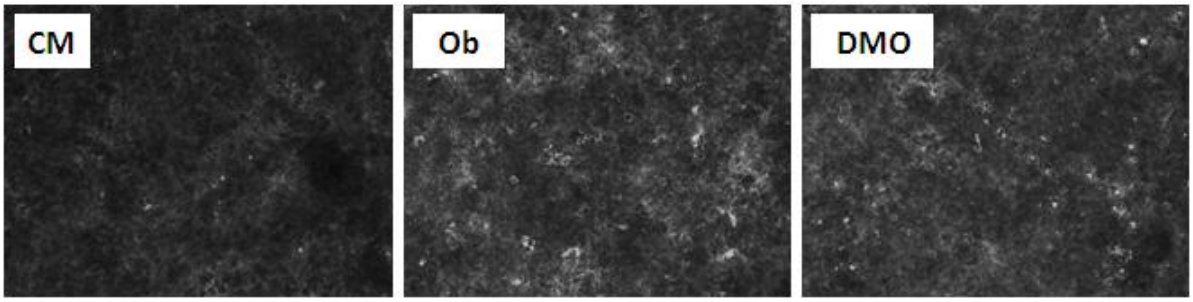


Fig.29 (A) Expression of **VCAM-1** and **ICAM-1** in EC stimulated for 1 day with control medium (CM), simple obese (Ob) and diabetic obese secretome (DMO). Characteristic images representative for both CAMs. **(B)** Quantification of obtained images: bars representing % surface covered in respect to the control. Data are expressed as mean fold ± SEM. n=6. ** $p < 0.005$ in the control compared to the conditions. Pictures obtained by immunogold assay.

2.1.2 Prothrombotic proteins expression in the ECM after exposure to DMO vs. Ob secretomes

VWF expression in the ECM after a week's stimulation was significantly higher when cells got stimulated with the Ob and DMO secretomes than with the regular growth medium (Fig.30), but there was no difference in the degree of the outcome, whether it was one or the other type of secretome.

A)



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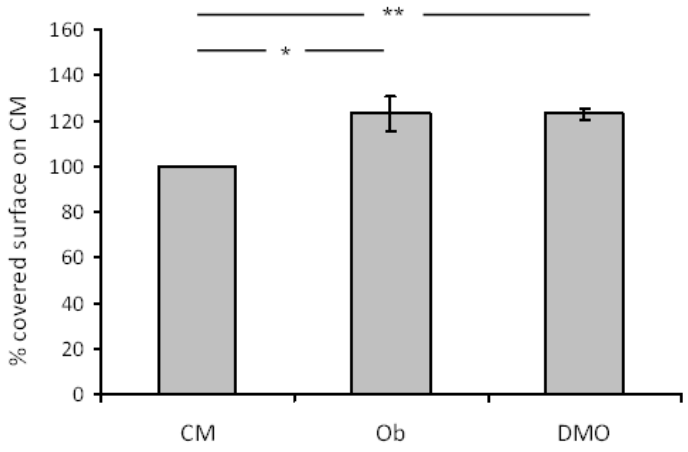


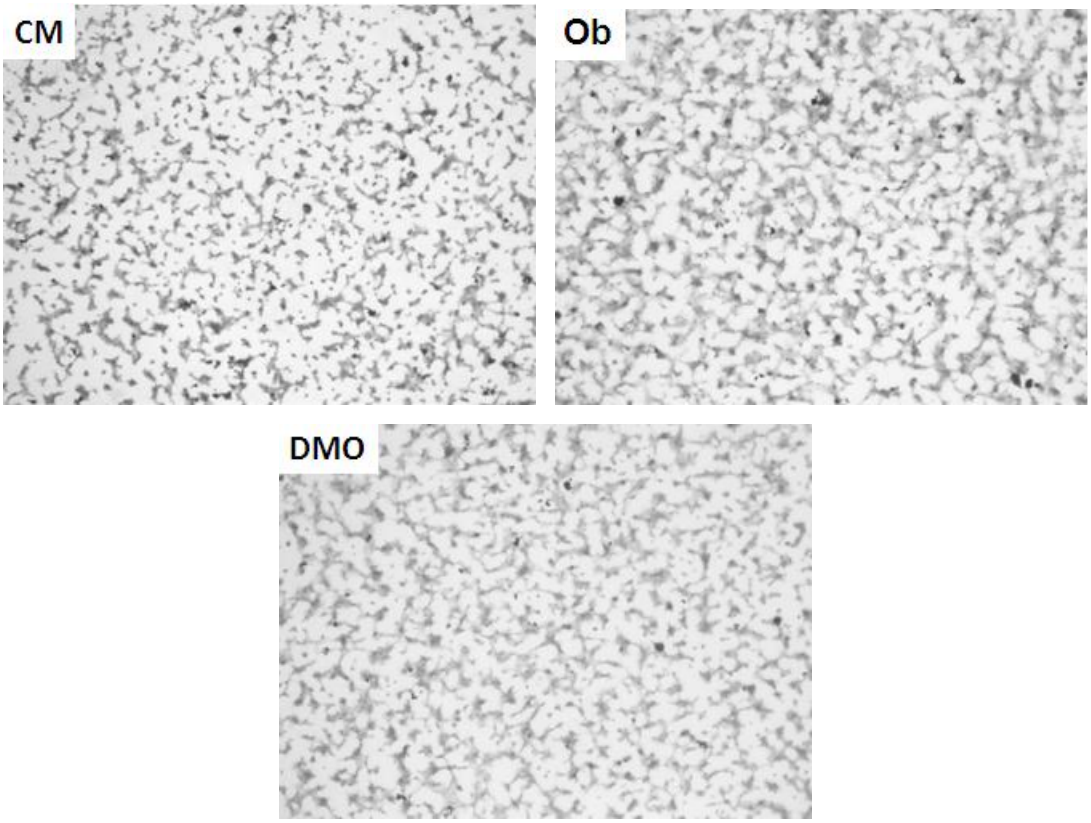
Fig.30 (A) Expression of **VWF** in EC stimulated for 7 days with control medium (CM), simple obese (Ob) and diabetic obese secretome (DMO). **(B)** Quantification of obtained images: bars representing % surface covered in respect to the control. Data are expressed as mean fold ± SEM. n=6. *$p < 0.05$ and **$p < 0.005$. Pictures obtained by immunofluorescence assay.

2.1.3 Thrombogenicity of the subendothelial cell matrix: Platelet adhesion on the ECM created from EC, after exposure to DMO vs. Ob secretomes

The ECM was synthesized by incubation of the HUVECs under the conditions of interest for one week. Then it was perfused under mentioned conditions with blood from healthy subjects, and the platelet adhesion, as final consequence of the thrombogenicity of the subendothelial matrix, was detected.

Again, endothelial cells formerly stimulated with both the obese or diabetic obese secretomes presented an increased platelet adhesion with respect to the control condition (Fig.31).The Ob secretomes induced a statistically significant increased platelet adhesion while platelet adhesion to the ECs exposed to the DMO presented an intermediary level, higher than the control medium but lower than the diabetic secretome.

A)



B)

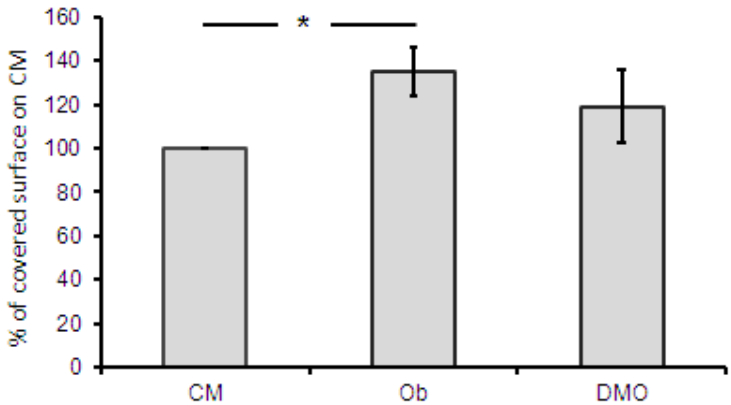


Fig.31 (A) Platelet adhesion in HUVECs after perfusion of citrated blood (800s^{-1}, 5min) in HUVECs cultivated for 7 days in control condition (CM), simple obese (Ob) and diabetic obese (DMO) secretome. **(B)** Quantification of platelet deposition. Data are expressed as mean fold ± SEM as % of change regarding the control. n=6. *$p < 0.05$. Pictures obtained by blood perfusion assay.

2.1.4 Summary Ob vs. DMO

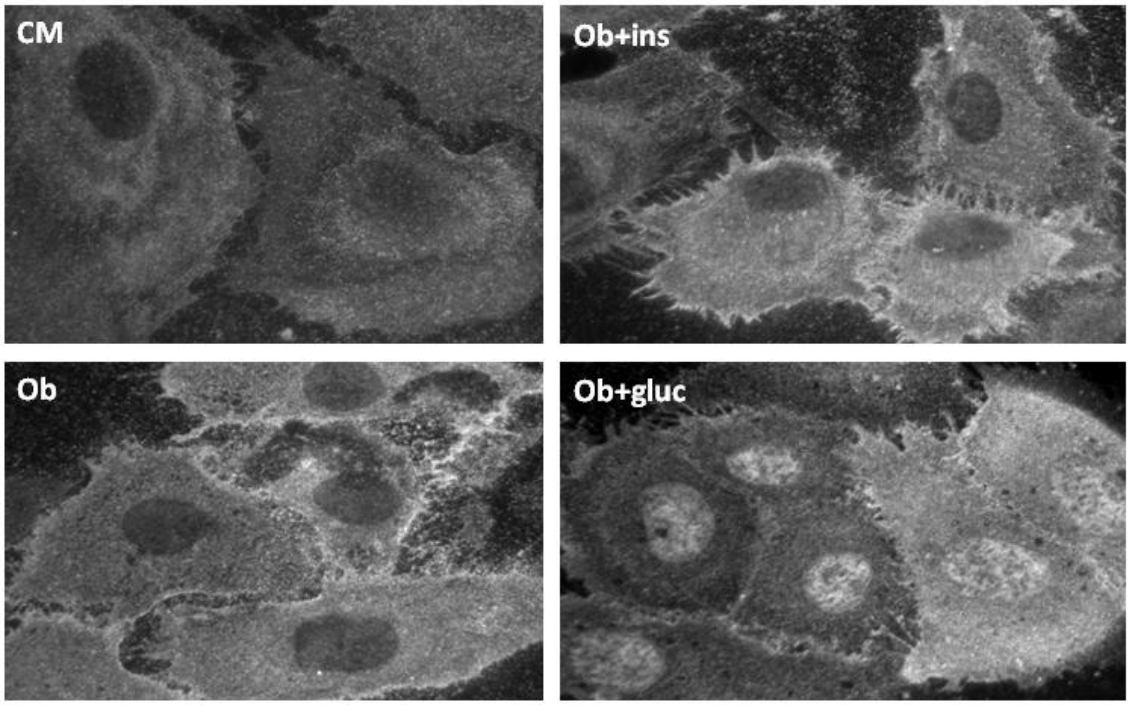
Ob and DMO secretomes acted in a similar way on endothelial cells at stimulation, regarding the expression of the adhesion receptor proteins VCAM-1 and ICAM-1 and the expression of the subendothelial prothrombotic protein VWF. Subendothelial thrombogenicity, as quantified by platelet adhesion, was lower in the secretomes of the diabetic metformin-treated subjects. The observed behavior was higher compared to the control condition.

2.2 Ob secretomes supplemented with insulin or glucose

2.2.1 Adhesion receptor expression on ECs: simple obese secretomes supplemented with various concentrations of insulin or glucose

Tracking the expression at 24h of the surface receptors of ICAM-1 on HUVECs by immunocytochemistry, we observed macroscopically a significant augmentation in their expression in EC exposed to the Ob secretomes as compared with the ones exposed to the control medium. Regarding data obtained after the addition of insulin or glucose at maximal concentrations to the secretomes, results showed no significant differences between ICAM-1 expression in EC exposed to the Ob secretomes alone or in the presence of glucose/insulin. (Fig.32 results for ICAM-1). Besides direct optic microscopy analysis, results were confirmed by the computed quantification analysis.

A)



B)

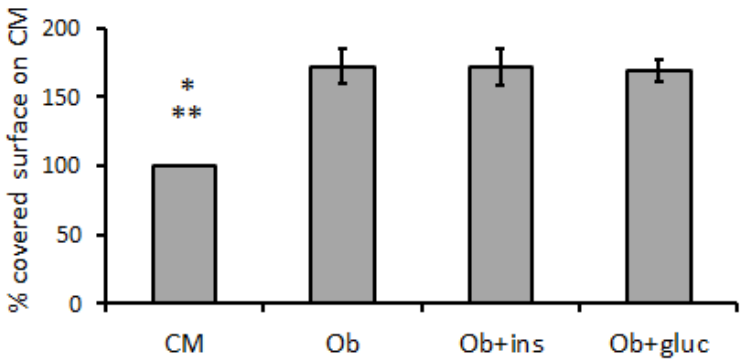
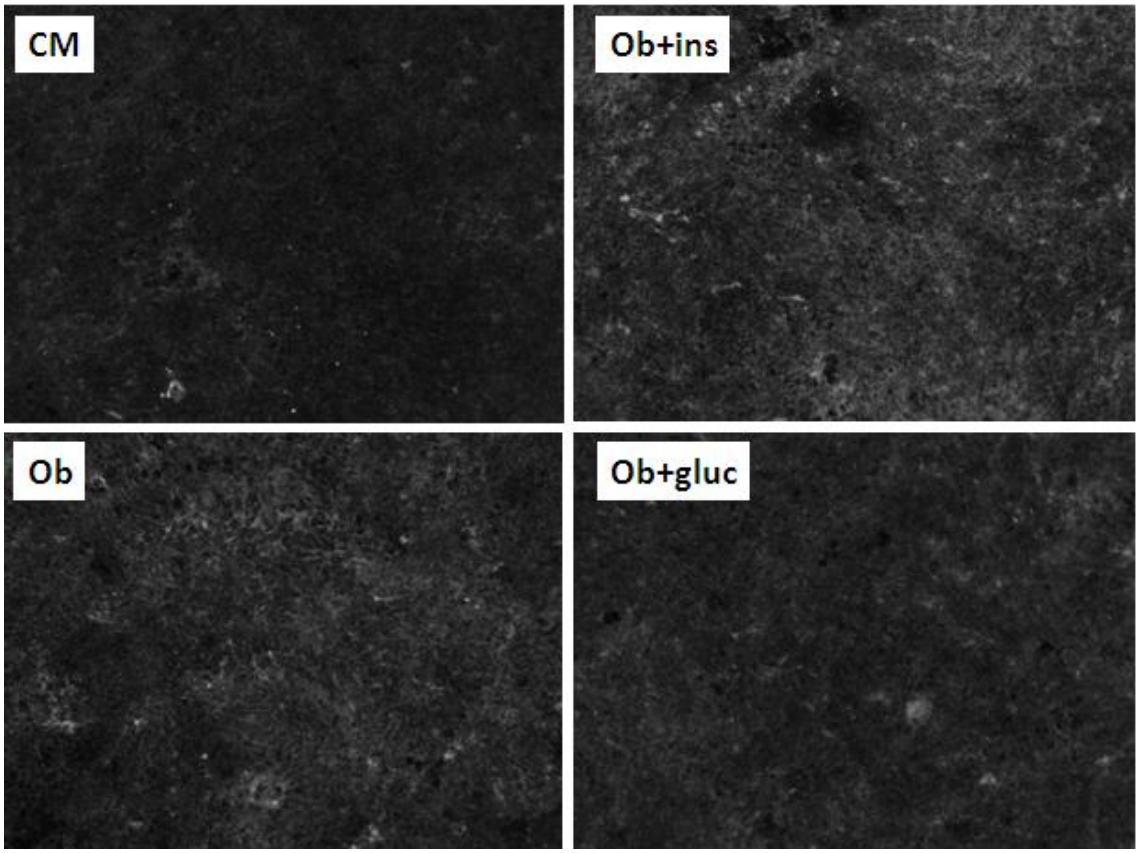


Fig.32 (A) Expression of **ICAM-1** membrane receptors in cells stimulated 24h with control medium (CM) and simple obese secretome, alone (Ob), with high insulin at 10^{-9} M (Ob+ins) or high glucose concentration at 16mM (Ob+gluc). **(B)** Quantification of obtained images: bars representing % total surface covered. Data are expressed as mean fold ± SEM. n=4. *$p < 0.05$ for CM in respect to Ob+gluc and **$p < 0.005$ for CM in respect to the other conditions. Images obtained by Immunogold assay performance.

2.2.2 Thrombogenicity of the subendothelial matrix: Prothrombotic subendothelial response after exposure to simple obese secretomes supplemented with glucose or insulin

In continuation we wanted to quantify the expression of the subendothelial **VWF** when cells were stimulated with simple obese secretomes supplemented with maximal concentrations of insulin and glucose. Their response to the simple obese secretome remained unchanged by addition of insulin at highest concentration. After exposure to glucose, endothelial cells presented a tendency to reduce the expression of the prothrombotic VWF (Fig.33).

A)



B)

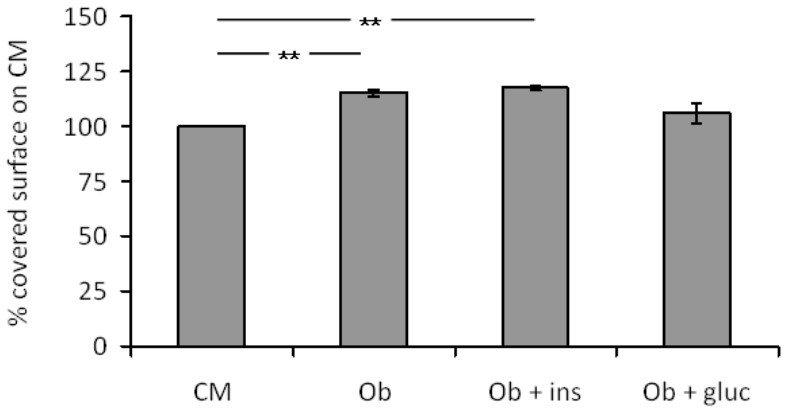


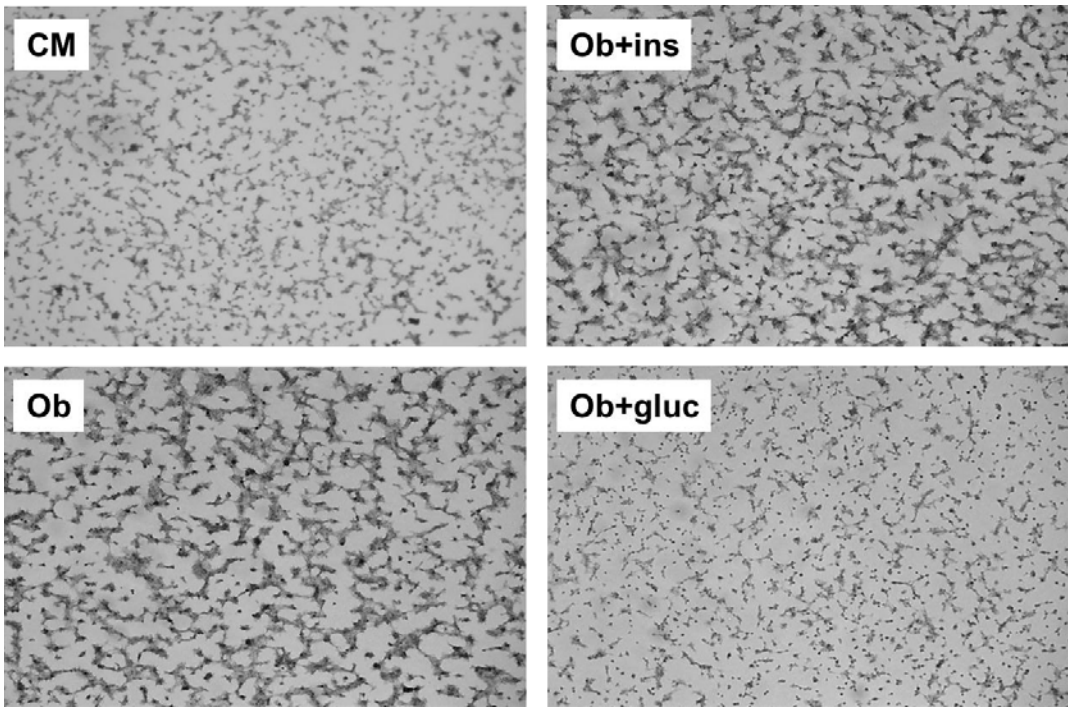
Fig.33 (A) Expression of **VWF** in EC stimulated for 7 days with control medium (CM), simple obese secretome (Ob), obese secretome plus insulin at 10^{-7}M (Ob + ins) and obese secretome plus glucose at 16mM (Ob + gluc). **(B)** Quantification of obtained images: bars representing % surface covered in respect to the control. Data are expressed as mean fold ± SEM. n=4. $^{**}p \leq 0.005$ where indicated. Pictures obtained by immunofluorescence assay.

Although the TF gene analysis indicated an increased expression in response to the conditions under study, no changes were detected regarding the expression of this protein at the extracellular matrix level. These results suggest that TF is not released by endothelial cells under the conditions explored.

2.2.3 Thrombogenicity of the subendothelial matrix: Platelet adhesion on the ECM created from ECs after exposure to simple Ob secretomes complemented with insulin or glucose

Blood perfusion of the ECM of HUVECs showed an equal platelet adhesion on the ECM generated from endothelial cells that had been cultured with the simple Ob secretomes and with or without insulin, in respect to the ones incubated with control medium (Fig.34). Interestingly, the ECM synthesized in the presence of glucose induced a significantly decreased platelet adhesion.

A)



B)

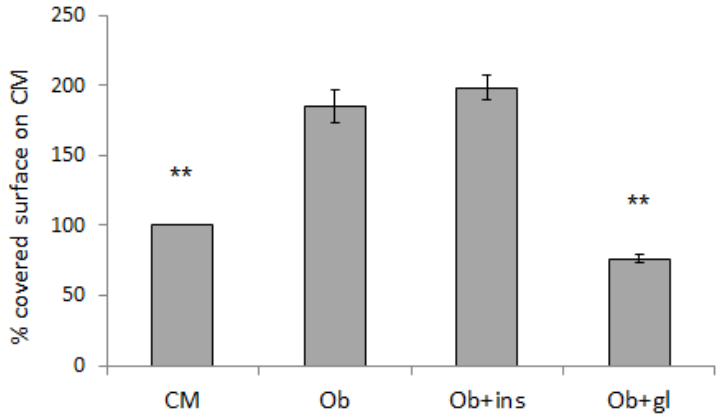


Fig.34 (A) Platelet adhesion in HUVECs after perfusion of citrated blood ($800s^{-1}$, 5min) in HUVECs cultivated for 7 days in control condition (CM), simple obese secretome (Ob) stimulation and secretome plus insulin at 10^{-7} M (Ob + ins) or glucose at 16mM (Ob + gluc). **(B)** Quantification of platelet deposition. Results are expressed as mean fold ± SEM as % of change regarding the control. n=3. For CM, $^{**}p < 0.005$ for the rest of the conditions. For Ob+gl, $^{**}p < 0.005$ for the rest of the conditions and CM.Pictures obtained by blood perfusion assay.

2.2.4 Summary Ob supplemented with insulin or glucose

Regarding the addition of insulin or glucose to the secretome, our results in general confirmed that insulin did not induce a surplus of injury on the endothelial cells, while very high concentrations of glucose provoked a significant decrease in platelet adhesion and a similar tendency in the expression of the VWF.

3. Comparative analysis of the cytokines from simple Ob vs. DMO secretomes

The obtained results indicated that both the obese (Ob) and diabetic obese (DMO) secretomes from metformin-treated patients induced an increased proinflammatory and prothrombotic endothelial injury. For that reason, in order to understand the changes in cytokine composition of the secretomes of the DMO patients treated with metformin, we comparatively examined their pattern with the adipose tissue secretomes of the simple Ob subjects. To this purpose, we used a flow cytometry determination method that was able to measure the levels of 6 specific adipokines that have been previously reported to be associated with the atherothrombotic disease in obesity and in the general population: interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-1b (IL-1b), tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12) and one described as protective cytokine, respectively interleukin-10 (IL-10).

Moreover, in this experiment we used as negative internal control the secretomes of adipose tissue from normo-weight patients (CT).

Overall, adipokines were present in a very low level in the CT secretome samples. This concerns interestingly also IL-10 which seems to have a protective role on the endothelium.

Except from IL-8, investigated adipokines were present in statistically significant greater amounts in the Ob secretomes as in the diabetic obese ones. IL-8 presented an opposite pattern, being considerably higher in the DMO secretomes. IL-12 level, although respecting the same pattern, did not reach statistical significance probably due to the high intersample variability (Fig.35).

To note that IL-8 and IL-6 were found in very high levels in the secretomes. Inflammatory IL-1b and anti-inflammatory IL-10 were inside the detection limits of less than 100 pg/ml, whereas the inflammation indicators TNF-α and IL-12 were present at the lowest levels, that was, at less than 10 pg/ml (Fig.35).

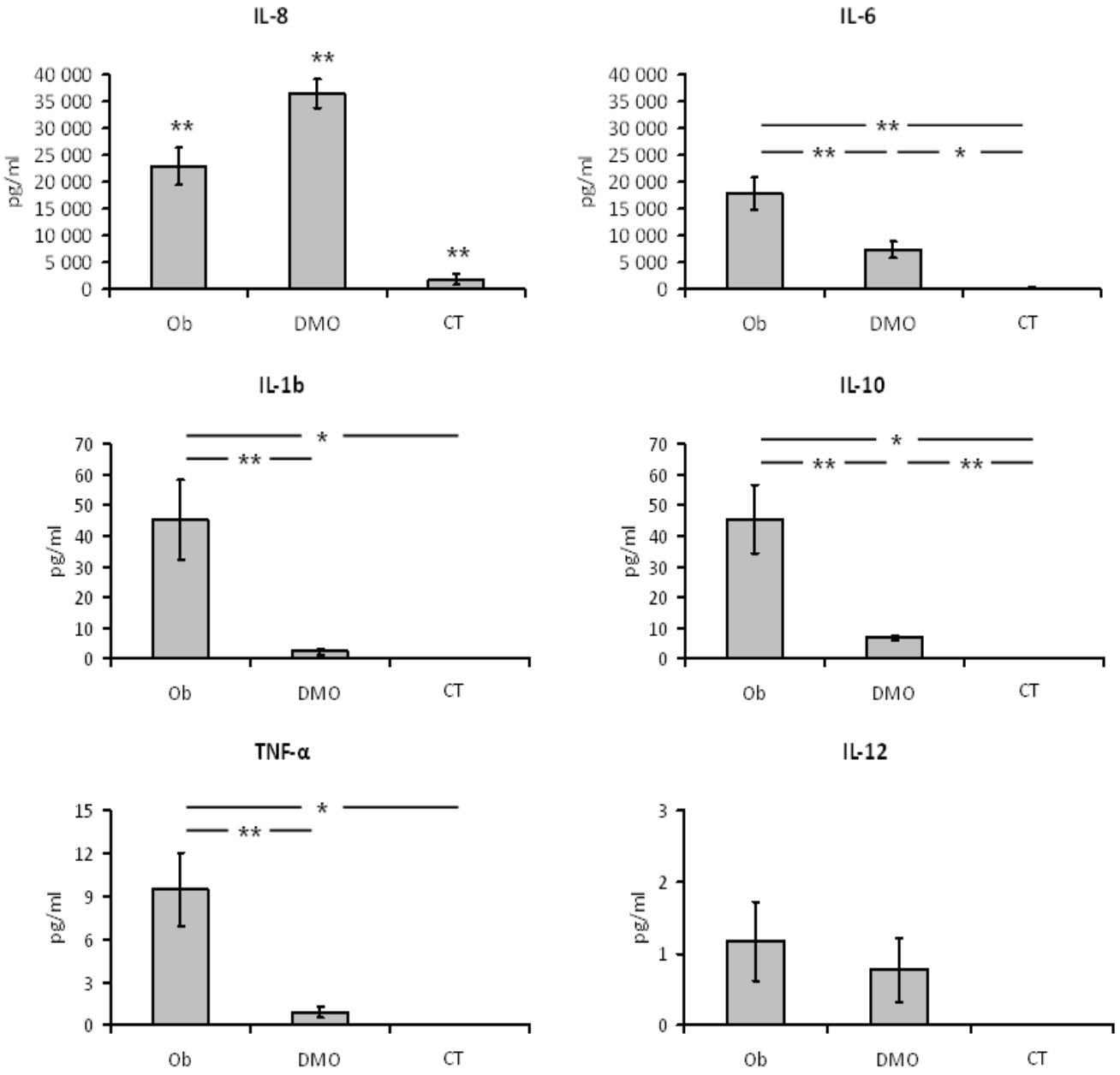


Fig.35 Cytokines expression in secretomes of obese (Ob), diabetic obese (DMO) and lean/normo-weight (CT) patients. Data are expressed as mean of obtained values ± SEM, representative of n=7 individuals for Ob and DMO, and n=4 for CT. *$p < 0.05$ and **$p < 0.005$ wherever indicated but from IL-8 referring to all conditions.

Summary/Conclusions

Compared to the diabetic obese secretomes originating from metformin-treated patients, the simple obese secretomes were, except from IL-8 and IL-12, the ones to have the majority of our studied cytokines elevated (including the protective IL-10).

4. Additional data concerning insulin uptake from the endothelial cells

As demonstrated above, insulin was not able to induce inflammatory and prothrombotic harm in the endothelial cells. In an attempt to find out about the grade of absorption of insulin from the medium by the endothelial cells, we performed an ELISA assay.

Therefore, we left the endothelial cells in their growth medium (containing human serum), adding insulin at an elevated concentration (10^{-9}M) on one hand and without addition on the other. Samples of the medium with and without insulin were obtained after 30 minutes, 3 hours and 24 hours, and measured with the ELISA method.

High insulin concentration was detected after 30 minutes of addition to the cells and declined with the pass of time (Fig.36), indicating its uptake by the endothelial cells. Insulin metabolism began after 30 min and reached the lowest level rapidly, at 3h.

Finally, this validated our decision to measure genetic expression after 24 hours of stimulation, since insulin starts to affect the cells at the beginning of its addition.

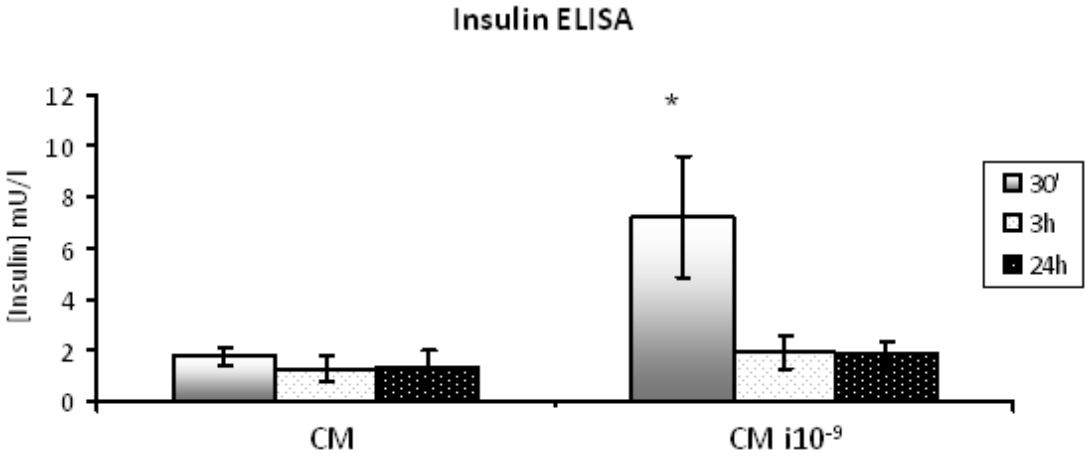


Fig.36 Insulin presence in normal growth medium (CM) at time specific measurements: 30 minutes, 3 and 24 hours after its addition to HUVECs. Null addition of insulin (CM) and at 10^{-9}M (CM i10^{-9}). Data are expressed as mean of obtained values ± SEM. n=4-6. *p < 0.05 in comparison to 3h and 24h at 10^{-9}M.

Summary/Conclusions

Insulin is greatly consumed by the endothelial cells already after half an hour of addition.

5. Metabolomics study of the obese adipose tissue secretomes

In the attempt to characterize the contribution of the obese adipose tissue secretomes to the metabolic systemic complications we performed a first metabolomic study of the visceral and subcutaneous obese adipose tissue secretomes in comparison with the ones from nonobese

subjects. The study of the diabetic obese secretomes is still under work and will not be subject of the present analysis.

Therefore, in the investigation performed for this thesis we compared the metabolic profile of visceral (VIS) and subcutaneous (SC) adipose tissue secretomes from nonobese (BMI= 24-26 kg/m^2) and obese subjects (BMI > 40 kg/m^2) with no other major metabolic risk factor than their own BMI.

After raw data filtering, retention time correction, peak alignment in the time domain and peak integration, XCMS generates a table containing the resulting integrated intensities for each m/z-retention time pair spectral feature detected. A feature is defined as a molecular entity with a unique m/z and a specific retention time. This data table was scaled to unit variance and used to fit four different PCA (Principal Component Analysis) multivariate models aimed at investigating the effect of obesity and fat pad regional differences on the secretomes metabolic profiles.

PCA is a method used for the unsupervised exploratory analysis of multivariate datasets deriving from high throughput analysis technologies. It involves a mathematical transformation of a number of possibly correlated input variables into a smaller number of uncorrelated variables called principal components. The projection of sample data into this new lower-dimensional variables subspace is called the PCA scores plot. Figure 37 shows the PC1/PC2 scores plots derived from the comparison of the obese and nonobese metabolic profile secretomes for either VIS-AT or SC-AT. This scatter plot provides a simplified qualitative overview on how secretome samples are related to each other in terms of their metabolic profiles i.e., similarity or dissimilarity among the metabolic profiles of secretome samples entering the study.

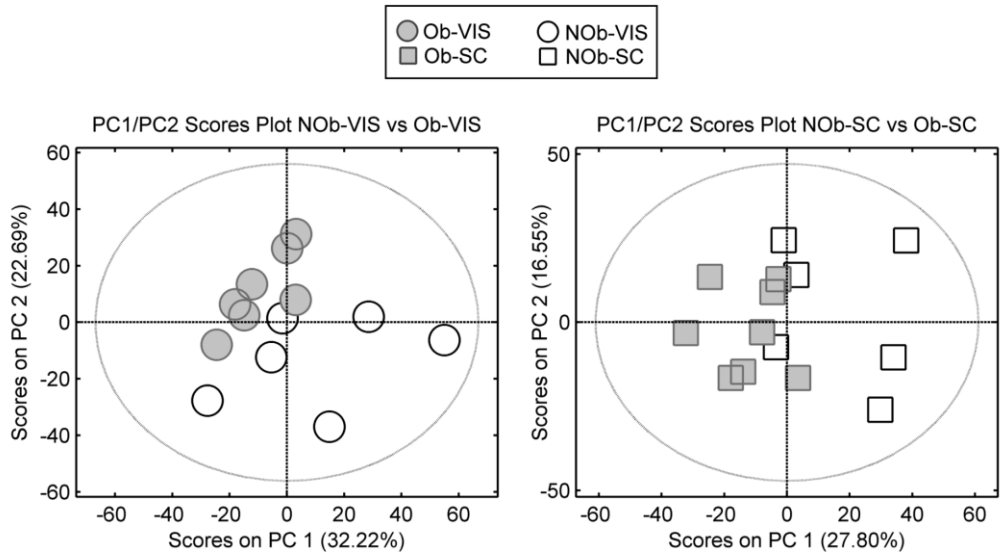


Fig.37 Principal component analysis (PCA) scores plot resulting from either VIS- (left panel) or SC-AT (right panel) secretomes metabolic profile comparison. Each point in the plot represents an individual secretome sample measurement. Samples close to each other present similar metabolic properties whereas those far from each other are dissimilar in terms of their metabolic profile. n=8 for Ob and n=6 for NOb.

Therefore, secretome samples corresponding to obese subjects presented a prevailing trend to cluster altogether and apart from secretome samples belonging to nonobese individuals for either VIS-AT or SC-AT. Nevertheless, PCA scores plot did not reveal any trend when comparing VIS-AT and SC-AT fat depots neither for obese nor for nonobese subjects. This first overview already indicated in a qualitative manner that obesity induced differential secretory metabolic patterns, an effect which resulted more pronounced for the VIS-AT depot.

We also analyzed the influence of obesity and the anatomical provenance of the fat pads on the metabolic profile of the secretomes from a univariate data analysis perspective. Thus, we applied multiple paralleled 2-way ANOVA on the entire list of metabolic features retrieved from XCMS. Data were rank-transformed before being submitted to 2-way ANOVA. After FDR correction for multiple testing, 31% and just 19% out of the initially detected features resulted significantly different due to obesity and regional fat origin, respectively.

Overall, the results show that obesity induces higher metabolic variation in secretomes than the regional provenance of fat *per se*. Next, we focused on the identification of those metabolites responsible for the higher differences related to obesity.

Obese and nonobese secretome samples from either VIS-AT or SC-AT were compared using the Mann-Witney test and FDR correction Features displaying significant differences were selected for further metabolite annotation.

In the case of the SC-AT secretome, we observed significantly lower levels of 2-KIC (2-ketoisocaproic acid) for the obese subjects as compared to the nonobese ones. Strikingly, all the other obesity-induced significant changes in the VIS-AT secretome were annotated as amino acids, namely essential ones (alanine, lysine, methionine, threonine), the branched-chain amino acid (BCAA) leucine, as well as glutamine and serine. We subsequently sought to determine the levels of the mentioned amino acids on the non-supplemented culture medium.

It should be considered that, unlike proteomics, metabolomic analysis of secretomes presents inherent challenges since media *per se* contain up to several dozen different metabolites. Glutamine and alanine were exclusively determined on secretome samples and were not detected in the non-supplemented culture medium. Therefore, they were considered as a product of the fat pads.

On the other hand, leucine, lysine, threonine, methionine and serine were detected in both the secretome and non-supplemented culture medium. Regardless of obesity, we consistently detected lower levels of these five amino acids in the secretomes as compared to the non-supplemented culture media, and we therefore considered them as uptaken metabolites.

Metabolite identification parameters together with statistical analysis details are summarized in Table 12.

Table 12. *Identification parameters and statistical summary for metabolites varying significantly in the Ob vs NOb comparison of VIS- and SC-AT secretomes‡*

	Retention Time (min)	Quantitative Ion (m/z)	p-values	q-values	Fold Change Ob vs NOb
Secreted Metabolites					
Alanine**	11.2	188	0.005	0.036	↑2.1
Glutamine**	14.4	246	0.022	0.081	↑2.8
2-KIC*, **	9.1	216	0.020	0.082	↓8.1
Uptaken Metabolites					
Leucine**	8.3	188	0.008	0.047	↑1.9
Serine**	9.8	219	0.014	0.062	↑1.6
Threonine**	10.2	130	0.022	0.081	↑2.2
Methionine**	11.8	61	0.035	0.098	↑1.5
Lysine**	17.0	174	0.004	0.036	↑3.1

‡Fold changes were calculated considering intensities of each quantitative ion corrected by internal standard, as the ratio of the median corrected intensities in the Ob group (n=8) relative to the NOb (n=6) group median. Arrow ↑ indicates significantly elevated and arrow ↓ significantly low levels of the metabolites in the Ob group secretomes. *Statistical differences among groups for SC-AT, and **comparison of retention times and spectral data to the corresponding pure standard compounds. $p < 0.05$ and $q < 0.1$.

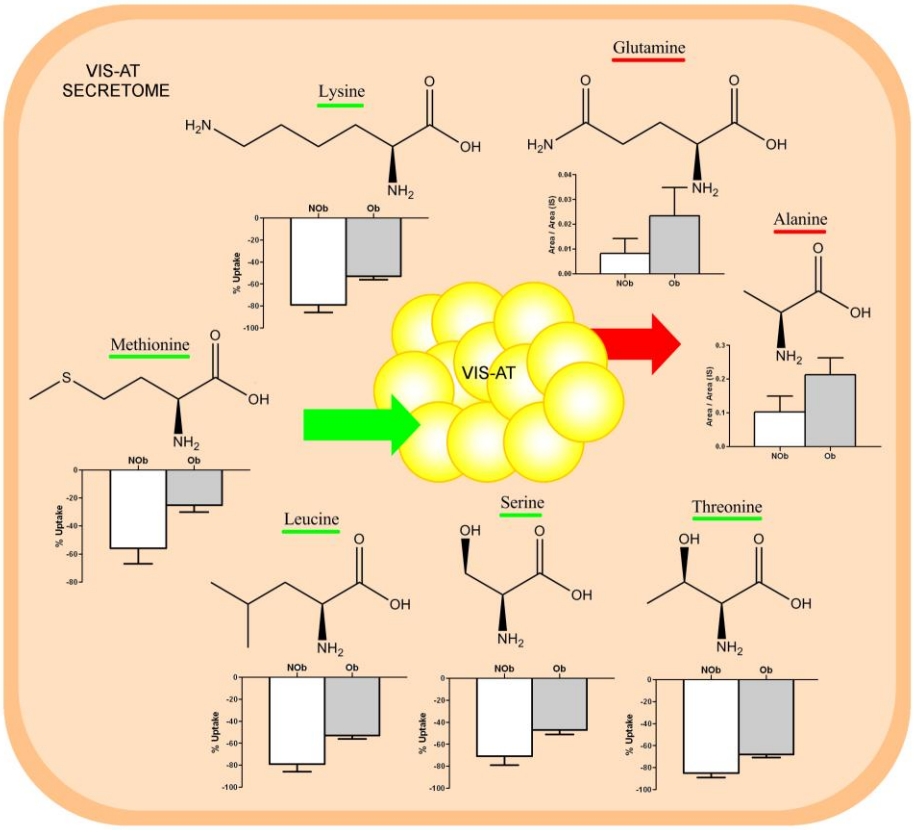


Fig.38 Significant metabolic changes detected in the VIS-AT secretomes of obese subjects. **Red**-underlined metabolites represent VIS-AT **secretion** and **green**-underlined metabolites represent VIS-AT **uptake**. For secreted metabolites, data is presented as mean ± SEM of the internal standard-corrected intensities for the corresponding quantitative ions. For uptaken metabolites, data is presented as mean ± SEM of the uptake percentage across all individuals in either Ob or NOb groups. The uptake percentage was calculated as the difference in percentage of the metabolite levels determined in the secretomes respective to the untreated media. Grey bars represent the obese group (Ob), and white bars represent the non obese group (NOb). n=8 for Ob and n=6 for NOb.

An overview of the significant metabolic changes detected in the VIS-AT secretomes of obese subjects is depicted in Fig.38. Higher released levels of glutamine and alanine were detected in the VIS-AT depot of obese subjects. On the other hand, VIS-AT of obese subjects presented a lower net uptake of leucine, lysine, threonine, methionine and serine.

Summary/Conclusions

Obesity rather than fat regional origin affects the metabolic signature of adipose tissue secretomes, and visceral obese fat pads present the greatest differences in the metabolite pattern of adipose tissue secretomes.

V. Discussion

The concept for the realization of this thesis was to further decipher the effect of the cytokines secreted by the visceral adipose tissue of obese individuals with type 2 DM on the endothelium, and moreover to contribute to the understanding of the complementary effect of hyperinsulinemia and hyperglycemia to this injury. As from ethical reasons it was not possible to obtain secretomes from diabetic patients before the initiation of their therapy, we have redirected our interest to patients treated with only metformin, the first line type 2 diabetes oral agent of worldwide use. Moreover, to a better understanding of the contribution of the adipose tissue's secreted cytoadipokines to the metabolic disease, we performed for the first time the comparative metabolomic analysis of obese and lean adipose tissue secretomes, the next step of our future investigation being the comparison of secretomes of obese *versus* obese diabetic, metformin-treated patients.

During the investigation regarding this thesis I had the opportunity to be involved in a translational research and therefore to develop not only my technical skills in molecular biology, but also to be initiated in the clinical and physiopathological knowledge of human diseases. Moreover, through the participation in the metabolomics study, I had the chance to work in an emerging -omics science.

Like in any scientific approach, the results presented have their own limitations and are open to debate. In the following pages I will discuss and decipher the major aspects and conclusions which emerge from the research we have done for this thesis.

As a result to the work performed during the thesis, two scientific papers have emerged. The first one, regarding the metabolomics study, is under review. The second one, regarding the effect of secretomes of visceral adipose tissue from obese diabetic metformin-treated patients on the endothelium, is yet in manuscript form.

1. Effect of the visceral obese adipose tissue secretomes of metformin-treated diabetic patients *versus* secretomes of simple obese subjects on the endothelial cells: is metformin changing the proinflammatory and thrombogenic effect of the obese adipose tissue secretomes?

As described in the introduction, obesity is characterized as a chronic state of inflammation, which not only deals with the effects straightforwardly connected to that state, but also drags with itself a number of other comorbidities. One of the negative outcomes strongly associated to obesity is diabetes mellitus type 2, a chronic disease with long-term important macrovascular complications[56].

Being cardiovascular morbidity and mortality the first-line complications in type 2 DM and obesity, risk factors for cardiovascular disease are most important nowadays in the clinical observation and account for several surveys. Complications in the macroendothelium comprise a state that has to be looked into regularly with acute and chronic prognosis, and undeniably portrays a disturbed life quality as well as life expectancy in the affected population.

We challenged ourselves in contributing to this field of investigation. The idea was to try to resemble situations where endothelial cells experience an obesogenic environment, alone, or accompanied by the diabetic state. Our laboratory had already introduced the concept of endothelial cell stimulation with the adipose tissue's secretome for the conduction of similar kinds of experiments, and what we did with this assignment was to bring the investigation on the matter

a step further, comparatively analyzing the injury induced on endothelial cells of the obese *versus* obese diabetic secretomes. As mentioned in the aims, unfortunately, for ethical reasons, we couldn´t obtain secretomes of untreated diabetic obese subjects. Therefore, we reoriented our investigations with the goal to understand the glucose lowering and insulin sensitizer independent effect of metformin, the actual first-line worldwide employed oral medical drug in type 2 diabetes, on the endothelial proinflammatory and prothrombotic activation determined by the cytoadipokines secreted from the visceral obese adipose tissue.

Metformin has been generally reported to reduce CV disease due to its proprieties to diminish endothelial oxidative stress, endothelial dysfunction, arterial stiffness and carotid arterial wall thickness [315,316]. But in obesity-associated diabetes, the aggression of the arterial wall has a multifactorial etiology. The endothelial injury determined by cytoadipokines emerging from the inflamed obese visceral adipose tissue maintains a proinflammatory systemic ambient and is a chronic stimulus for further activation of endothelial inflammatory pathways. As previously reported[206], cytoadipokines secreted from the obese adipose tissue directly determine the synthesis of adhesion molecules, prothrombotic factors and an increased platelet adhesion on the endothelium. In this sense, metformin has been reported to reduce systemic inflammation in obese insulin resistant and prediabetic patients [320,322,323] but controversy persists about its actions on inflammation and on the cardiovascular outcome in type 2 diabetic patients[321,324,337]. Metformin increases the fibrinolytic activity, slightly diminishes PAI-1 and decreases platelets' response to proaggregating agents [296]. *In vitro*, there is sustained evidence that metformin inhibits cytokine-induced nuclear factor-κB activation via PI3K-dependent AMPK activation in all vascular wall cells (HUVEC, vascular smooth cells and macrophages) [308,310,311,313]. Regarding its action on the synthesis and cytokine secretion of the obese adipose tissue's proinflammatory and prothrombotic cytokines, there is evidence from studies in animal models and human adipose tissue that metformin diminishes the local oxidative stress and reduces local inflammation and expression of chemerin and MCP-1 by activating the local diminished AMPK [330,338,339].

In our *in vitro* model we analyzed the proinflammatory and prothrombotic effect of the diabetic visceral obese adipose tissue secretomes, originating from metformin-treated patients, on the endothelium, compared to the exposure to secretomes of simple obese subjects.

The important limitation of our study remains the fact that we do not have the possibility to apply our initially proposed study model to the secretomes of diabetic obese patients, that is, before receiving any treatment. In this sense, we´ve based our comparison on the reported research data present in the literature. There is no comparative report on cytokine secretion between visceral adipose tissue from obese *versus* obese diabetic subjects. On the contrary, there is a heterogeneous abundance of data suggesting that inflammation increases with the increase in tissue mass and that obese tissue inflammation and insulin resistance increase in the diabetic obese visceral adipose tissue [340]. Moreover, data from various sera studies from type 2 diabetic patients report different serum profile of adipokines in function of the association with obesity or not [261,340,341,342,343]. Maybe the more complete data arrive from the study of Doupis *et al* who reported that diabetes and obesity equally affect the endothelial cell function, while smooth muscle cell function is affected only by diabetes, probably due to the different observed sera profile of cytokines and growth factors in the two entities. Interestingly, the obese diabetic patients from that study, all with a significant lower BMI (36.4 *versus* 38.1kg/m^2 for simple obese subjects) present, excluding IL-6 and IL-8 (probably due to the lower BMI and consequent visceral

adipose tissue), higher levels of all measured sera cytokines, like CRP, TNF-α, MCP-1, E-selectin, soluble (s) ICAM-1 and sVCAM-1 etc than the simple obese subjects. At the same time, the nonobese type 2 diabetic patients present, like in other studies, the lowest values of the mentioned markers [261,343]. Therefore, it seems that, once initiated, diabetes and obesity potentiate their deleterious effect on inflammation.

Regarding our clinical data from the obese diabetic patients treated with metformin, we observed that in this group the systemic inflammation parameter hs-CRP and the leukocyte count, although higher as in the obese group, were in fact lower than cumulative ones from BMI- and diabetes-expected values as compared to the study of Doupis mentioned above [261] (table 8). These lower values of hs-CRP can be due to the direct insulin sensitizer effect of metformin on the liver but can also reflect a lower hepatic cytoadipokine aggression in the metformin-treated obese patients.

Concerning our *in vitro* experiments, the results regarding the expression and synthesis of proinflammatory adhesion receptors, after exposure to the secretomes of the obese *versus* metformin-treated diabetic obese visceral adipose tissue, indicate that although at gene expression level there is a tendency to an increased endothelial inflammatory injury in the endothelium exposed to the secretomes of the diabetic obese subjects treated with metformin, at protein level there are no significant changes. Intriguingly, NF-κB presents an increased expression, but this remains without echo at the synthesis level of proinflammatory adhesion molecules. Nevertheless, overall both types of secretomes induce a significantly increased activation of endothelial inflammatory pathways with respect to the control media.

Expression and synthesis of prothrombotic subendothelial proteins present no differences between EC cultured with the distinct secretomes, remaining equally higher as compared to the control medium. Contradictorily, secretomes of diabetic obese subjects treated with metformin determine the synthesis of a less thrombogenic subendothelial matrix, as quantified by the reduced platelet adhesion, than the ones from simple obese individuals.

To understand the biological relevance of this setting, we investigated the cytokine content of both types of visceral adipose tissue secretomes (the ones deriving from the visceral adipose tissue of obese diabetic patients treated with metformin and the ones deriving from simple obese subjects), by quantifying some of the cytokines most relevant to cardiovascular injury.

Hence, we observed that metformin treatment had a heterogeneous effect on the cytokine profile of the obese adipose tissue, decreasing secretion of proinflammatory TNF-α, IL-12, IL-1b and IL-6 but also of the anti-inflammatory IL-10, while only increasing the synthesis of the deleterious IL-8. The same adipokines were, as predicted, very low or (nearly) absent in the secretomes originating from nonobese patients.

Generally we expected that proinflammatory cytokines would appear much more elevated in the secretomes of diabetic obese subjects, due to the deleterious supplementary effect of diabetes on obesity. In this meaning, our results are indicating that metformin determines a significant decrease in TNF-α, IL-12, IL-1b and IL-6, although IL-8 increases, maybe compensatorily. As mentioned above, it has been reported that in HUVECs metformin inhibits dose-dependently the TNF-α- and IL-1b- induced NF-κB activation, and IL-6 and IL-8 production. This effect takes place through the PI3K-dependent activation of AMPK, the basal mechanism of action of

metformin [317,318,319]. Our data indicate that metformin has a distinct effect on the cytokine secretion of the obese adipose tissue than that on the endothelium *in vitro* under a specific proinflammatory stimulus. Therefore, despite low levels of TNF-α and IL-1b in the secretomes of obese diabetic metformin-treated patients, IL-8 was higher than in the simple obese secretomes, while IL-6 diminished. The decrease of IL-10 could be interpreted as consequence to the diminution of the defence of the adipose tissue in front of the longstanding local inflammation.

Metformin-treatment diminished but did not completely inhibit the deleterious cytoadipokine secretion from the obese adipose tissue. Nevertheless, as repeated above, the cytoadipokines studied are only a representative selection of a higher amount of cytokines documented to be present in the obese adipose tissues' secretomes. That means that there could have been discrepancies in the presence of the adipokines, like the boost of IL-8 in DMO, and in this way we can explain the differences in the maintained endothelial inflammatory injury observed in HUVECs exposed to the secretomes of the diabetic patients treated with metformin. This is also confirmed by the fact that NF-κB activation is still increased. Anyway, it is important to note that the observed endothelial injury is similar to the one induced by the simple obese secretomes, being lower than predicted by the BMI and the diabetic state of the researched subjects. It seems that the changes in the cytoadipokines' pattern induced by metformin-.treatment blocked the repercussion of the endothelial activation of NF-κB by the diabetic secretomes at the level of VCAM-1 and ICAM-1. Not to forget that the significant increase in NF-κB activation could be due to the action of other metabolites of the obese secretomes due to the fact that NF-κB is implicated also in other stress dependent proinflammatory cellular responses in obesity and type 2 diabetes [340,344,345,346].

Corroborating the clinical and analytical data of the patients from our study with the experimental ones, our investigations are strongly indicating that metformin inhibits the secretion of a part of the deleterious cytoadipokines from the visceral obese adipose tissue, maintaining the proinflammatory endothelial activation as regarding proinflammatory adhesion receptors at the prediabetic obese level.

Going back to, and associating the data regarding the effect of metformin on the thrombogenicity of the subendothelial matrix, we observed, interestingly, that the highest antithrombotic effect of metformin was detected in our investigation regarding the thrombogenicity of the subendothelial matrix synthesized under the influence of the secretomes of the diabetic obese patients treated with metformin. Thus, our work provides further evidence that metformin has a beneficial role on thrombogenicity, not only regarding platelet action and synthesis of PAI-1 as found in the literature, but also regarding the synthesis of a less thrombogenic subendothelial matrix, even lower than that induced by the simple obese secretomes. This observation is sustained from the recent evidence from *in vivo* sera studies that have reported that metformin ameliorates platelet volume and function in treated type 2 diabetes patients [305]. The mechanism of this effect remains actually under study.

In **conclusion**, metformin presents a partially beneficial action on the deleterious secretion of cytokines from the visceral obese adipose tissue of type 2 diabetic patients.

Nevertheless, both secretomes of simple obese and diabetic obese patients treated with metformin induced a comparative inflammatory injury on endothelial cells, but the secretomes of

the obese diabetic patients treated with metformin induced the synthesis of a less thrombogenic subendothelial matrix.

2. Glucose and insulin proinflammatory and thrombogenic effect on the endothelial cells: is there an additional effect to the one induced by the visceral obese adipose tissue secretomes of simple obese subjects?

2.1 Effect of insulin on the macroendothelial cell model

In the last few years, the chronic low state of inflammation associated with obesity has emerged as a new risk factor for the obesity-associated cardiometabolic comorbidities. The debate about the role of inflammation *versus* hyperinsulinemia and insulin resistance in the genesis of macroendothelial injury is, as depicted in the introduction, despite the high number of studies performed on endothelial cells regarding the effects of hyperinsulinemia, still going on, as results have not been conclusive. Moreover, the consequences of hyperinsulinemia on a macroendothelial cell model, existing additionally in a high whole-adipogenic-derived inflammatory environment, has not been yet investigated *in vitro*. We attempted to enlighten the subject by studying the role of insulin in low to high concentrations in the complementary endothelial injury induced by the secretomes of the visceral adipose tissue, originating from morbidly obese patients.

Here we observed that simultaneous stimulation of ECs with insulin and secretomes for 24h induced only at the very high level of 10^{-7} M a slightly increased expression of the adhesion molecules VCAM-1, ICAM-1 and of the transcription factor NF-κB, with respect to the stimulation induced by the simple obese secretomes. Interestingly, these changes were not paralleled by the ones in the expression of the genes of the prothrombotic factors VWF and TF, as insulin appeared to have protective prothrombotic effects. Anyway, these results at gene expression level have not been confirmed at the protein synthesis level, as the synthesis of ICAM-1 was lower than in the ECs exposed only to the simple obese secretomes. Moreover, the synthesis of VWF and the platelet adhesion present equal levels between insulin supplemented or not to obese adipose tissue secretomes.

As previously commented, the increase in the expression of the nuclear transcription factor-κB could be determined by insulin by the latter's implication in other pathways than activation of cellular adhesion receptors.

Our data are in contradiction with the ones emerging mostly from the group of Madonna *et al* regarding the proinflammatory role of insulin. Even so, our results are mostly in accordance with publications reporting that insulin can exert potential anti-inflammatory and antiatherogenic effects in clinical trials and in experimental models, hence likely to be cardioprotective and to improve clinical outcomes [243,245,347].

To note that the kinetics of the experiments and the insulin concentration studies have been equivalent to the ones of Madonna *et al*, ranging from 10^{-11} to 10^{-7} mol/l which are extending from 1.7mU/l to $1.7x10^{4}$mU/l, as mentioned before. This span covers from pathophysiological to pharmacological levels of insulinemia, and are far over the insulin concentrations in fasting and postprandial states of obese hyperinsulinemia in the presence of insulin resistance [332,333]. Moreover, although the evidence of an endothelial cell-insulin receptor has been recognized long

before [333,334], we verified in our experimental model as earlier described the insulin uptake in HUVECs.

Our **conclusion** is that insulin was not able to affect in a distinct degree the already high proinflammatory and prothrombotic response of the endothelium to the obese secretome.

2.2 Effect of glucose on the macroendothelial cell model

In the introduction we mentioned that hyperglycemia can be a reason for cardiovascular complications, but that its levels of adjustment are a subject that has not been resolved. Even if glycemic control prevents the onset of microvascular complications in diabetic patients, recent clinical trials reported that low glucose levels can result worthless for the vasculature. Moreover, high glucose variations and the metabolic memory seem to have more echo on the arterial outcome than individual values *per se*. Data from *in vitro* experiments regarding the capacity of glucose to induce the inflammatory activation of ECs are controversial, as presented in the introduction.

Similarly as for insulin, we decided to make an evaluation of the effect of hyperglycemia on the activation of the endothelial injury mechanisms, in the presence of the deleterious cytoadipokines secreted from the obese adipose tissue. Therefore, we co-exposed ECs to the obese secretomes and glucose concentrations of 11 and 16mM, corresponding to 198 and 288 mg/dl glucose and characterize states of hyperglycemia.

Increasing concentration of glucose seemed to not result overall in any proinflammatory or prothrombotic additional damage to that already induced by the obese visceral secretomes. In detail, there was a tendency for glucose to peak at 11mM for VCAM-1, ICAM-1 and TF genes but afterwards descending to the values of simple obese secretomes, while VWF gene expression remained unchanged. NF-κB expression remained also unchanged following concomitant exposure to glucose. Interpretation of results is challenging due to the high variations between samples. Maybe in order to quantify more precisely the effects of glucose, the number of samples should have been increased. Anyway there were no clear changes.

Results were paralleled at protein expression level with the absence of difference in ICAM-1 in the presence of high concentration of glucose. As for the VWF, it seemed to display a tendency towards lowering down its expression with glucose. Intriguingly, platelet adhesion to the subendothelial matrix generated by the EC exposed to the secretomes and glucose was strongly diminished, less than the level of the control medium. Explication of this decreased platelet adhesion could be on one side a positive effect of glucose on EC, or, on the other, the fact that glucose exerts toxic effects on ECs determining the alteration of their normal growth. At optic microscopy level, ECs presented in both conditions a similar aspect, without changes in their morphology and without intracytoplasmic inclusions, reaching confluence after the same number of days in both conditions.

Therefore our data are supporting previous ones from the literature that provide evidence about the fact that hyperglycemia is not sufficient to directly promote endothelial inflammatory activation [262,263,265-267]. There are scarce data in the literature about the thrombogenicity of the macroendothelial ECM under hyperglycemic conditions (most researches concerning platelet

volume and reactivity), which seem not to be affected in type 2 diabetes. Anyway there have been made no studies about the impact of the glycemic control on this finding [348,349,350].

Our experiments brought us to the **conclusion** that high glucose was incapable of producing a surplus of inflammatory injury to the one determined by the cytoadipokines of the secretomes on the endothelial cells. Probably, endothelial damage of hyperglycemia is mediated predominantly through the oxidative stress generated by the advanced glycation end-products.

3. Metabolomic comparative analysis of obese and lean visceral and subcutaneous adipose tissue secretomes: is the metabolic obese secretome pattern of the visceral adipose tissue indicative of the implication of the adipose tissue-emerged metabolites in the systemic metabolic disease?

In order to understand the contribution of the obese adipose tissue-emerged metabolites to the systemic metabolic disease associated with obesity, we performed a comparative analysis of the secretomes of visceral and subcutaneous fat pads of obese and nonobese subjects, by applying an untargeted metabolomics approach. To our knowledge, this is the first comprehensive differential report on metabolomics of secretomes of different regional adipose depots isolated from obese subjects. Study participants presented no other metabolic disturbances than their BMI, and because of that, they presented signs of systemic inflammation, such as increased high sensitive C reactive protein, insulin resistance, and decreased adiponectin. The goal of our next investigation, actually under work, is to further decipher the comparative metabolomics of the secretomes of simple obese and diabetic obese subjects, but is not the topic of the actual thesis.

Strikingly, although untargeted GC-MS-based metabolomics allows for the nonbiased screening of a wide range of chemical classes (sugars, short and medium chain fatty acids, steroids, amino acids or organic acids, among others), our results demonstrated that amino acids accounted for the main differences observed in the secretory pattern of visceral and subcutaneous fat depots of nonobese and obese individuals with no metabolic risk factors other than their own BMI. Supporting data from previous studies [351], our work reports significant differences in essential, branched-chain amino acids (BCAA) and nonessential amino acids, pointing out that obesity overall blunts amino acid metabolism in adipose tissue and profoundly affects BCAA catabolism. Moreover, our results demonstrate that the effect of obesity upon the secretory metabolic pattern of the adipose tissue is more profound than the effect of the regional provenance of the fat tissue, being the more affected the metabolism of obese visceral adipose tissue, in line with our previously reported results regarding holistic changes in obese adipose tissue [206].

Already decades before, the obese state has been associated with an increased plasmatic level of amino acids [352]. Recently the plasma amino acid profile has been found to be altered according to visceral fat accumulation [353]. It is also a fact that amino acids contribute directly to hyperinsulinemia through direct pancreatic beta cell stimulation and gluconeogenesis [354,355,356]. What is more, hyperaminoacidemia affects insulin action and glucose cellular uptake [232,357,358,206,359,360]. Nowadays, it seems reasonable to conclude that the plasmatic amino acid excess in obesity is at least in part the consequence of widespread hyperproteic and hyperlipidic diet**.** At any rate, the role of chronic exogenous amino acid overload, with particular emphasis on the highly represented BCAAs in the etiopathogenesis of metabolic alterations associated with obesity,

has been under debate for decades [289,361]. Recent metabolomic sera studies of obese individuals reported notable differences in the amino acid metabolic signatures between obese, mild insulin-resistant but nondiabetic subjects and lean individuals [289], while a decrease in a cluster group of metabolites comprising BCAAs and related analytes predicts improvement in insulin resistance (HOMA-IR), independent of the amount of weight lost [362]. Moreover, recent clinical evidence from the Framingham offspring study indicated increased levels of a group of five BCAA and aromatic amino acids (namely isoleucine, leucine, valine, phenylalanine and tyrosine) as candidate metabolic risk markers for diabetes in obesity, predating the clinical onset of diabetes by years [293]. On the contrary, a low glutamate to glutamine ratio would exert a preventive role on the cardiometabolic status [294].

Amino acids are not stored in the body. There is a highly active interorgan transport, and dietary amino acids in excess of those required for protein synthesis or gluconeogenesis, are rapidly catabolized [363]. BCAA metabolism in adipose tissue has recently gained renewed interest, as it has been demonstrated that coordinated regulation of adipose tissue BCAA enzymes in fasting and in feeding may modulate circulating BCAA levels. In this sense, a transcriptional study on subcutaneous adipose tissue biopsies from a cohort of monozygotic twins discordant for obesity reported decreased mitochondrial BCAA catabolism for the obese twins in parallel with the onset of systemic and local adipose tissue inflammation. The down-regulation of BCAA oxidation enzymes was paralleled by decreased levels in plasma of 2-KIC acid and increased levels of leucine [206,364]. These changes correlated with elevated fasting insulin levels and insulin resistance [206,365]. Contrarily, another recent study on type 2 diabetic and obese youth infirms these findings, indicating the presence of an adaptive metabolic plasticity in youth that equilibrates fatty acid and amino acid metabolism [366].

We found both decreased secreted levels of 2-KIC and diminished leucine uptake in the obese subcutaneous or visceral fat depots, respectively. 2-KIC is the transamination product of leucine, i.e, a leucine breakdown product. This confirms data from indirect functional in vivo and ex vivo proteomic and transcriptional studies on adipose tissue in the fasting state [351,367,368]. The dysregulation of leucine metabolism seems to be higher in the visceral obese adipose tissue, eluding the formation of 2-KIC.

On the other hand, we have determined increased released levels of both glutamine and alanine in the obese visceral adipose tissue secretomes. Adipose tissue alanine and glutamine released in quantities sufficient to make a significant contribution to the whole body economy of these amino acids has been previously reported [369,370]. Catabolic pathways of BCAA leucine through the KIC acid route involve the formation of important amounts of alanine, glutamine and glutamate. These pathways are the route for disposal of amino groups released in the transamination of BCAA[232,289,206,369]. Therefore, since alanine and glutamine are highly gluconeogenic amino acids, the increased amount of alanine released by the visceral adipose tissue to the systemic circulation could contribute to hyperinsulinemia and to the development of insulin resistance.

It is worth mentioning that in the visceral adipose tissue of obese subjects, the uptake of leucine and other reported glucogenic (methionine, serine, threonine) and exclusively ketogenic (leucine) amino acids was found to be diminished. This is indicative of profound deviations in local amino acid metabolism and results in increased release in the circulatory flux of amino acid metabolites with gluconeogenic potential, as well as unmetabolized BCAA with demonstrated direct insulin-resistant cellular consequences.

Altogether, **our findings point to** a blunted amino acid metabolism and overload of BCAA catabolism in the inflamed and lipid-overcharged obese adipose tissue. This would, in turn, independently if primary or secondary to the exogenous overload, essentially contribute to the altered and increased plasmatic amino acid metabolite pool, sustaining the deleterious actions of amino acids on the onset of metabolic carbohydrate disturbances in obesity.

Our results demonstrate that the early stages of obesity, including the presence of systemic inflammation and insulin resistance, before the clinical onset of significant metabolic alterations other than the BMI, are characterized by a markedly affected adipose tissue- amino acid metabolism and secretion. Further combined functional and metabolomic approaches are awaited to understand the dynamic alteration of amino acid metabolism in the obese state and the specific contribution of the adipose tissue-emerged metabolites to the metabolic systemic disease.

VI. Conclusions

Taking on account the results obtained from this thesis under the conditions applied, we conclude following:

1. ***Treatment with metformin*** presents a partially beneficial action on the deleterious secretion of cytokines from the visceral adipose tissue of type 2 diabetic obese patients.

   Both secretomes of simple obese and diabetic obese patients treated with metformin induced a comparative inflammatory injury on endothelial cells, but the secretomes of the obese diabetic patients treated with metformin induced the synthesis of a less thrombogenic subendothelial matrix.

2. ***Insulin*** does not increase the expression of proinflammatory adhesion molecules and the thrombogenicity of the subendothelial matrix of HUVECs by the cytokines secreted from the obese visceral adipose tissue.

   Pharmacological levels of insulin induce the increased expression of NF-κB.

3. ***Glucose*** does not determine an increase of the endothelial proinflammatory and prothrombotic injury provoked by the cytoadipokines of the visceral obese secretome in HUVECs.

   High levels of glucose, though, seem to decrease importantly the plaquetary adhesion capacity when acting in combination with the secretome.

4. **Obesity,** more than the regional distribution of fat pads, markedly affects the ***metabolic profile*** of **adipose tissue secretomes** before the clinical onset of other significant metabolic alterations aside from BMI. Visceral obese adipose depots present the greatest alterations.

   Amino acid metabolism is blunted and BCAA catabolism overloaded in the obese adipose tissue. This may contribute to the altered and increased plasmatic amino acid metabolite pool, sustaining the deleterious actions of amino acids on the onset of metabolic carbohydrate disturbances in obesity.

VII. References

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Resumen

Introducción

1. La obesidad: una enfermedad en todo el mundo

La obesidad se define como la acumulación de tejido adiposo excesivo o anormal que puede perjudicar la salud. El estado de obesidad designa un aumento desproporcionado de peso con respecto a la altura, el sexo y la edad. Una cuantificación simple de esta relación utilizada comúnmente para clasificar el sobrepeso y la obesidad es el índice de masa corporal (IMC), calculado dividiendo el peso de una persona en kilogramos (kg) por el cuadrado de su altura en metros (m) (**IMC = kg / m^2**).

Según la Organización Mundial de la Salud (World Health Organization, WHO), la obesidad en los seres humanos se clasifica en base a un IMC ≥ 30. La misma organización sostiene que el principal responsable de la obesidad es el desequilibrio energético entre las calorías consumidas y las calorías gastadas, aparte del impacto sobre este balance a los efectos dietéticos, económicos, psicológicos, reproductivos, cultural y farmacológicos. Se han reconocido más de 50 genes vinculados o no con la herencia de la obesidad, pero la obesidad sigue siendo predominantemente una enfermedad poligénica compleja. Además, la obesidad es atribuible también a los cambios epigenéticos.

Sin dudas, la obesidad comenzó como un problema de salud pública en las sociedades con un alto estatus socioeconómico, principalmente los Estados Unidos y Europa. Pero tan pronto como los países en desarrollo experimentaron cambios en su dieta, la carga de la obesidad se desplazó hacia las sociedades más pobres. Países tan diversos como México, China, Tailandia y Sudáfrica muestran aumentos espectaculares en la obesidad. Preocupante es también la incrementada prevalencia de sobrepeso en los jóvenes, alcanzando hasta el 25,6% en Estados Unidos y el 10-36% en el continente europeo.

La distribución de la grasa es un factor importante a tener en cuenta, y la medición de la grasa visceral es un mejor factor de predicción de riesgos para la salud que la grasa corporal total. Muchas técnicas se han establecido para la medición de la distribución de la grasa corporal, incluyendo el IMC, la circunferencia de cintura, impedancia bioeléctrica, etc.

Las comorbilidades asociadas a la obesidad son debidas al exceso de tejido adiposo en sí, y a las alteraciones metabólicas y / o psicosomáticas inducidas por el exceso de la masa adiposa. Reducen la esperanza de vida y suponen aumento en la hipertensión arterial, las enfermedades cardiovasculares (ECV), la dislipidemia y la diabetes .

2. Diabetes Mellitus Tipo 2

Según la Asociación de Diabetes Americana (ADA), diabetes mellitus (DM) se define como un grupo de trastornos metabólicos caracterizados por hiperglucemia crónica resultante de defectos en la secreción y/o acción de la insulina. Los criterios para el diagnóstico de la diabetes se basan en cifras de glucosa en plasma en ayunas ≥ 126 mg/dl.

La diabetes tipo 2 representa el 90-95% de los casos de diabetes. Presenta una prevalencia creciente y subyacen defectos que pueden variar desde resistencia a la insulina predominante con deficiencia de insulina relativa, a un defecto predominante de secreción de insulina de las células beta con resistencia a la insulina. Se presenta con mayor frecuencia en los adultos que en los niños y aparece asociada a obesidad, falta de actividad física, en hipertensión, dislipidemia, etc. Se han

estudiado varios genes candidatos como posiblemente implicados en la etiología de la DM2, pero la evidencia actual indica que se trata de una enfermedad genética compleja.

En las últimas tres décadas el número de personas con DM en todo el mundo se ha duplicado. La prevalencia mundial está aumentando rápidamente en poblaciones que han sido industrializadas en un corto período de tiempo. Asia se ha convertido en el "epicentro de diabetes" en el mundo, como resultado de su rápido desarrollo económico, seguida por la región del Golfo y África. La diabetes tipo 2 es cada vez más frecuente en adolescentes obesos. 25% de los niños y adolescentes de 21% con grado severo de la obesidad, independientemente de la etnia. En Europa, la prevalencia de la enfermedad en los países europeos también se estima que aumentará en los próximos 10 años.

La DM tipo 2 es una enfermedad crónica con una alta morbilidad y mortalidad cardiovascular. Los pacientes tienen un mayor riesgo de retinopatías y nefropatías entre otras complicaciones. El tratamiento de la DM tipo 2 además de medicación requiere cambio en los hábitos de vida. Para el tratamiento farmacológico de DM tipo 2, se usan agentes orales, tales como las biguanidas (por ejemplo metformina), sulfonilureas, inhibidores de las alfa-glucosidasas, sensibilizadores de la insulina (como pioglitazona), inhibidores de DPP-IV y agentes miméticos de incretina.

3. Aterotrombosis

El endotelio vascular constituye el revestimiento interior celular de los vasos sanguíneos. Ha surgido como un regulador clave de la homeostasis vascular y alteraciones en su función pueden conducir a tales disfunciones.

EL subendotelio subyace del endotelio e implica la matriz extracelular sintetizada por las células endoteliales, que contiene numerosas proteínas, como el colágeno, factor de von Willebrand (VWF, importante en la cascada de la coagulación), la laminina, metaloproteinasas (MMPs) y el factor tisular (TF, implicado en el mantenimiento de la hemostasis vascular).

La **adhesión celular** es un proceso esencial necesario para el correcto funcionamiento de los organismos multicelulares. Moléculas de adhesión celular (CAMs) están involucrados en una variedad de procesos: la adhesión célula-célula y célula-matriz, la migración celular, la activación de los linfocitos y la iniciación de la respuesta inmune. La superfamilia **Ig** de receptores de adhesión consiste en las proteínas de la superficie celular que están implicadas en la fase final de la adhesión celular de los granulocitos y monocitos al endotelio. Los miembros de esta familia expresadas por las células endoteliales, implicadas en la adhesión de leucocitos, son las moléculas de adhesión intercelular (ICAM)-l y II-y la molécula de adhesión celular vascular-l (VCAM-1).

<u>El endotelio en la trombosis arterial</u>

Aterotrombosis arterial consiste en la disminución progresiva del calibre de la pared del vaso como consecuencia de la acumulación local de colesterol y la formación de las placas ateroscleróticas. La placa abruptamente puede romperse o el endotelio puede erosionar, lo que resulta en la exposición de material trombogénico, incluyendo FvW y TF, y la formación de un trombo en el lumen. Si el trombo es lo suficientemente grande, se bloquea la arteria induciendo un síndrome coronario agudo o infarto de miocardio o un accidente cerebrovascular. Si la placa no se rompe y la lesión continúa creciendo, la lesión puede invadir el lumen y consecuentemente

reducir la cantidad de sangre suministrada al tejido adyacente, lo que conduce a la hipoxia sintomática de este último.

Papel de la inflamación en la aterogénesis

La aterosclerosis hoy en día no es sólo debido a la hipercolesterolemia, sino también a complejidad de factores tales como la hiperglucemia crónica y la inflamación, local y sistémica. Las causas patógenas de la inflamación local y sistémica han sido subestimadas durante mucho tiempo, pero la presencia de inflamación sistémica es evidente incluso antes de la detección del infarto de miocardio. La inflamación inicia la lesión aterosclerótica y contribuye a la formación de la placa compleja, debilita la capa fibrosa (que causa la ruptura de la placa fácilmente) y mejora la trombogenicidad del núcleo lipídico.

Como marcadores potencialmente útiles de la inflamación han sido reconocidos entre otros las CAMs, interleucinas (IL-6,-1β, -8, -10, -12), TNF-α, MCP-1 y biomarcadores de la matriz extracelular (VWF, TF).

4. Los factores que contribuyen a la enfermedad cardiovascular en la obesidad y la diabetes mellitus tipo 2

El tejido adiposo blanco es un sitio de almacenamiento de energía principalmente para triacilglicéridos. Hoy en día se ha demostrado poseer un papel metabólico complejo, que consiste en secretar y liberar una variedad de proteínas y biomoléculas, interaccionando con otros tejidos y órganos. Además, se le ha atribuido una actividad pro-inflamatória (secreción de citoquinas) que puede activar directamente los mecanismos de lesión endotelial.

La obesidad es capaz de estimular tanto la inflamación crónica como el desarrollo de resistencia a la insulina. Sin embargo, la insulina se ha demostrado que tiene efectos anti-inflamatorios, así como efectos proaterogénicos, proinflamatorios y otros. No obstante, el impacto de la hiperglucemia es objeto de controversia. En base a ello, investigar, **como la glucosa, la insulina y la DM tipo 2 alteran el efecto de las adipoquinas secretadas por el tejido adiposo obeso, ha sido uno de los objetivos de esta tesis.**

La **metabolómica**, un método relativamente nuevo de la biología molecular, se centra en la medición del estado fisiológico de los metabolitos de un organismo causada por factores tales como la dieta, estilo de vida, ambiente, efectos genéticos y fármacos. Es principalmente funcional en fenotipificación, en el descubrimiento de biomarcadores, en el diagnóstico de enfermedades, y hasta la fecha, se sabe poco de su contribución a la caracterización de los metabolitos en suero, en la aparición de la diabetes y de la obesidad.

5. El papel de la metformina en el tratamiento de la diabetes tipo 2

El principal modo de acción de la metformina -el fármaco de primera línea como antidiabético oral- es la supresión de la gluconeogénesis hepática. Esto reduce los niveles circulantes de glucosa, aumenta la sensibilidad hepática a la insulina, y reduce la hiperinsulinemia asociada con la resistencia a la insulina.

El mecanismo molecular por el cual la metformina actúa es inhibir la producción de glucosa hepática celular mediante la activación de la proteína quinasa activada por AMP (AMPK),

una enzima hepática importante en la señalización de la insulina, en el balance de energía de todo el cuerpo y en el metabolismo de la glucosa y grasas .

Como se sugiere por los datos de los ensayos clínicos, la metformina reduce la mortalidad cardiovascular y también la morbilidad cardiovascular, debido a su efecto reductor de glucosa. Además, la terapia con metformina también disminuye la sensibilidad a los agentes de agregación plaquetaria, probablemente debido a la reducción de los niveles de glucosa en la sangre.

Objetivos

Con el fin de avanzar en la comprensión de los procesos fisiopatológicos implicados en la aterotrombosis y la diabetes tipo 2 en la obesidad, hemos propuesto los siguientes objetivos:

1. Investigar los efectos de las concentraciones fisiológicas y fisiopatológicas de insulina y glucosa en la lesión endotelial proinflamatoria y protrombótica inducida por las citoquinas secretadas por el tejido adiposo obeso visceral.

2. Investigar el posible efecto protector de la metformina sobre la lesión endotelial, inducido por citoquinas provenientes del tejido adiposo visceral de los sujetos obesos con diabetes tipo 2 de diabetes establecida.

3. Estudiar el impacto de la obesidad sobre el metabolismo de los depósitos de tejido adiposo regionales, o sea, visceral y subcutáneo, comparando el perfil metabólico de secretomas de tejido adiposo obeso visceral y subcutáneo con otros similares aislados de sujetos delgados.

Debe considerarse que los pacientes obesos con diabetes establecida se han sometido a cirugía bariátrica sólo después de la iniciación del tratamiento con metformina. Por esta razón, el estudio de los secretomas de tejido adiposo visceral de los pacientes obesos con diabetes establecida sin ningún tratamiento fue, por razones éticas, no posible.

Resultados

1. Expresión génica en células endoteliales (CE)

La expresión de genes se realizó con el fin de evaluar los cambios proinflamatorios y protrombóticos introducidos por el secretoma de tejido adiposo visceral de obesidad mórbida u obesidad mórbida de sujetos diabéticos tipo 2 tratados con metformina, en las CE. Los genes relacionados, cuya expresión se analiza en esta tesis, después de 24 horas de estimulación, son los VCAM-1, ICAM-1, FVW, TF y NF-κB.

La respuesta endotelial a la estimulación con secretomas viscerales de pacientes con obesidad simple (Ob) vs. secretomas de obesos diabéticos tratados con metformina (DMO):

Ambos secretomas Ob y DMO activaron las vías inflamatorias y protrombóticas, como se demuestra por la comparación con la condición del medio control (CM). En nuestros experimentos hemos observado que los secretomas del tejido adiposo de los sujetos DMO parecían activar la cascada inflamatoria en un grado ligeramente mayor que los obtenidos por secretomas de tejidos Ob, marcado por la mayor expresión de VCAM-1, NF-κB. FVW aumentó ligeramente sin alcanzar significación estadística. De todos modos los resultados de ICAM-1 y TF son opuestos, siendo bastante similares entre ambos grupos de secretoma, aunque este resultado debe tomarse con

precaución debido a la alta variabilidad en el grupo de obesos. En general se puede concluir que el secretoma DMO provoca una ligera expresión aumentada de los genes proinflamatorios y protrombóticos estudiados, mientras que ambos secretomas inducen una activación importante de las CE con respecto al CM.

La estimulación con secretomas Ob suplementados con insulina:

La hiperinsulinemia es una característica común de la DM tipo 2 asociada a la obesidad y todavía persiste el debate sobre sus efectos sobre el endotelio. La potencia protrombótica y proinflamatoria de secretomas obesos viscerales de tejido adiposo en CE ha sido descrita anteriormente y publicada (resultados previos de nuestro grupo). Por lo tanto hemos decidido evaluar en nuestro modelo experimental *in vitro* el efecto complementario sobre las CE de las concentraciones de insulina fisiopatológicas inducido por los secretomas Ob.

Hemos observado que la estimulación simultánea de las CE con insulina y secretoma durante 24 horas indujo (sólo en concentraciones de 10^{-7} M) un incremento en la expresión de las moléculas de adhesión VCAM-1, ICAM-1 y el factor de transcripción NF-κB. Curiosamente, estos cambios no fueron acompañados por cambios en la expresión de los genes protrombóticos FVW y TF, ya que parecían tener efectos protectores protrombóticos.

Estimulación con secretomas Ob suplementados con glucosa:

Con el fin de realizar una evaluación global de un efecto aditivo de la glucosa en el endotelio a uno inducido por los secretomas obesos, se añadió glucosa en forma concomitante. Hemos empleado en nuestros experimentos el estado de glucosa basal en el cultivo de HUVECs, o sea, 5 mM, y dos condiciones fisiopatológicas de la glucosa: 11 mM y 16 mM.

El aumento de la concentración de glucosa parece no producir ningún daño adicional proinflamatorio o protrombótico a los que ya inducidos por la obesidad del secretoma Ob. De todos modos los resultados son difíciles de evaluar debido a la gran variabilidad observada.

2. Expresión de proteínas en CE

La expresión de genes era necesario ser confirmada en la proteína. La expresión del receptor se cuantificó después de 24 horas de estimulación, mientras que la expresión de las proteínas ECM se cuantificó después de 7 días de cocultivo, el tiempo requerido para la expresión en el subendotelio. Debido a la complejidad de la técnica, las CE fueron expuestas a las condiciones extremas de nuestro diseño experimental, a las concentraciones basales y máximas de insulina y glucosa, respectivamente.

Secretomas Ob y DMO actuaron de una manera similar sobre las CE, con respecto a la expresión de los receptores de proteínas de adhesión VCAM-1 e ICAM-1 y a la expresión del subendotelial protrombótico FVW. La trombogenicidad subendotelial, cuando se cuantificó por la adhesión de plaquetas, fue menor en los secretomas de los sujetos diabéticos tratados con metformina. La respuesta observada fue superior al de la condición control.

En cuanto a la adición de insulina o de glucosa al secretoma, nuestros resultados confirmaron que la insulina no indujo un excedente de lesión en las CE, mientras que concentraciones muy altas de glucosa provocaron una disminución significativa en la adhesión de las plaquetas y una tendencia similar en la expresión del FVW.

3. Análisis comparativo de las citoquinas de secretomas Ob vs DMO

Con el fin de entender los cambios en la composición de citoquinas de los secretomas de los pacientes DMO tratados con metformina, se examinó su patrón con los secretomas de tejido adiposo de los sujetos Ob. En comparación con los secretomas DMO procedentes de pacientes tratados con metformina, los secretomas obesos eran, excepto para IL-8 e IL-12, los que tenían la mayoría de citoquinas elevadas (incluyendo la protectora IL-10).

4. Estudio metabolómico de los secretomas de tejido adiposo obesos

En el intento de caracterizar la contribución de los secretomas de tejido adiposo obeso en la aparición de las complicaciones sistémicas metabólicas, se realizó un primer estudio metabolómico de secretomas de tejido adiposo obeso visceral y subcutáneo en sujetos obesos y no obesos. De acuerdo con nuestros resultados, la obesidad *per se*, y no la grasa de origen regional, modificó la firma metabólica de secretomas de tejido adiposo, y los especímenes de grasa visceral obesa presentaron las mayores diferencias en el patrón de metabolitos en los secretomas del tejido adiposo.

Discusión

Esta tesis pretende avanzar en el intento de descifrar el efecto de las citoquinas secretadas por el tejido adiposo visceral de los individuos obesos con DM tipo 2 sobre el endotelio y a su vez contribuir a la comprensión del efecto complementario de la hiperinsulinemia y la hiperglucemia en este tipo de lesiones.

En nuestro modelo se analizó *in vitro* el efecto proinflamatorio y protrombótico en el endotelio de los secretomas DMO procedentes de pacientes tratados con **metformina**, frente a secretomas Ob. Dado que no era posible obtener secretomas de pacientes DMO antes de recibir cualquier tratamiento, basamos nuestra comparación en los datos descritos en la literatura. Hay una abundancia heterogénea de datos, lo que sugiere que la inflamación aumenta con el aumento de la masa del tejido y que la inflamación del tejido obeso y la resistencia a la insulina aumentan en los tejidos adiposos DMO viscerales. Por otra parte, datos de estudios de diversos sueros de pacientes diabéticos tipo 2 reportan un perfil diferente de adipoquinas en el suero, en función de la asociación con la obesidad o no.

Nosotros esperábamos que las citoquinas proinflamatorias estuvieran mucho más elevadas en los secretomas DMO, debido al efecto deletéreo complementario de la diabetes en la obesidad. Nuestros datos indican que la metformina tiene un efecto distinto sobre la secreción de citoquinas del tejido adiposo obeso y su impacto sobre el endotelio del que se describe en la literatura,y ello a pesar de los niveles generales más bajos de las citoquinas en los secretomas DMO. La disminución de la IL-10 podría interpretarse como consecuencia de la disminución de la defensa del tejido adiposo frente a la inflamación local de larga duración. Sin embargo, los citoadipocinas estudiadas son sólo una selección representativa de una mayor cantidad de citoquinas documentadas que puede estar presentes en secretomas de tejidos adiposos obesos.

Los secretomas DMO indujeron una expresión ligeramente mayor de los genes proinflamatorios y protrombóticos estudiados, mientras tanto secretomas Ob y DMO indujeron

una activación importante de las CE con respecto al medio de control. Además, nuestro trabajo proporciona evidencia adicional de que la metformina tiene un papel beneficioso en la trombogenicidad, no sólo con respecto a la acción plaquetaria y la síntesis de PAI-1 (datos de la literatura), sino también con respecto a la síntesis de una matriz subendotelial menos trombogénica.

Estimulación simultánea de las CE con **insulina** y secretomas durante 24 horas indujo a altas concentraciones de insulina una expresión ligeramente mayor de VCAM-1, ICAM-1 y de NF-κB, con respecto a la estimulación inducida por los secretomas Ob . Sin embargo, la insulina parece tener efectos protectores protrombóticos. De todos modos, estos resultados en la expresión génica no han sido confirmados a nivel de síntesis protéica.

Nuestros datos están en contradicción con los emergentes en su mayoría del grupo de Madonna *et al* sobre el papel proinflamatorio de la insulina. Aún así, los resultados de la mayoría de publicaciones sugieren que la insulina puede ejercer potenciales efectos anti-inflamatorios y antiaterogénicos en ensayos clínicos y en modelos experimentales, por lo es un tanto probable que sea cardioprotector y mejore los resultados clínicos.

De manera similar a la insulina, co-expusimos CE a secretomas Ob y concentraciones **hiperglucémicas** de 11 y 16mM. El aumento de la concentración de glucosa no provocó daño adicional proinflamatorio o protrombótico al ya inducido por los secretomas Ob. En algunos casos, la interpretación de los resultados fue difícil debido a las variaciones entre las muestras.

Por lo tanto, nuestros datos apoyan los anteriores de la literatura que proporcionan evidencia sobre el hecho de que la hiperglucemia no es suficiente para promover directamente la activación inflamatoria endotelial. Existen pocos estudios en la literatura sobre la trombogenicidad macroendothelial de la matriz extracelular endotelial bajo condiciones de hiperglucemia, que parecen no verse afectados en la diabetes tipo 2. No obstante no se ha realizado ningún estudio sobre el impacto del control glucémico en este descubrimiento.

Con el fin de comprender la contribución de los metabolitos surgidos del tejido adiposo obeso a la enfermedad metabólica sistémica asociada con la obesidad, se realizó un análisis comparativo de los secretomas de especímenes grasos viscerales y subcutáneos de los sujetos obesos y no obesos, mediante la aplicación de un enfoque **metabolómico**.

En conjunto, nuestros resultados apuntan a un metabolismo elevado de aminoácidos y a una sobrecarga del catabolismo de aminoácidos BCAA en el tejido adiposo obeso inflamado. Esto, a su vez, independientemente, contribuye a la alteración y el aumento de aminoácidos metabolizados, sosteniendo las acciones perjudiciales de los aminoácidos en la aparición de complicaciones en la obesidad.

Nuestros resultados demuestran que las primeras etapas de la obesidad, incluyendo la presencia de inflamación sistémica y la resistencia a la insulina, antes de la aparición clínica de importantes alteraciones metabólicas, se caracterizan por un incremento en el metabolismo de aminoácidos y su secreción. Además, gracias a la combinación de análisis funcionales y metabólicas se espera comprender la alteración dinámica del metabolismo de aminoácidos en el estado de obesidad y su contribución específica de sus metabolitos en la enfermedad sistémica metabólica.

Conclusiones

Teniendo en cuenta los resultados obtenidos en esta tesis y bajo las condiciones aplicadas, se concluye lo siguiente:

1. El ***tratamiento con metformina***, un método ampliamente utilizada para normalizar la glucosa de pacientes afectos de diabetes tipo 2, actúa sólo de manera parcial en la reducción de las citoquinas nocivas secretadas por el tejido adiposo visceral de pacientes diabéticos tipo 2.
2. La ***insulina***, añadida al secretoma obeso visceral, no amplia ni reduce el daño inducido en el endotelio por el propio secretoma.
3. La ***glucosa*** no es capaz de inducir un incremento en la lesión endotelial, adicional al daño inducido por las citoadipoquinas del secretoma obeso visceral.
4. La ***huella metabólica*** de los secretomas del tejido adiposo se ve alterada por el estado de la obesidad en general. Los depósitos viscerales obesos presentan las mayores diferencias en el patrón de metabolitos de los secretomas. Las etapas tempranas de la obesidad, incluyendo la presencia de inflamación sistémica y la resistencia a la insulina, se caracterizan por una alteración en el metabolismo y la secreción de aminoácidos por el tejido adiposo.