UNIVERSITÀ DEGLI STUDI DI PADOVA

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CIRCULATING MICROPARTICLES AND HYPERCOAGULABILITY IN OBESITY

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ABSTRACT

Obesity has been associated with hypercoagulability and increased risk of both arterial and venous thromboembolic events. Many different and complex changes in plasma coagulation factors have been described in patients with obesity. Conventional plasma recalcification times don't assess the entire process in physiological and holistic manner.

In a case-control study, the presence of hypercoagulability was evaluated in free of metabolic syndrome overweight and obese patients by measuring different subtypes of microparticles (MPs), thrombin generation, whole blood rotation thromboelastometry (ROTEM®) and impedance aggregometry (Multiplate®). Furthermore, the levels of MPs were prospectively measured in a group of 20 III degree obese patients before and after 12 months bariatric surgery in order to investigate the effect of weight loss.

Twenty overweight patients (body mass index [BMI] range 25–29.9 kg/m^2), 20 with I degree (30-34.9 kg/m^2), 20 with II degree (35–39.9 kg/m^2) and 20 with III degree obesity (< 40 kg/m^2) were enrolled and compared to 40 age and gender-matched normal weight individuals.

Microparticles: a significant increase in median levels of all MP subtypes was observed in the three degrees of obese patients compared to controls. All MPs, except for endothelial-derived MP, which had significantly decreased at T3, all MPs subtypes had significantly decreased at T12. *Thrombin generation:* obese patients had a significantly shorter median lag time, higher median peak thrombin and increased median endogenous thrombin potential compared to controls. *Thromboelastometry:* in INTEM and EXTEM tests MCF and AUC were significantly increased in III degree obese compared with controls; MCF in FIBTEM was significantly higher in I, II and III degree obesity than controls. *Impedance aggregometry:* a significant difference in platelet aggregation was found between III degree obese subjects and healthy controls in each of the tests considered.

We conclude that obesity is associated with overproduction of procoagulant MP and increase thrombin generation. A relationship between hypercoagulability detected by whole blood thromboelastometry and aggregometry and increased fat mass is shown. Assessment of global hemostasis tests may be helpful in the early characterization of the prothrombotic state in obese patients.

1. INTRODUCTION

1.1. Definition of Obesity and Body Weight Categories

The World Health Organization (WHO) defines obesity as 'a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health may be impaired'.[1]

Measurement of height and weight was the initial step in the clinical assessment of overweight and obesity.

The first concept used was percent of ideal body weight. Remote studies suggested that terms like "healthy or good weight ranges" were associated with decreased mortality. Afterwards, body mass index (BMI) substituted the assessment of obesity, calculated as body weight (in kg) divided by height (in meters) squared. The initial use of the index was in 1842 by Quetelet, a Belgian mathematician, who noticed that in people he considered to have a "normal frame" the weight was proportional to the height squared.

With the help of experts from around the world who formed 'The International Obesity Task Force', in 1997 the World Health Organization (WHO) came up with the definition of obesity as a BMI ≥ 30 kg/m^2, using data from the Third Health and Nutrition Examination Survey found that the optimal BMI for longevity in whites was 23–25, and that in blacks was 23–30 kg/m^2.[2] Overweight or pre-obesity as the weight category encompassing the BMI range between 25 and < 30 kg/m² and obesity is defined as a BMI ≥ 30 kg/m²; obesity in turn is subdivided into classes I, II and III (Table 1).

Classification	BMI kg/m^2 [general cut off point]	BMI kg/m^2 [cut off point for Asian population]
Underweight	< 18.5	< 18.5
Normal range	18.5 - 24.9	15.5 – 22.9
Pre-obese	25.0 – 29.9	23.0 – 27.4
Obese class I	30.0 – 34.9	27.5 – 32.4
Obese class II	35.0 – 39.9	32.5 – 37.4
Obese class III	≥ 40.0	≥37.5

Table 1: The classification of weight category by BMI, adapted from WHO.[3]

The definitions and classifications of body weight categories have changed over time.[4] BMI is widely used to indirectly assess adiposity, but over the last 30 years there have

been several new concepts challenging the simplistic concept that obesity can be diagnosed based on weight and height.

Obesity is associated with a reduced life expectancy, largely because obese individuals are at an increased risk of type 2 diabetes, cardiovascular disease (CVD), and several types of cancer.

The use of BMI alone as a marker of overweight and obesity is limited for, although it correlates well with total body fat content in adults, it fails to consider the distribution of that fat. In view of the relationship between central obesity and coronary heart disease (CHD), other methods of defining obesity have been used; for example, the waist circumference (WC) and waist-to-hip ratio.

Two patterns of obesity have been distinguished for the purposes of risk factor assessment; namely, central (visceral) obesity and peripheral obesity. Central obesity produces a characteristic body shape which resembles an apple and thus is also referred to as “apple shaped” obesity: it is defined as deposition of fat around the trunk and in the visceral adipose tissue. It is more common in men and carries a higher risk of CHD, as well as various metabolic derangements including dyslipidemia and glucose intolerance.[5] In “pear shaped” obesity or peripheral obesity accumulation of fat is predominantly in the gluteo-femoral area. It is more common in women and is less strongly associated with cardiovascular risk.

Men with a WC of > 102 cm and women with a WC > 88 cm have a higher risk of CHD, and a WHR of > 0.92 carries a nearly 3-fold increased risk (Figure 1).[6]

Much interest has recently focused on the concept of “all obesity is not created equally”. Obese individuals without the metabolic abnormalities that commonly accompany excess adiposity, a condition known as metabolically healthy obesity (MHO), account for a substantial proportion of the obese adult population. Numerous possible mechanisms underlying MHO have been suggested, including adipose tissue distribution and inflammation.[7]

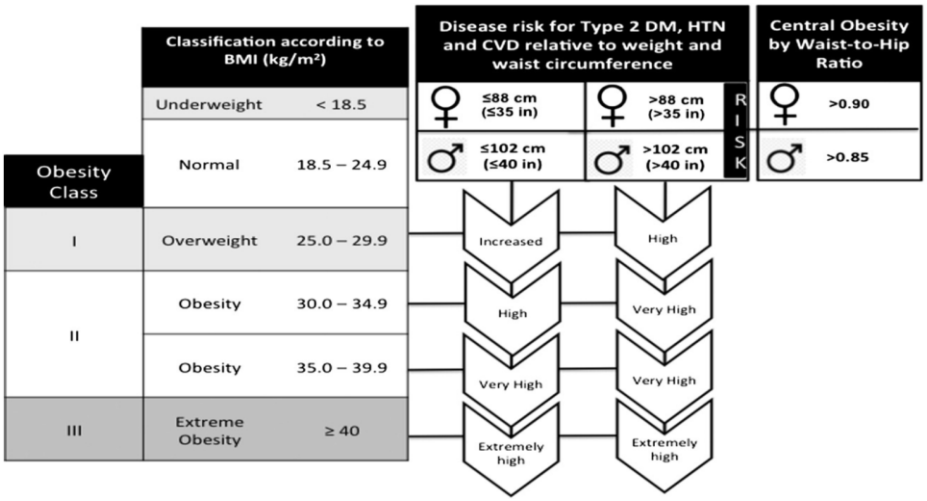


Figure 1: Classification of obesity developed by the National Heart, Lung and Blood Institute task force, along with the associated disease risk with increasing BMI, waist circumference and waist to hip ratio.[8]

1.2. Prevalence Rates and Trends

Over the last few decades, overweight and obesity increased worldwide and became a major public health challenge not only in high-income countries but also in middle- and low-income ones.

The more recent US data are from the National Health and Nutrition Examination Survey, 2011–2012. More than one-third (35%) of adults were obese and the prevalence of obesity was higher among middle-aged adults (40%). Data from NHANES II (between 1976 and 1980) and NHANES III (between 1988 and 1994) demonstrate that the prevalence rates of obesity increased considerably, to about 21% in men and to about 26% in women. By 2003–2004 the prevalence had increased to almost 32% in men and 34% in women.[9]

Among European countries, Italy had relatively favorable obesity prevalence and trends in adults The prevalence of overweight and obesity modestly increased between the early 1980s (27% of adult population was overweight and 7% obese in 1983) and the late 1990s and levelled off thereafter (31–34 % overweight and 8–9% obese between 1999 and 2004).

According to a recent epidemiological study published in Lancet, on 2013 in Italy the prevalence of overweight-obesity and obesity in > 20 year old male was respectively

58.3% and 18.6% and > 20 years old female was respectively of 41.4% and 17.7% (Figure 2).[10]

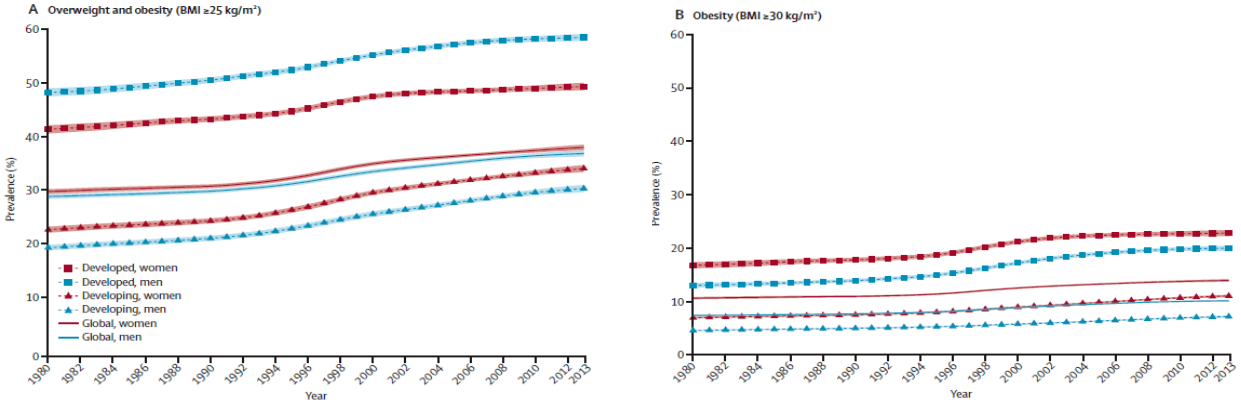


Figure 2: Age-standardized prevalence of overweight and obesity and obesity alone, ages ≥ 20 years, by sex, 1980−2013, modified from [10].

1.3. Pathogenesis

Many different hypotheses have been proposed to explain the origin of the obesity epidemic. It's broadly accepted that the two major putative contributors are specific food marketing and consumption habits, and decrease in physical activity. However, there are others contributors, such as infections and microbiota [11], maternal age, sleep debt, endocrine disruptors (such as industrial chemicals), reduction in ambient temperature variations, intrauterine and intergenerational effects, and drug-induced weight gain. Despite the importance of all these contributors, it is known that genetic factors have a key role in the risk of one becoming obese.

Environmental factors are likely to be major contributors to the obesity epidemic, together with biological predisposition. It is certain that obesity develops when there is a positive imbalance between energy intake and energy expenditure, but the relative contribution of these factors is poorly understood. Evidence supports the contribution of both excess energy intake and decreased energy expenditure in determining obesity:

1. Dietary data from four consecutive NHANES studies, consisting of 39,094 adults in the USA, have shown that the temporal trends in the increase of the quantity and energy

density of foods consumed by adults parallel the increasing prevalence of obesity in the US population.[12]

2. Data from the Central Statistical Office show that car ownership and television viewing, proxy measures of physical inactivity, closely parallel the rising trends in obesity in England.[13]

3. Using data from NHANES, Dietz et al. demonstrated that the prevalence of obesity increased by 2% for each additional hour of television viewed. [14]

4. There is also evidence that the relative availability and price of different food products affect food consumption and that the built environment, such as quality of local parks, affects the level of physical activities in a community.

These findings not only emphasize the impact of environmental factors on the obesity epidemic, but also indicate that policies affecting the availability of high caloric-density food, the cost of fruits and vegetables, and the built environment may contribute to the alarming prevalence rates of overweight and obesity.

Common forms of obesity are caused by a combination of environmental factors with many gene variants of minor effect. However, at least ~ 7% of non-syndromic early-onset severe obesity are monogenic, thereby caused by gene variants of major effect (Figure 3).[15]

It is well-characterized that the obesity epidemic is not caused by single gene disorders, but actually has a complex genetic background. Obese children under three years of age without obese parents are at low risk for obesity in adulthood, but among older children, obesity is an increasingly important predictor of adult obesity, regardless of whether the parents are obese. Parental obesity more than doubles the risk of adult obesity among both obese and non-obese children under 10 years of age.[16]

The effect of genes on the pathogenesis of obesity is more frequently stronger when combined with other genes and the environment. The ‘thrifty genotype hypothesis’ argues that the ancestors of present day humans in countries plagued by obesity underwent positive selection for genes that favored “thrift” or energy storage.[17] These so-called ‘thrifty genes’ are defined as those that bestow superior energy efficiency such that the energy balance equation is shifted heavily toward energy intake as opposed to energy expenditure. It is postulated that survival of a population necessitated the selection for ‘thrifty genes’ that enabled extra fat reserves to be laid down during times of energy surplus to be utilized during harder times.

Gene ID	Gene name	Chromosome location	Inheritance	Protein function	Additional features	Reference
LEP	Leptin	7q32.1	Autosomal recessive	Stimulates anorexigenic pathway and inhibits the orexigenic pathway	Low levels of leptin, insulin resistance, dyslipidemia, susceptibility to infection diseases and hypogonadotropic hypogonadism	Paz-Filho et al., 2010[24]; Montague et al., 1997[25]
LEPR	Leptin receptor	1p31.3	Autosomal recessive	Leptin receptor	Immune dysfunctions, hypogonadotropic hypogonadism, growth hormone deficiency and hypothyroidism	Clement et al., 1998[26]; Saeed et al., 2014[27]; Farooqi et al., 2007a[28]
PCSK1	Proprotein convertase subtilisin/kexin type 1	5q15	Autosomal recessive/ dominant	Proteolytic processing of prohormones and proneuropeptides	Diabetes insipidus, growth hormone deficiency, malabsorptive diarrhea, hypogonadism, adrenal insufficiency and hypothyroidism	Farooqi et al., 2007b[29]
POMC	Pro-opiomelanocortin	2p23.3	Autosomal recessive	Protein precursor of melanocortin family that transmits leptin effects to MC4R and regulates the adrenal growth	Adrenal insufficiency and pigmentation abnormalities	Kühnen et al., 2016[30]
TUB	Tubby bipartite transcription factor	11p15.4	Autosomal recessive	Modulates anorexigenic neuropeptides*	Retinal dystrophy	Borman et al., 2014[31]
SH2B1	Src homology 2 B adapter protein 1	16p11.2	*	Modulates cell signaling in response to leptin and other hormones	Insulin resistance, maladaptive behavior, delayed speech and language development	Doche et al., 2012[32]; Rui, 2014[33]
MC4R	Melanocortin-4 receptor	18q21.3	Autosomal co-/ dominant	Transmits the anorexigenic and orexigenic effects, controlling satiety and energy expenditure	Hyperinsulinemia, increased linear growth or isolated obesity phenotype	Doulla et al., 2014[34]; Farooqi, 2003[35]; Melchior et al., 2012[36]; Farooqi, 2015[37]
MRAP2	Melanocortin 2 receptor accessory proteins 2	6q14.3	Autosomal dominant	Regulates melanocortin receptors	*	Asai et al., 2013[38]; Schonnop et al., 2016[39]
KSR2	Kinase suppressor of ras 2	12q24	*	Involved in cellular fuel oxidation	Low heart rate, reduced basal metabolic rate and insulin resistance	Pearce et al., 2013[40]; Costanzo-Garvey et al., 2009[41]
SIM1	Single-minded homolog 1	6q16.3	Autosomal dominant	Regulates the development and function of the paraventricular nucleus	Accelerated linear growth, impaired concentration, memory deficit, emotional lability and autistic spectrum behavior	Ramachandrappa, 2013a[42]; Holder et al., 2000[43]
BDNF	Brain-derived neurotrophic factor	11p13	Autosomal dominant	Regulates the development, survival and differentiation of neurons	Hyperactivity, impaired memory, reduced nociception, delayed speech and language development	Gray et al., 2006[44]
NTRK2	Neurotrophic tyrosine kinase receptor type 2	9q22.1	Autosomal dominant	BDNF receptor	Development delay as well as short-term memory and nociception impaired	Yeo et al., 2004[45]
LRP2	Low-density lipoprotein receptor 2	2q31.1	Autosomal dominant	Binds to the long-form of LEPR and activates STAT3 signaling	Puberty delay, decelerated linear growth, hypothyroidism, insulin resistance and elevated levels of prolactin	Paz-Filho et al., 2014[46]

Notes: *Not yet elucidated.

Figure 3: Rare non-syndromic forms of early-onset obesity, modified from [18].

1.4. Complications

Numerous studies have demonstrate d increased mortality above a certain threshold of BMI. In the Framingham heart study, a prospective cohort study, male and female non-smokers aged 40 year who were obese lived 5.8 and 7.1 years less than their non-obese counterparts.[19] Another study by Fontaine et al. which used data from the National Health and Nutrition Examination Survey (NHANES I and II) and the NHANES III Mortality Study found a marked reduction in life expectancy in obese young adults compared to non-obese adults.[20]

Obesity increases risk for many disorders that are associated with high mortality and morbidity, including diabetes, hypertension, coronary heart disease, dyslipidemia, gallbladder disease, and certain malignancies (Table 2). Not only does excess weight increase the risk of these disorders, but the pattern of fat distribution is important in many of these conditions. Men are more likely to have abdominal or upper-body obesity,

whereas women are more likely to have a gluteo-femoral or lower-body pattern of fat distribution. However, as women gain weight, they become more likely to develop abdominal and upper-body fat.

Insulin resistance/hyperinsulinemia
Type 2 diabetes
Hypertension
Dyslipidemia
Coronary heart disease
Stroke
Gallbladder disease
Cancer (postmenopausal breast, ovarian endometrial, gastric and esophageal, colon, kidney, prostate)
Osteoarthritis
Chronic venous insufficiency
Thromboembolism
Asthma
Obstructive sleep apnea
Breathing difficulties
Complication of pregnancy
Menstrual irregularities
Hirsutism
Increased surgical risk
Psychological distress

Table 2. Disorders associated with obesity, modified from [21].

1.4.1. Arterial and venous vascular complications

In 2015 Dagenais et al. studied the impact of increased BMI and waist circumference on the occurrence of cardiovascular death, myocardial infarction, stroke, heart failure, and total mortality among 8802 men and women with cardiovascular disease enrolled in the Heart Outcomes Prevention Evaluation (HOPE) study and observed during a median period of 4.5 years. In these individuals with CVD, elevated BMI was an independent predictor only for myocardial infarction when not adjusted for abdominal obesity indexes. Similarly, increased WC was independently related to cardiovascular death, myocardial infarction, and total mortality. Abdominal obesity and increased BMI to a lesser degree worsen the prognosis of patients with CVD.[22] The mechanisms by which increasing body weight contributes to higher risk of CVD, independent of other prognostic factors, are not fully understood. It is possible that obesity, particularly adipocytes, activates

coagulation, inflammation, and the sympathetic and renin-angiotensin aldosterone system.[23] Being overweight and obese are also associated with coronary endothelial dysfunction, hyperinsulinemia, and other metabolic disorders.[24]

In previous studies, only obesity has consistently been demonstrated to be an independent risk factor for venous thromboembolic events. It's known that a positive association between diabetes and deep vein thrombosis,[25] arterial hypertension and the risk of pulmonary embolism,[26] and dyslipidemia and venous thromboembolism (VTE).[27] Elevated levels of triglycerides and low high-density lipoprotein (HDL) seem to increase the risk of VTE, whereas increased HDL levels may protect against VTE.[28] Finally, it was described the association between the metabolic syndrome, a cluster of risk factors for atherosclerosis, and unprovoked deep vein thrombosis.[29]

It is generally believed that the genesis of venous thromboembolism differs from atherosclerotic cardiovascular disease.[30] The concept that venous thromboembolism and atherosclerosis are two completely distinct entities has recently been challenged because patients with spontaneous VTE have been found to have a higher prevalence of atherosclerosis, defined by the presence of asymptomatic carotid atherosclerotic lesions, than patients with VTE secondary to known risk factors and control subjects.[31]

In 2008 it was performed a systematic review of the literature to assess the relation between VTE, atherosclerosis and atherothrombosis: meta-analysis was conducted on 21 case-control studies that evaluated the prevalence of cardiovascular risk factors, such as BMI, hypertension, diabetes, dyslipidemia and smoking, in subjects with prior venous thromboembolism. The results suggest that major risk factors for atherothrombotic disease also are significantly associated with VTE.

In the past, only obesity has been consistently shown to be a minor risk factor for venous thrombosis, whereas conflicting results have been reported for hypertension, dyslipidemia, diabetes mellitus, and smoking. Ageno et al. confirmed the association between obesity and VTE but also demonstrated for the first time an association between VTE and diabetes mellitus, hypertension, low HDL cholesterol and high triglycerides.[32]

From a laboratory perspective, an association between venous and arterial events is plausible because they share common characteristics such as activation of both platelets and coagulation.[33]

The risk of arterial thrombosis in patients with major cardiovascular risk factors is most likely mediated by the presence of an inflammatory state and hypercoagulability. Both

increased inflammation and coagulation also may predispose these patients to develop venous thromboembolic events. Obesity, in particular central and abdominal obesity, is associated with increased thrombin formation and decreased fibrinolysis.[34] [35] Obesity also is associated with immobility and increased abdominal pressure, another risk factor for thrombosis. Diabetes mellitus often is associated with increased levels of procoagulant factors and the inhibition of endogenous fibrinolysis.[36] Dyslipidemia also is associated with hypercoagulability, endothelial dysfunction, and increased platelet aggregation.[37]

It is therefore no surprise that these risk factors also predispose to VTE and can be considered part of the underlying disease process that is associated with cardiovascular disease and venous thromboembolism.[M1] This association between VTE and atherothrombosis has great clinical relevance with respect to individual screening, risk factor modification, and the primary and secondary prevention of VTE.

1.5. Coagulation tests

There is a potential for significant paradigm shift in the assessment of hemostasis from the conventional plasma recalcification times, which correspond to artificially created compartments of hemostasis to tests that assess the entire process in a more physiological and holistic manner. Primary hemostasis consists of the interaction between platelets and vessel wall. Human platelets are critically involved in both normal hemostasis and pathological bleeding and thrombosis. These particular cells contribute greatly to vessel constriction and repair, host defense, and tumor growth/metastasis. In addition, platelets acting together with other cells - white, endothelial, or smooth muscle cells - play a part in inflammation, in related pathologies, and in the promotion of atherosclerosis. The rapid progression of these different capacities causes the activated platelets to form a hemostatic plug that occludes the site of injury to prevent blood loss.[38] An increased risk of bleeding could be present when platelet count is reduced and/or one of their functions is defective. Conversely, improper thrombus formation could be due to a growth in platelet count or reactivity. In particular, activated platelets adhere and aggregate within atherosclerotic lesions, forming occluding arterial thrombi that may result in thromboembolic disease such as stroke or myocardial infarction, two of the major causes of morbidity and mortality in the Western world.[38, 39] These different functions of platelets may be reliably detected with a wide spectrum of tests. The history of platelet

function testing begins with the development of the evaluation of the bleeding time by the Duke procedure.[40] For many years, it has been the unique screening test to identify both congenital or acquired platelet disorders. In the 1960s, the revolutionary platelet aggregation test in platelet rich plasma - ie, light transmission aggregometry - according to Born's studies was the key method used to diagnose platelet function.[41] Since the late 1980s, other platelet function testing methods, such as platelet aggregometry in whole blood, the study of activated platelets ex vivo by flow cytometry, the measurement of specific compounds released by platelets, and the assessment of platelet nucleotides have become available (Figure 4).

Method	Sample	Method application	Method principle
Bleeding time	Native WB	Screening test (obsolete)	In vivo measurement of bleeding block
Tests based on platelet aggregation			
Light transmission platelet aggregation (LTA)	Citrated PRP	Screening test for bleeding tendency Diagnostic for platelet defects Monitoring antiplatelet treatment effect	Photo-optical measurement of light transmission increase in relation to agonist-induced platelet aggregation
Impedance platelet aggregation	Citrated WB	Screening test for bleeding tendency Diagnostic for platelet defects Monitoring antiplatelet treatment effect	Measurement of electrical impedance increase in relation to agonist-induced platelet aggregation
Lumiaggregometry	Citrated WB	Detection of storage/release disorders	LTA or WB aggregometry combined with luminescence
Plateletworks	Citrated WB	Monitoring of the platelet response to antiplatelet agents	Platelet counting pre- and postactivation in whole blood
Tests based on platelet adhesion under shear stress			
PFA-100; Innovance PFA-200	Citrated WB	Assessment of bleeding risk and drug effects Searching severe platelet dysfunctions, revealing of VWD	Time evaluation of high shear WB flow blocked by platelet plug into a hole in activated surface
Impact; Cone and Plate(let) Analyzer	Citrated WB	Screening of primary hemostasis	Shear-induced platelet adhesion–aggregation upon specific surface
Global thrombosis test (GTT)	Native WB	Evaluation of platelet function and thrombolysis	Measurement of time cessation of WB flow by high shear-dependent platelet plug formation
Platelet function methods combined with viscoelastic test			
TEG/platelet mapping system	Citrated WB	Assessment of global hemostasis plus monitoring antiplatelet treatments effect	Evaluation of rate of clot formation based on low shear-induced and agonist addition
ROTEM platelet	Citrated WB	Assessment of global hemostasis plus diagnostic of platelet defects plus monitoring antiplatelet treatments effect	Measurement of electrical impedance increase in relation to agonist-induced platelet aggregation
Platelet analysis based on flow cytometry			
Flow cytometry	Citrated WB, PRP, W-Plt	Cell counting, detection platelet activation by extent of expression of surface and/or cytoplasmic biomarkers	Engineering laser-based detection of suspending fluorescent label platelets in a flowing solution
Evaluation of Thromboxane metabolites			
Radio- or enzyme-linked immune assays	Serum, urine, citrated Pls	Measurement of TxA2 metabolites (and Beta-TG, PF4, soluble P-selectine)[a]	Ligand-binding assays

Note: [a]Not planned in this review.
Abbreviations: Beta-TG, beta-thromboglobulin; Pls, plasma; PRP, platelet-rich-plasma; ROTEM, rotational thromboelastometry; TEG, thromboelastography; TxA2, thromboxane A2; WB, whole blood; W-Plt, washed platelets; VWD, Von Willebrand disease.

Figure 4: Different platelet function tests, modified from [42].

1.5.1. Coagulation cascade and fibrinolytic system

Hemostasis and its abnormalities have been traditionally assessed by plasma clotting times, such as the prothrombin, activated partial thromboplastin and thrombin times.

The coagulation cascade has two initial pathways which lead to fibrin formation. These are the contact activation pathway (also known as *the intrinsic pathway*), and the tissue factor pathway (also known as the *extrinsic pathway*) which both lead to the same fundamental reactions that produce fibrin. The pathways are a series of reactions, in which a zymogen (inactive enzyme precursor) of a serine protease and its glycoprotein co-factor are activated to become active components that then catalyze the next reaction in the cascade, ultimately resulting in cross-linked fibrin. Anionic phospholipid, especially phosphatidylserine, was required for the assembly and optimal function of most of the coagulation complexes.

A "Cascade model'' was a great advance in our understanding of coagulation. This resulted from work that was aimed at elucidating the identity, function and interactions of the individual procoagulant proteins. [43] The coagulation "Cascade" models very well the screening coagulation laboratory tests, the prothrombin time (PT) and activated partial thromboplastin time (aPTT), which correspond to the extrinsic and intrinsic pathways (Figure 5).

However these tests have several limitations. These include the fact that all of them are performed under conditions that are far from physiological, and they split the process of coagulation into artificial segments thus not assessing the potential impact of other components of the hemostatic system.[44]

Furthermore, clotting times are insensitive to mild hemostatic disorders (e.g. carriers of hemophilia, mild liver disease) or to increased coagulability that leads to increased thrombosis risk (e.g. obesity, diabetes and metabolic diseases).

High factor VII and factor VIII levels correlate with measures of obesity and an increased risk of CHD and stroke. High triglycerides and low high density lipoprotein (HDL) cholesterol, the most common lipid disturbances found in obesity, are also found with high factor VII and VIII levels. Fibrinogen promotes arterial and venous thrombosis through increased fibrin formation, platelet aggregation and plasma viscosity; and promotes atherosclerosis through vascular smooth muscle and endothelial cell proliferation.[45] [46]

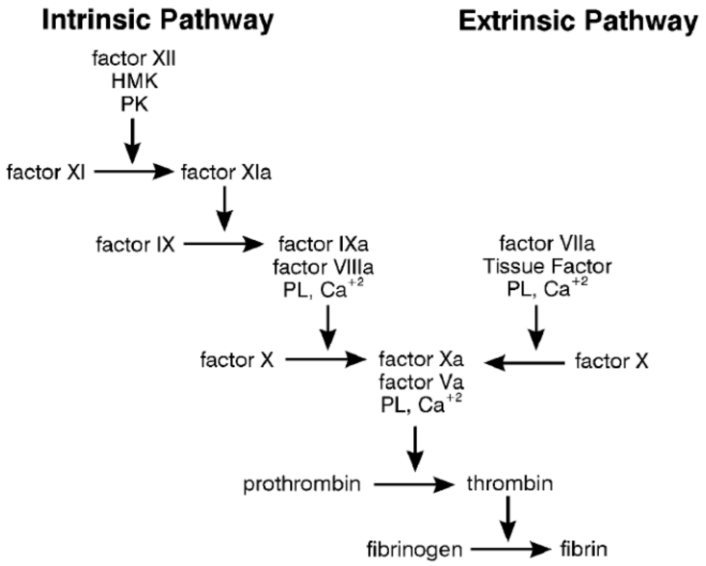


Figure 5 : The cascade model of coagulation. The “intrinsic” and “extrinsic” pathways are reflected in the clinical laboratory tests aPTT and PT, respectively, adapted from[47]

Obese patients exhibit increased tissue factor (TF)-mediated coagulation, with raised adipocyte and monocyte TF expression secondary to elevated levels of C-reactive protein (CRP), transforming growth factor-beta (TGFβ), tumor necrosis factor-alfa (TNFα), angiotensin II and insulin.[48]

Even if there were no differences in conventional plasma recalcification times, it is known that obesity and other metabolic diseases are correlated with prothrombotic state: conventional coagulation tests, such as PT and aPTT, do not reflect the suspected hypercoagulable state.

If the main event in coagulation is considered to be fibrin formation by endogenously generated thrombin, the analogous step in fibrinolysis is fibrin degradation by plasmin. Fibrinolytic system is a parallel system which is activated along with activation of coagulation cascade and serves to limit the size of clot. The conversion of the inactive proenzyme plasminogen into the active enzyme plasmin is the central step in the fibrinolytic system. Plasmin degrades fibrin into soluble fibrin degradation products. Two physiologic plasminogen activators are capable of catalyzing the conversion of plasminogen: tissue-type and urokinase-type plasminogen activator (Figure 6).[49]

As long as blood coagulation and fibrinolysis remain in equilibrium, response to injury, such as blood vessel penetration, is appropriately self-limited. However, alterations in this balance may lead to a prothrombotic state or a bleeding tendency.

PAI-1 is produced in several tissues including liver, spleen and adipocytes. Elevated PAI-1 levels compromise the normal clearance of fibrin and consequently promote thrombosis.[48]

PAI-1 levels are positively correlated with obesity (measured by BMI, WC and WHR), insulin resistance, and triglyceride levels.[50] High PAI-1 concentrations have been found in patients with recent myocardial infarction, chronic coronary heart disease and peripheral arterial disease.[51]

A variety of methods have been proposed to assess fibrinolytic activity in blood or its components, but due to the complexity of the system, the design of a "gold standard" assay has remained an elusive goal. Assessment of fibrinolysis in whole blood is truly "global."

Finally, it is indispensable to remember that the hemostasis process is not only constituted by bioumoral coagulation factors but also different cell types play a key role.

A cell-based model might describe the role of different kind of cells and reflects the pathways of hemostasis *in vivo*. Hemostasis occurs in three (overlapping) phases. The initiation of coagulation takes place on tissue factor (TF)-bearing cells, such as the fibroblast illustrated in Figure 7. TF is expressed on a variety of extravascular cells under normal conditions, and can also be expressed by blood monocytes and endothelial cells in inflammatory states. If the procoagulant stimulus is sufficiently strong, enough factors Xa, IXa and thrombin are formed to successfully initiate the coagulation process. Amplification of the coagulant response occurs as the "action" moves from the TF-bearing cell to the platelet surface. The procoagulant stimulus is amplified as platelets adhere, are activated and accumulate activated cofactors on their surfaces. Finally, in the propagation phase, the active proteases combine with their cofactors on the platelet surface – the site best adapted to generate hemostatic amounts of thrombin. The activity of the procoagulant complexes produces the burst of thrombin generation that results in fibrin polymerization. Most conventional tests of blood coagulation explore specific stages of clotting cascade in plasma. This may represent a limitation for the study of coagulation processes in which the interactions between plasma factors and phospholipid surfaces act together with other blood components in clot formation. It is possible that

tests that assess global hemostasis may be better reflective the overall hemostatic potential of blood.

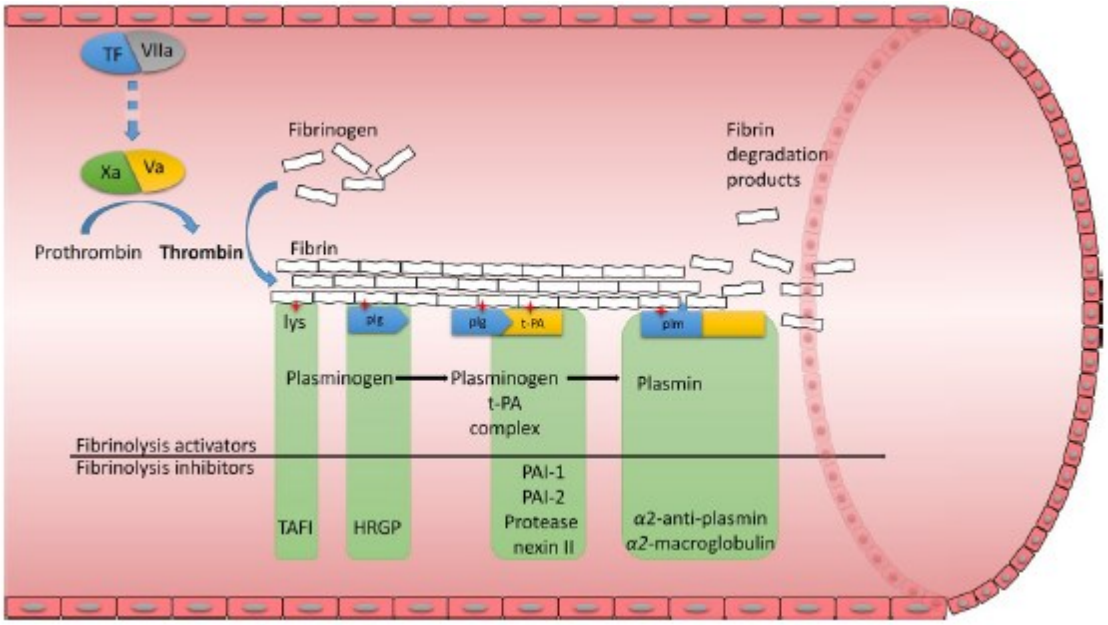


Figure 6: Simplified fibrinolysis scheme, from [52].

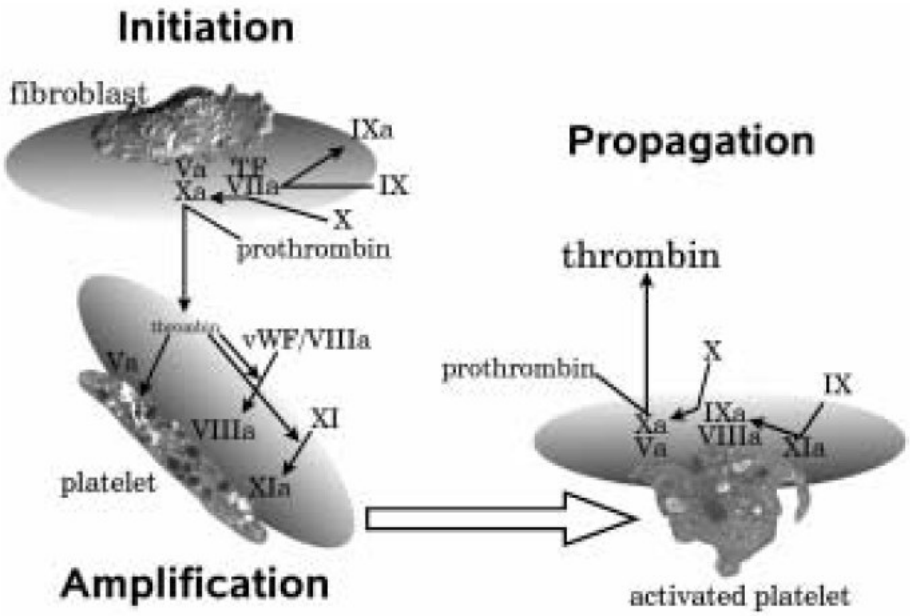


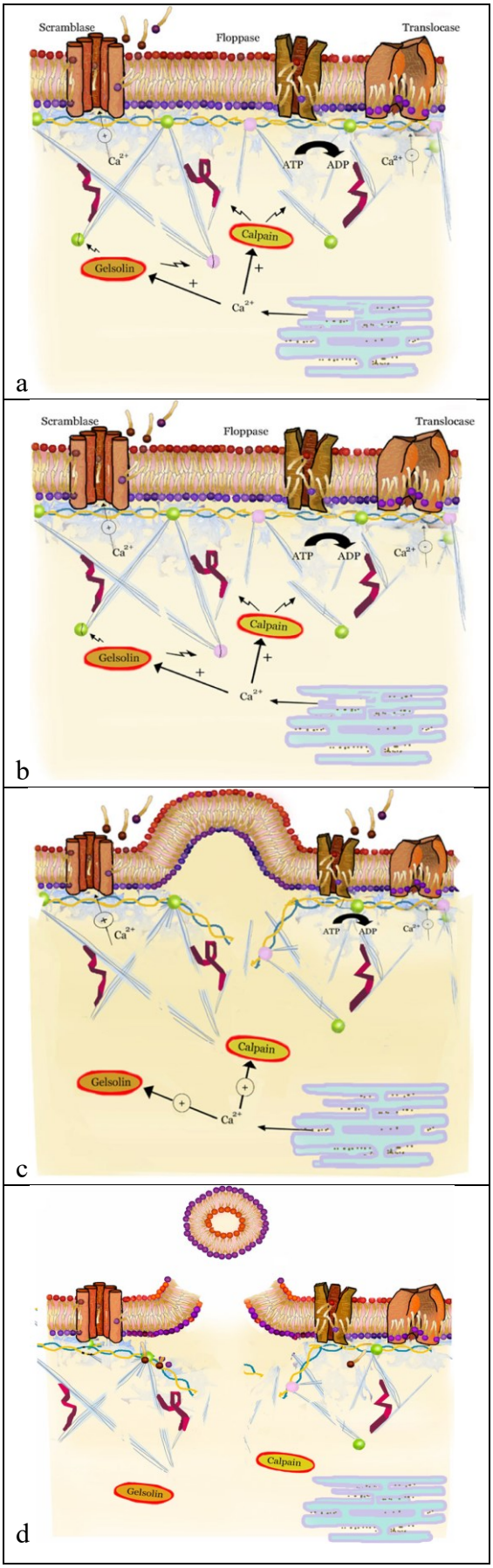
Figure 7: A cell-based model of coagulation, adapted from [53].

1.5.2. Microparticles

'Microparticles' (MPs) are fragments released from stimulated or apoptotic cells after plasma membrane remodeling. They consist of a cell-derived vesicle that is formed from the outward blebbing of the plasma membrane and subsequent shedding into extracellular space.[54] MPs range in size from 100 to 1000 nm in diameter, but are typically ~ 200 nm in size. Hardly detectable in the peripheral blood of healthy individuals, procoagulant MPs circulating at elevated levels are often associated with different diseases.[55] The composition and the distribution of cell membrane phospholipids are highly specific: phosphatidylcholine and sphingomyelin are located on the external membrane layer, while phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE) are found on the inner side of the cell membrane.[56]

The preservation of this asymmetry is essential and is maintained through a complex transmembrane enzymatic balance. Five enzymes are involved in regulation of cell membrane phospholipid asymmetry and are implicated in microparticle generation: gelsolin (present only in platelets), aminophospholipid translocase, floppase, scramblase and calpain. These enzymes maintain a dynamic asymmetric steady state and allow membrane phospholipids to move to the outside of the cell membrane while at the same time, the aminophospholipids are redirected to the inner side of the cell membrane. When the concentration of calcium rises in the cytosol, for example during platelet activation, this steady state is changed and results in the expression of PS on the exterior membrane surface. Loss of phospholipid asymmetry arises during platelet activation, apoptosis and necrosis and results in exposure of PS on the outer cell surface and this leads in turn to a prothrombotic state. MPs contribute to hemostatic and inflammatory responses, vascular remodeling and angiogenesis, cell survival, and apoptosis, well-known processes involved in atherothrombosis (Figure 8).[57]

MPs are enriched with bioactive proteins and nucleic acids, and there is growing evidence that they form a network of biological vectors able to transfer cellular components to remote areas of the body in order to mediate specific changes in their target cells, thus mediating cell activation, phenotypic modification, and reprogramming of cell function. Because microparticles readily circulate in the vasculature, they may serve as shuttle modules and signaling transducers not only in their local environment but also at remarkable distance from their site of origin.



a)

Calcium is stored in the endoplasmic reticulum. Scramblase is inactive, while Translocase is active. Translocase transports anionic phospholipid, such as phosphatidylserine and phosphatidyl-ethanolamine, from the outside layer of the cell membrane to the inside, maintaining PS and PE inside. Each PS is transported with one molecule of ATP. Floppase is an ATP-dependent protein that contributes to maintaining the physiological membrane asymmetry.

b)

Intracellular calcium increases released by endoplasmic reticulum. Calcium activates calpain and gelsolin. Calpain cleaves long actin filaments. Gelsolin cleaves the actin capping proteins. The raised cytoplasmic Ca^{2+} also activates scramblase and inactivates translocase. Phospholipid asymmetry begins to be compromised.

c)

Spectrin and actin are cleaved. At this point protein anchorage to the cytoskeleton is disrupted allowing membrane budding.

d)

Generated microparticle expose phosphatidylserine on the external surface

Figure 8: Schematic representation of the resting cytoskeleton, modified from [57].

Although microparticle formation represents a physiological phenomenon, MPs are hardly detectable in the peripheral blood of healthy individuals. They are released from cells under conditions of stress state. A multitude of pathologies are associated with a considerable increase in circulating microparticles, including inflammatory and autoimmune diseases, atherosclerosis and malignancies.[58]

Believed to be formed by all cell types, MP formation has been observed in cells of the vasculature (endothelial cells, platelets, leucocytes and vascular smooth muscle cells), erythrocytes, cardiomyocytes and podocytes, as well as cancer and progenitor cell populations.[59]

1.5.2.1. Platelet-derived microparticles

Both the megakaryocytes and platelets can generate microparticles. Greater than 90% of circulating MPs are thought to be platelet derived, present in the plasma of normal healthy individuals at an estimated concentration of up to 50 mg/m. Platelet activation is a prerequisite in the afore mentioned mechanisms leading to microparticle production.[60] Platelet-derived microparticles (PMP) contain a unique subset of proteins derived from the parent cell: they express GpIb (CD42b), platelet endothelium adhesion molecule (PECAM-1; CD31) and P-selectin (CD62P) [61], CD63, CD41a and CD 61.

PMP can transfer receptors from platelet membranes to different cell types and enhance engraftment of hematopoietic stem/progenitor cells, as well as confer adhesive properties with circulating tumor cells.[60] PMP promote monocyte and neutrophil adhesion to the endothelium and stimulate COX-2 expression in monocytes and endothelial cells. Furthermore, it was established that PMP could not only passively carry various proteins and receptors, but also chemoattract hematopoietic cells and stimulate their adhesion, survival, and proliferation.

Higher concentrations of PMP have been documented in thrombophilic diseases in which platelets play a role, such as heparin-induced thrombocytopenia, thrombosis, idiopathic thrombocytopenic purpura,[61] but also in cardiovascular disease, for example in patients with peripheral arterial disease, unstable angina, myocardial infarction, cerebrovascular accident and diabetes.[62]

Many studies have explored associations for platelet activation markers to gain insights on the pathogenesis of obesity and atherosclerotic disease.[63] Csongrádi et al. report on a large cross-sectional cohort study that explored relationships for platelet activation

markers in obese subjects, including those with and without common co-morbidities for atherosclerotic disease.[64]

The extent of atherosclerotic disease, and the metabolic derangements associated with obesity and atherosclerotic disease, influence the generation of platelet microparticles. This possibility is supported by a study that showed weight loss-induced improvements in insulin resistance, among women with obesity, reduced markers of platelet activation. [65]

1.5.2.2. PMP exposing P-selectin

Soluble P selectin (sPsel) belongs to the selectin family of cell adhesion molecules. It is a membrane component of the platelet alpha granule and endothelial cell Wiebel - Palade body. It mediates binding to specific carbohydrate containing ligands, which is present on leukocytes and in smaller amounts on platelets.[66]

Expression of P-selectin provide additional prothrombotic potential to microparticles. PMP exposing P-selectin (P-selectin+ MP) reflect platelet activation. Increased concentrations of circulating PMP P-selectin+ are found in peripheral arterial disease and myocardial infarction.[62]

Platelets from obese individuals seem to be more reactive both at baseline and after aspirin, suggesting an innate platelet hyperaggregability.[67] Csongrádi et al. found “independent” associations between platelet-derived microparticle levels with BMI, and between soluble P-selectin and fibrinogen, and platelet P-selectin+ and both carotid intimal medial thickness and PAI-1: this provides evidence that platelet activation is influenced by the complex metabolic and inflammatory parameters that participate in the pathogenesis of atherosclerotic disease.[64]

1.5.2.3. Endothelial-derived microparticles

Endothelial cell microparticles (EMP) account for only 5% to 15% of the total MP. [68] They express different antigens, such CDs 31 and CD34, CD62E (E-selectin)[69], CD 105 (endoglin) and CD146. EMP have different markers (qualitatively and quantitatively) depending on whether they are generated by cell activation processes or by apoptosis. It was found that in apoptosis, constitutive endothelial cell markers were increased on

microparticles (CD31 > CD105), whereas inducible endothelial microparticles markers were markedly increased during activation processes (CD62E > CD54 > CD106).[70]
EMP, which play an important role in cell-to-cell communication and function, have confirmed procoagulant, proinflammatory, angiogenic and other functions, affecting pathological processes.
When EMP expressing tissue factor, which is the initiator of the extrinsic coagulation pathway, they induce TF-dependent thrombin formation in vitro and in vivo.[71]
EMP not only as a surrogate marker of endothelial dysfunction or injury but also as effectors able to amplify a pre-existing vascular dysfunction. Elevations in EMP are negatively correlated with flow-mediated vasodilation and positively correlated with pulse wave velocity and carotid intima-media thickness.[72] High levels of circulating MPs, from platelet, monocyte or endothelial lineages, were observed in patients with hypertension, EMP being features of both systolic and diastolic pressures. EMP in peripheral blood may be sensitive markers of the thrombo-occlusive vascular process developing in the coronary arteries of STEMI patients. EMP increase also in patients with congestive heart failure, in cerebral ischemia: EMP act as novel diagnostic and therapeutic biomarkers of circulatory hypoxia-related diseases.

1.5.2.4. Leukocyte derived-microparticles

Leukocyte-derived microparticles (LMP) may originate from neutrophils, monocytes and macrophages, and B- and T lymphocytes. They carry markers from their parental cells (Figure 9) and harbor membrane and cytoplasmic proteins as well as bioactive lipids implicated in a variety of mechanisms, maintaining or disrupting vascular homeostasis.
When they carry tissue factor or coagulation inhibitors, they participate in hemostasis and pathological thrombosis. Both proinflammatory and anti-inflammatory processes can be affected by LMP, thus ensuring an appropriate inflammatory response. LMP also play a dual role in the endothelium by either improving the endothelial function or inducing an endothelial dysfunction. LMP are implicated in all stages of atherosclerosis. They circulate at a high level in the bloodstream of patients with high atherothrombotic risk, such as smokers [73], diabetics and subjects with obstructive sleep apnea [74], where their prolonged contact with the vessel wall may contribute to its overall deterioration. LMP modify the endothelial function and promote the recruitment of inflammatory cells

in the vascular wall, necessary processes for the progression of the atherosclerotic lesion. In addition, LMP favor the neovascularization within the vulnerable plaque and, in the

Cellular Origin of Microparticles	Marker
Leukocyte	CD45*
Neutrophil	CD15
	CD64
	CD66b
	CD66e
Monocyte	CD14
	CD11a
	CD18
Lymphocyte	CD2
	CD3
	CD4
	CD8
	CD19
	CD20

Figure 9: Cellular origin of LMP, modified from [75].

ruptured plaque, they take part in coagulation and platelet activation. Finally, LMP participate in angiogenesis.[75]

They circulate at a high level in the bloodstream of patients with increased atherothrombotic risk or CVD: LMP might be useful in predicting cardiovascular events.

1.5.2.5. Tissue factor-positive microparticles

Tissue factor (TF) is a transmembrane protein that binds plasma factor VII/ VIIa. The TF:FVIIa complex activates both factor X and factor IX to initiate blood coagulation (Figure 5).[76]

TF is normally expressed by extravascular cells or present in the blood in an encrypted form. It is constitutively expressed by certain cells within the vessel wall and cells surrounding blood vessels, such as vascular smooth muscle cells, pericytes, and

adventitial fibroblasts: this provides a primary hemostatic barrier around all blood vessels that rapidly activates blood coagulation after injury.

TF is also expressed in a tissue-specific pattern with high levels in different organs, in particular, by astrocytes in the brain, epithelial cells in the lung, cardiomyocytes in the heart, and trophoblasts in the placenta to give additional hemostatic protection to these tissues. This distribution of TF is consistent with its essential role in hemostasis, because this limits inappropriate activation of the blood coagulation cascade.

Pathologic conditions lead to induced TF expression by variety of vascular cells, and this expression plays an important role in thrombosis. In sepsis, TF is expressed by vascular cells, such as monocytes and endothelial cells.

The presence of TF on MPs dramatically increases their procoagulant activity. TF has a high affinity for FVII/FVIIa, and therefore TF+MPs in blood will readily bind FVII/FVIIa.[77]

Levels of TF+MPs are elevated in patients with a variety of diseases, including cardiovascular disease, diabetes, cancer, sickle cell disease, and endotoxemia. In cancer, TF+MPs are derived from tumors and may serve as a useful biomarker to identify patients at risk for venous thrombosis.[77]

Many cell types can generate circulating TF+MPs. For instance, leukocytes, endothelial cells, platelets and vascular smooth muscle cells have all been shown to produce TF+MPs. The contribution of these different cell types to the pool of circulating TF+MPs may depend on the underlying disease.[76]

1.5.2.6. Microparticles CD36+

CD36 is a transmembrane glycoprotein expressed on a variety of cells and tissues, including platelets, erythrocytes, monocytes, endothelial cells and leucocytes. It’s a multifunctional signalling molecule recognized by several known ligands, such as thrombospondin-1, long chain fatty acids, and lipoproteins, including HDL, LDL, and VLDL. Various studies described that CD36 is intimately involved in lipid metabolism and homoeostasis and implicated in pathological conditions associated with metabolic dysregulation, including obesity, insulin resistance, diabetes, diabetic nephropathy and atherosclerosis. Soluble CD36 (sCD36) was elevated in prediabetic conditions, including polycystic ovary syndrome, and was identified as a novel marker of liver injury in patients with altered glucose metabolism, so sCD36 could be used as a marker of the

metabolic syndrome and atherosclerosis. Alkhatatbeh et al. described that sCD36 was associated with the plasma MP fraction, suggesting that sCD36 in the plasma of normal subjects is a product of circulating MPs. Furthermore, CD36+MPs are readily detectable in diabetes mellitus, occurring at levels corresponding to approximately half that of PMPs and the origin was mainly from erythrocytes in mellitus diabetes as compared with endothelial cells in healthy controls.[78, 79]

1.5.3. Thrombin generation

Thrombin is a key enzyme in the coagulation process and leads to the conversion of fibrinogen to fibrin and clot formation. In the hemostatic mechanism there are no pathways that bypass thrombin. The measurement of the *in vitro* thrombin generation (TG) describes the potential of a sample to generate thrombin and may quantify the composite effects of multiple parameters of the coagulation system, so it reflects much, if not all, of the thrombotic-haemostatic function of the blood.[80]

The Calibrated Automated Thrombogram (CAT) method is considered a test of global hemostatic capacity. This method describes the potential of a sample to generate thrombin and may quantify the composite effects of multiple parameters of the coagulation system, so it can reflect both thrombotic (high TG) and hemorrhagic (low TG) phenotype, therefore indicating both the increased risk of thrombosis and bleeding.[81]

Specifically, it generates a thrombin generation curve that mimics the overall plasma coagulability potential when a thrombogenic stimulus appears (tissue factor, phospholipids and calcium chlorid) (Figure 10).[82] The thrombin activity can be measured in platelet-poor plasma by continuous cleavage of a low-affinity fluorogenic substrate for thrombin: the first derivative of the intensity of fluorescence is the TG curve. This curve is characterized by a lag (initiation) phase, followed by a thrombin burst (propagation), which is then eventually completely inhibited by plasma protease inhibitors (termination).[82]

From this curve, various parameters can be inferred that describe thrombin activity, including:

- lag time: the time until thrombin burst;
- peak height: peak amount of thrombin generation (peak thrombin);
- peak time: time thrombin takes to reach the peak;

- endogenous thrombin potential (ETP): the total amount of thrombin generated, represented by the area under the curve (AUC).[83]

Shorter lag time and higher ETP and thrombin peak are indicator of a hypercoagulable state, on the other hand prolonged lag time and reduced ETP and peak are indicator of a hypocoagulable state. [44]

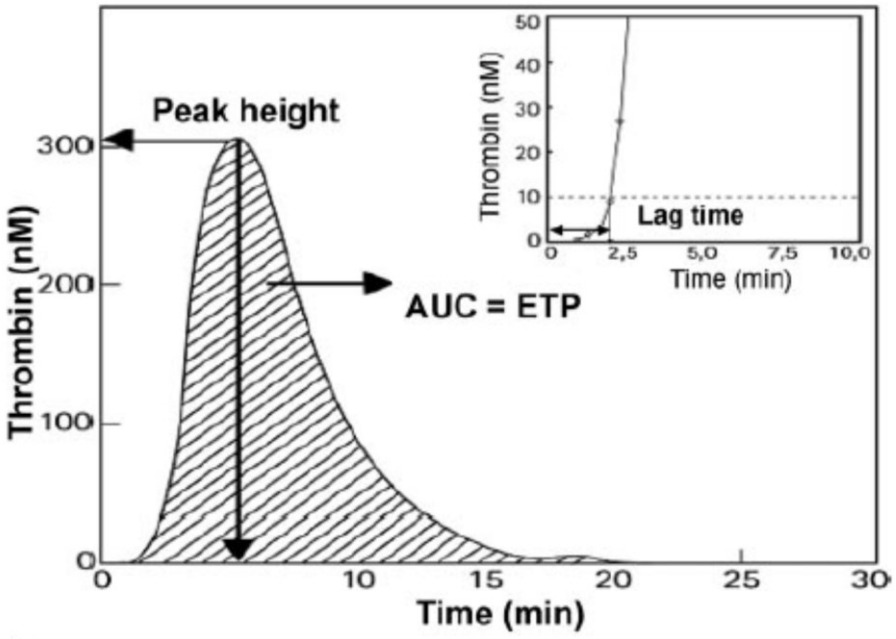


Figure 10: Example of a thrombin generation curve obtained by means of the CAT method in normal individuals, adapted from [82].

Increase of thrombin generation is observed in deficiency of antithrombin as well as in congenital hyperprothrombinaemia. When thrombomodulin is added, thrombin generation becomes sensitive to all congenital and acquired (also oral contraceptives) disorders of the protein C system.[81] The thrombin generation test has great clinical potential, such as in monitoring patients taking anticoagulants and antiplatelet drugs, screening for genetic or acquired thrombotic disorders, and evaluating bleeding risk control in patients with hemophilia using bypass agents or replacement therapy.

1.5.4. Platelet function methods in conjunction with viscoelastic methodologies: thromboelastography and thromboelastometry

Thrombelastography was first described by Hartert on 1948. The viscoelastic changes that occur during coagulation were recorded, providing a graphical representation of the fibrin polymerization process. The rate of fibrin polymerization as well as the overall clot strength is assessed.[84]

The basic principle of thromboelastography involves incubation of whole blood or platelet rich plasma in a heated sample cup into which is suspended a pin. The pin and the cup or the cup alone oscillates, and as the blood clots, the motion of the cup is transmitted to the pin which is recorded via a computer. To perform thromboelastography, special equipment is needed, either a thromboelastograph (TEG®**;** Haemoscope Corporation, IL, USA) or rotation thromboelastometer (ROTEM®; Sysmex, Milton Keynes, UK). In the TEG®**,** a sensor (pin) is connected with a torsion wire, and clot formation generates a physical connection between the cup and sensor, which is recorded via a mechanical-electrical transducer. In the ROTEM®, the pin (sensor) is fixed on the tip of a rotating shaft, whereas the sample cup is stationary and the position of the axis is detected by reflection of light on a small mirror on the axis.[52]

The in vitro addition of specific reagents and activator to whole blood provokes the hemostatic activation followed by modifications in hematic viscoelastic forces: the entire process is instantly recorded during all clotting formation and also visualized in a typical curve. Different tests are available depending on the reagent used: tissue factor for extrinsic pathway (EXTEM test), different activators (glass, silica, kaolin, ellagic acid) for intrinsic pathway (INTEM test). The fibrin component of coagulation thromboelastometry (FIBTEM test) is used for the assessment of the specific role of fibrinogen in clot formation after the inhibition of platelets function.[85]

The advantage that the TEG®/ROTEM® offers is its bedside capability to deliver within 30 min a representation of the sum of platelet function, coagulation proteases and inhibitors, and the fibrinolytic system. The main uses of the TEG®/ROTEM® have been to monitor blood component therapy during surgery, such as liver transplantation, cardiac surgery and in trauma patients.[84]

Among available ROTEM® parameters (Figure 11), the following were selected:

a) Clotting Time (CT, sec.), the time from the beginning of the coagulation analysis until an increase in amplitude of 2 mm, corresponding to the initiation phase of the clotting process;

b) Clotting Formation Time (CFT, sec), the time in seconds between an increase in amplitude of thromboelastogram from 2 to 20mm;

c) Maximum Velocity (MaxV, mm/min), the peak of the first derivative of the thromboelastographic clotting curve; the CFT and MaxV reflect measures of the propagation phase of whole blood clot formation;

c) Maximum Clot Firmness (MCF, mm), the maximum amplitude in millimetres reached in the thromboelastogram which correlates with platelet count and function as well as with the concentration of fibrinogen; the MCF quantifies the maximum clot firmness of the established whole blood coagulum;

d) Area under curve (AUC, mm*100), defined as the area under the velocity curve, that is the area under first derivative curve ending at a time point that corresponds to MCF, was also evaluated.

'Hypercoagulable profile was defined as CT or CFT shorter and/or MCF, MaxV or AUC higher than in the healthy controls.[84]

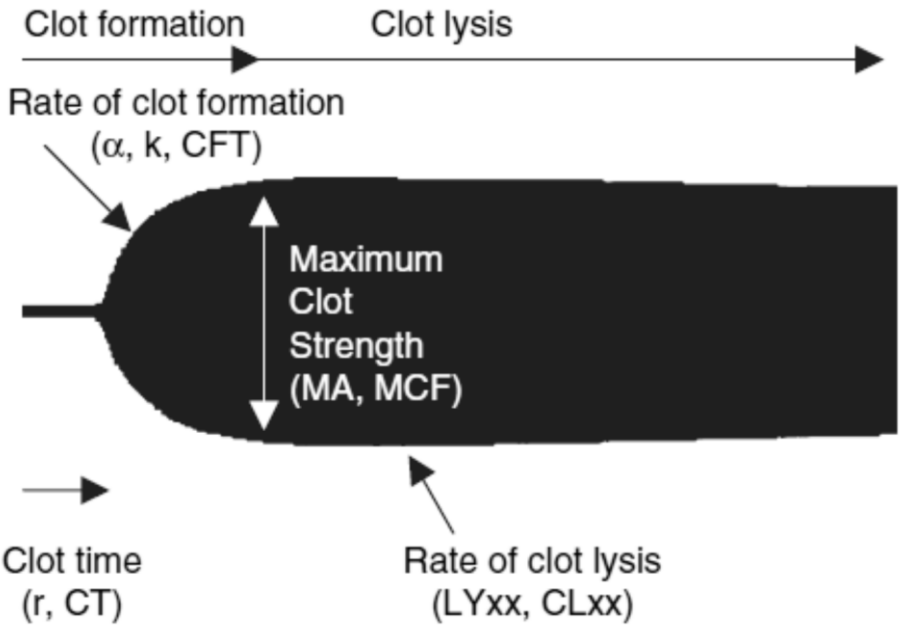


Figure 11. Diagrammatic representation of a TEG®/ROTEM® trace indicating the commonly reported variables, derived from [84].

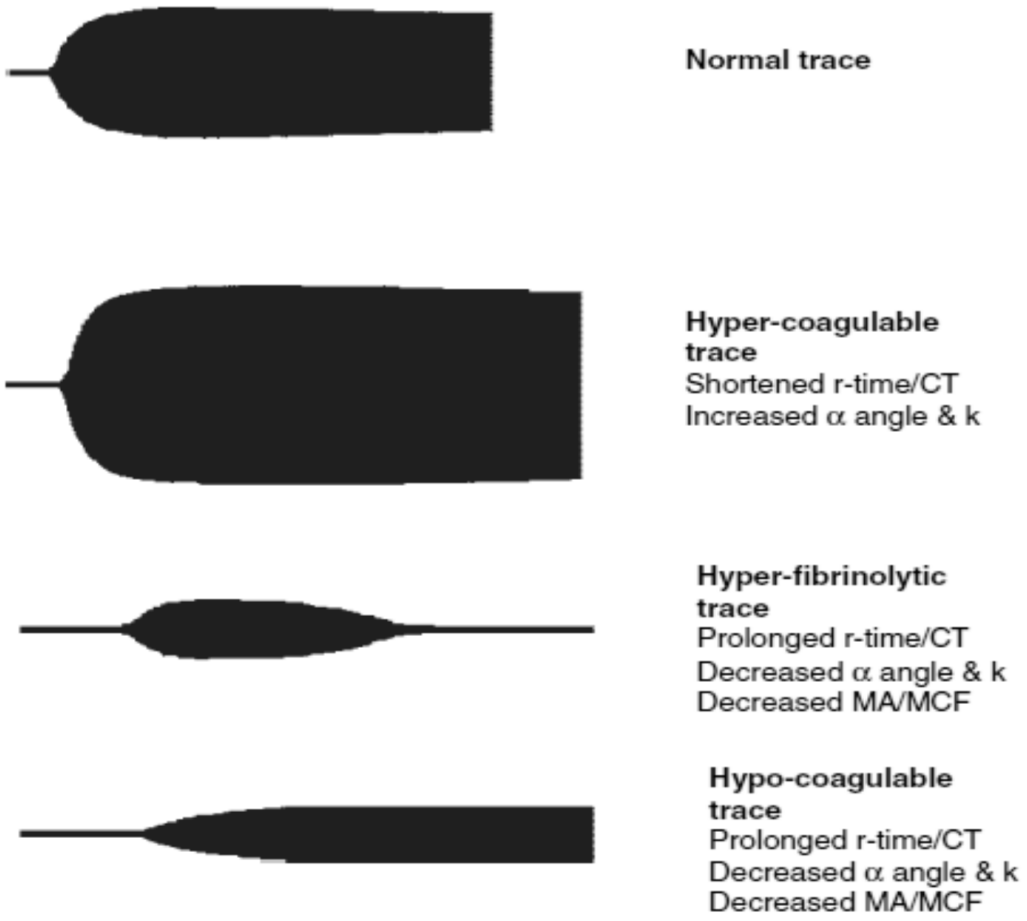


Figure 12. Examples of abnormal ROTEM® traces, derived from [84].

1.5.5. Test based on a platelet aggregation: impedance whole blood aggregometry

Impedance whole blood aggregometry allows one to assess platelet function by using the anticoagulated whole blood (WB) as milieu without any sample processing.

The most commonly used method is light transmission aggregometry ('Born' aggregometry) employing citrated or heparinized platelet-rich plasma (PRP).[86] The test measures the change in light transmission in real time when agonists are added to PRP or washed platelets. A typical panel of agonists includes adenosin diphosphate (ADP), collagen, arachidonic acid, adrenaline, PAR-1 peptide, U46619 and ristocetin. The nature of the response is dependent on the agonist used, its concentration and the role of the feedback agonists ADP and thromboxane A2. Disadvantages of this technique include the need of centrifugation to separate other blood cells from platelets, which are also known

to influence platelet function. Besides, PRP does not contain all blood platelets: this usually leads to the loss of giant platelets which may be both hypo- and hyperactive.

For these reasons, novel methods have been introduced to measure platelet aggregation in whole blood. One of these methods is impedance aggregometry, introduced by Cardinal and Flower.[87] As compared with PRP, impedance aggregometry in whole blood was more sensitive to the aggregating effect of thrombin, ristocetin and arachidonic acid, or to the inhibitory effect of prostacyclin or aspirin.

It evaluates platelet responses in disposable ready-to-use test cuvettes, each containing two pairs of electrodes. The platelets, after activation by different agonists, adhere to the two copper wires of each of the two measuring units. During analysis, the instrument continuously measures the changes of the electrical resistance (called "impedance") between the electrodes that is proportional to the amount of platelets adhering to each couple of electrodes. The impedance change determined by each sensor is recorded independently. Impedance aggregometry, as realized in commercial instruments (e.g. Chrono-log®) has some problems. The re-usable electrodes have to be cleaned between analyses, which is impractical and a possible source of error.

For these reasons, a novel instrument to measure platelet aggregation in diluted whole blood has been developed. Due to the use of four electrodes per test cell, the technique is called "multiple electrode aggregometry" (MEA). The instrument is named "Multiplate®", for "multiple platelet function analyzer", indicating the multiplicity of channels and sensors per channel of the device.

The mean values of the determinations are expressed in arbitrary "aggregation units" (AU). Increased electrical resistance is continuously monitored and reported as the area under the curve (AUC), that is used to express the aggregation response over the measured time (AU*min).[88] Monitoring antiplatelet therapies (e.g. aspirin) by using impedance has permitted the prediction of major adverse cardiovascular events in cardiovascular patients at high risk.[89] In addition, MEA has not only been able to identify cardiovascular patients not responding to antiplatelet agents and at risk of major adverse cardiovascular events, but has been considered a skill assay to distinguish those patients with a high inhibition of platelet function and at risk of bleeding.

1.6. Adipose tissue and low grade chronic inflammation

Physiologically, adipose tissue (AT) stores energy to support metabolic requirements in the times of need. From an evolutionary point of view, this is beneficial, but with increased nutrient intake and reduced energy expenditure in our modern world, adipose tissue expands and his function becomes altered.

AT secretes a variety of bioactive peptides, known as adipokines, which act at both the local (autocrine/paracrine) and systemic (endocrine). Moreover, it expresses numerous receptors that allow it to respond to afferent signals from traditional hormone systems. AT contains the metabolic mechanisms to permit communication with different organs. Through this interactive network, it is integrally involved in coordinating a variety of biological processes including energy metabolism, neuroendocrine function and immune function. Adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells. Although adipocytes secrete different kind of endocrine hormones, many secreted proteins are derived from the non-adipocyte fraction.[90] These components function as an integrated unit, making adipose tissue a true endocrine organ.

In obesity dysfunctional adipose tissue is considered one of the primary origins of the metabolic disturbances that are present in the metabolic syndrome, such as insulin resistance and hyperglycemia, dyslipidemia, hypertension and prothrombotic and proinflammatory state.[91] AT dysfunction is thus characterized by decreased release of homeostatic protective factors such as adiponectin, nitric oxide, or protective prostaglandins and increased activation of stress related pathways leading to pathological adipokine release (leptin, resistin, visfatin) and development of low-grade inflammation.

1.6.1. Leptin

Leptin is a 16-kDa polypeptide containing 167 amino acids with structural homology to cytokines and it is the product of the OB gene. Circulating levels of leptin parallel fat cell stores, increasing with over feeding and decreasing with starvation. It acts mainly at the level of the central nervous system to regulate food intake and energy expenditure: leptin levels rapidly decrease with caloric restriction and weight loss. This decline is associated with adaptive physiological responses to starvation including increased appetite and decreased energy expenditure. These same responses are observed in leptin-deficient mice and humans, despite massive obesity. Furthermore, these responses are readily normalized by low dose leptin replacement. In contrast, common forms of obesity are

characterized by elevated circulating leptin. Neither endogenously high leptin levels nor treatment with exogenous leptin is effective in ameliorating this obesity, consistent with a state of leptin resistance. [92]

The absence of leptin or a mutation in leptin receptor genes induces a massive hyperphagia and obesity in animal models, and humans [93], however, the prevalence of these mutations in obese humans is rare.

Adipocytes secrete leptin in direct proportion to adipose tissue mass as well as nutritional status. mRNA expression is higher in subcutaneous adipose tissue than in visceral adipose tissue in human Leptin's secretion is regulated by a variety of factors. For example, it is increased by insulin, glucocorticoids and tumor necrosis factor α and decreased by β3-adrenergic activity, androgens, and GH.

Although leptin acts mainly to regulate food intake, numerous other important endocrine effects of leptin include regulation of hematopoiesis, immune function, angiogenesis and bone development. The increased risk of cardiovascular disease with obesity makes adipokines, including leptin, an attractive instigator of atherosclerosis. In a large prospective study, leptin was independently associated with an increased risk of coronary artery disease. There is a relationship between leptin and the low grade inflammatory state in obesity, suggesting that it could exert peripheral biological effects as a function of its cytokine-like structure.[94] It was described that platelets express the leptin receptor and leptin potentiates the aggregation of platelets from ob/ob (knocked out for ob gene) mice in response to known agonists.[95] Furthermore, leptin can induce dose-dependent platelet stimulation in healthy subjects, but this effect was reduce in overweight and obese patients.[96]

1.6.2. Tumor necrosis factor

Tumor necrosis factor α (TNFα) is a 26-kDa transmembrane protein that is cleaved into a 17-kDa biologically active protein that exerts its effects via type I and type II TNFα receptors. TNFα is a pro-inflammatory cytokine produced by a variety of cell-types: within adipose tissue, TNF is expressed by adipocytes and stromal vascular fraction.[90]

Although initially suspected of playing a role in cachexia, TNFα has now been implicated in the pathogenesis of obesity and insulin resistance. Chronic exposure to TNFα induces insulin resistance both *in vitro* and *in vivo.*[97]

This effect is mediated by activation of serine kinases that increase serine phosphorylation of insulin receptor substrate-1 and -2, making them poor substrates for insulin receptor kinases and increasing their degradation. Moreover, TNFα influences gene expression in metabolically important tissues such as adipose tissue and liver. It represses genes involved in uptake and storage of nonesterified fatty acids (NEFAs) and glucose, suppresses genes for transcription factors involved in adipogenesis and lipogenesis, and changes expression of several adipocyte secreted factors including adiponectin and interleukin-6.[98]

1.6.3. Interleukin-6

Interleukin-6 (IL-6) circulates in multiple glycosylated forms ranging from 22 to 27 kDa in size.

IL-6 is produced by many cell types (fibroblasts, endothelial cells, monocytes), and many tissues including adipose tissue. Secretion is higher in visceral adipose tissue than in subcutaneous adipose tissue and macrophages contribute approximately 50% of white adipose tissue-derived IL-6.

It is now well known that IL-6 production by adipose tissue is enhanced in obesity. Adipose tissue IL-6 expression and circulating IL-6 concentrations are positively correlated with obesity, impaired glucose tolerance, and insulin resistance. Both expression and circulating levels decrease with weight loss.

IL-6 is a multifunctional cytokine acting on many cells and tissues. One of the main effects of IL-6 is the induction of hepatic C-reactive protein production, which is an independent major risk marker of cardiovascular complications. IL-6 also decreases insulin signaling in peripheral tissues by reducing expression of insulin receptor signaling components and inducing suppressor of cytokine signaling 3, a negative regulator of both leptin and insulin signaling.[90]

1.6.4. High-sensitivity C-reactive protein (hs-CRP)

C-reactive protein (CRP) has emerged as the main acute phase reactant inflammatory marker in clinical practice. It's produced by the liver and the serum levels of this protein increase over 100-fold in response to tissue injury, infection, inflammation and neoplastic proliferation. The measurement of serum CRP concentrations is inexpensive and is done

routinely to assess patients. Many studies have demonstrated the positive association between CRP with various measures of obesity, obesity related disease, and cardiovascular risk. In addition, the serum high-sensitivity C reactive protein (hs-CRP) level is known to be a predictive marker of the degree of atherosclerosis and future cardiovascular events. It's known that it has been associated with obesity, insulin resistance and cardiovascular dysfunction and several studies have also observed that elevated CRP predicts the prognosis of patients with CVD at the acute stage.[99]

1.7. Treatment of obesity

Patients with obesity needs a stepped intensification of care approach to weight management. If the patient is not able to achieve the weight and health goal by life-style alone and meets the indications for drug therapy, then addition of adjunctive pharmacotherapy should be considered. As a last step, bariatric surgery can be considered for subjects with complications and who meet its indications. A multidisciplinary approach that addresses psychological, social, environmental and biological factors of obesity is critical to ensure comprehensive care, as well as best practices and outcomes.

1.7.1. Lifestyle

The target of obesity care is assisting patients in making healthier dietary and physical activity choices that will lead to a net negative energy balance. Lifestyle education, provided in individual or group sessions by a trained health worker, is aimed to improve physical activity level, eating behaviors, healthy diet, stress management, and sleep. The initial goal is to achieve a 5% to 10% weight loss over the initial 6 months of treatment.[8] Caloric reduction is the most important component in achieving weight loss whereas increased and sustained physical exercise is particularly important in maintaining the lost weight. The dietary prescription will vary according to the patient's metabolic profile and risk factors. Weight loss is primarily dependent on reducing total caloric intake, not the proportions of carbohydrate, fat, and protein in the diet. Moreover, several studies support the evidence that promoting eating habits consistent with Mediterranean diet nutrients pattern may be a useful and safe strategy for the treatment of obesity.[100] A consultation with a dietitian for medical nutrition therapy is particularly useful along

with the importance of emphasizing collaborative care and self-management of chronic disease.

In addition to reducing caloric intake, patients are also encouraged to burn more calories. There is a distinction between physical activity and exercise and weight loss counselling should encourage both aspects as part of treatment. Whereas physical activity consists of any bodily movement that increases energy expenditure, for example activities of daily living like walking, climbing stairs and gardening, while exercise is defined as planned, structured and repetitive bodily movement done to improve or maintain one or more components of physical fitness. Studies have demonstrated that lifestyle activities are as effective as structured exercise programs in improving cardiorespiratory fitness and weight loss.[M2]

1.7.2. Pharmacotherapy

Non-pharmacological treatment of obesity can be effective, but the long-term success rate is low and regaining lost weight is a major problem. Randomized studies have shown that a greater initial weight loss achieved with changes in lifestyle associated with other strategies (for example, anorectic drugs) improves long-term weight maintenance, provided that it is followed by a 1-2 years of integrated weight maintenance program consisting of lifestyle interventions involving dietary change, nutritional education, behavior therapy and increased physical activity. A greater initial weight loss as the first step with a pharmacological intervention may result in improved sustained weight maintenance.

Pharmacotherapy is approved for patients with a BMI $\geq$ 30 kg/m^2 or $\geq$ 27 kg/m^2 when complicated by an obesity-comorbidity. It is clearly stated that medications should be approved by the regulatory agencies, and clinicians should know what the benefits and health risks of the prescribed medication are. Medications work to reinforce lifestyle change and should be prescribed only as an adjunct to a good quality lifestyle intervention.[101]

Factors leading to the development of obesity have been extensively studied in recent years. The central regulation of appetite, especially with regard to the hedonic appetite, is a field highly exciting. Recently, new medications have been approved for the treatment of obesity in the United States: orlistat, liraglutide, phentermine/topiramate, naltrexone/bupropion and lorcaserin.

1.7.3. Bariatric surgery

In adults, bariatric surgery is extremely effective compared to conservative treatment, resulting in adequate long-term weight loss and reduction of mortality. Surgical approaches to treat obesity were first reported in Sweden in 1952. Actually, indications for bariatric surgery are reserved for patients with BMI ≥ 40 kg/m^2 or with BMI ≥ 35 kg/m^2 with co-morbidities in which surgically induced weight loss is expected to improve the disorder (such as metabolic disorders, cardiorespiratory disease, severe joint disease, obesity-related severe psychological problems).

The former classification of operations according to their influence on food ingestion, defined as:

- limiting stomach capacity (restrictive surgery): adjustable gastric banding and sleeve gastrectomy (SLG),
- limiting absorption of nutrients (malabsorptive surgery): biliopancreatic diversion (BPD) and BPD/duodenal switch (BPD-DS);
- combined procedures: Roux-en-Y gastric bypass (RYGB).

Anyway, this classification not appropriately reflects the current level of knowledge about early and

weight-independent metabolic effects of these operations. Nowadays, most of the standard surgical interventions are being mostly referred to as metabolic operations.

1.7.3.1. Sleeve Gastrectomy

Sleeve gastrectomy (SLG) (Figure 13) was first performed by Hess in 1988 as part of his biliopancreatic diversion with the duodenal switch (BPD-DS) procedure, adapted from Scopinaro's biliopancreatic diversion (BPD) and DeMeester's duodenal switch (DS) procedures.[102] SLG consists of removing 80% of the stomach along the greater curvature, creating a tube-like stomach remnant, with the rest of the intestine intact. Weight loss induced by SLG was initially ascribed exclusively to a restrictive action: gastric volume reduction leads to reduced food intake. However, it has been demonstrated that both the resection of the gastric fundus and the rapid emptying of the stomach promote significant neuro-hormonal changes: by removing the gastric fundus, SLG induces a significant decrease in the circulating levels of ghrelin, the hormone of appetite, and an increased satiety through a food-mediated release of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) from the L cells of the small intestine. These changes

persist 1 year after surgery, showing that SLG can be considered a “food-limiting” operation rather than a restrictive procedure.

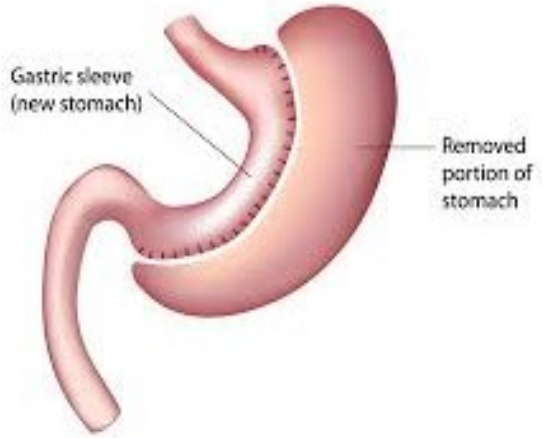


Figure 13: Sleeve gastrectomy.

2 AIM

The objectives of this study were to evaluate global hypercoagulability in overweight and obese patients with and without metabolic syndrome by measuring: a) the levels of MP of different cellular origin and TF-bearing MP as well as MP functional activity and the levels of FVIIa-AT complexes, as an indirect measure of TF activation; b) thrombin generation as a general marker of hypercoagulability by measuring thrombin generation by the calibrated automated thrombogram (CAT) method; c) altered platelet aggregability by whole blood rotation thromboelastometry (ROTEM®) and by impedence aggregometry (Multiplate®).

Furthermore, in order to evaluate the effect of weight loss, the aim of this study was to prospectively evaluate the levels of MP of different origin as well as their functional activity in a group of 20 II degree obese patients.

3 PATIENTS AND METHODS

3.1 Study participants

Eighty patients of both sexes referred to the Centre for the Study and the Integrated Treatment of Obesity of the Padua University Hospital between January 2011 and December 2012 for a cardiovascular evaluation were enrolled in this study.

Inclusion criteria were:

a) BMI ≥ 25 kg/m^2;

b) age between 18–70 years;

c) their informed consent.

These subsequent exclusion criteria were considered:

- acute or previous [namely, acute (< 3 months) or previous (> 3 months)] cardiovascular disease or ischemic complications;
- acute or previous venous thromboembolism;
- infections or inflammatory disease;
- severe blood hypertension (≥ 160/100 mmHg);
- diabetes mellitus (ongoing anti-diabetic medications or glycohemoglobin test ≥ 6.5%);
- cancer;
- recent surgery (≥ 3 months if major surgery, ≥ 1 month if minor surgery) or previous bariatric surgery;
- anticoagulation, antiplatelet or steroid therapy;
- pregnancy and/or puerperium, estroprogestinic therapy at the time of sampling or in the preceding three months.

Eighty consecutive patients were included in the study. Twenty (25%) patients were overweight (BMI = 25-29.9 Kg/m^2); 20 (25%) were I degree obese (BMI = 30-34.9 Kg/m^2); 20 (25%) were II degree obese (BMI = 35-39.9 Kg/m^2) and 20 (25%) were III degree obese (BMI ≥ 40 Kg/m^2).

A control group of 40 consecutive normal weight subjects (BMI < 25 kg/m^2) matched for age (± 3 years), sex and ethnic origin referred to our Centre for diet advice or for the evaluation of their cardiovascular risk was recruited. They were free from listed exclusion criteria.

In order to evaluate the levels of MP of different origins as well as their functional activity in obese patients, before and after weight loss, other 20 patients referred to our

Centre in the same period were enrolled in this study. Inclusion criteria were a BMI > 40 kg/m^2, age > 18 years and an informed consent. All subjects enrolled were free from exclusion criteria. Patients were examined at enrollment (T0) and after 3 and 12 months (T3 and T12) post-sleeve gastrectomy..

A detailed history was collected from patients and controls in order to assess dietary habits, physical activity and medication in the seven days prior to blood sampling. Active exercise was defined as completing at least 90 minutes (min) of primarily aerobic lower body exercise over 3 days per week. Physical activity was self-reported. Blood pressure (BP) was measured in the same way in all subjects.

The study protocol was approved by the Institutional Ethical Committee of the Padua University Hospital.

3.2 Anthropometric parameters

Anthropometric parameters such as BMI, waist circumference, systolic and diastolic blood pressure, after overnight fasting, were measured in all cases and control. Weight and height were measured by standard scales. BMI was calculated by dividing body weight (kg) by height squared (m) and classified according to the cutoff points proposed by the World Health Organization (WHO).[3] Waist circumference was measured between the last rib and the iliac crest using a flexible and inelastic tape measure. BP was measured in both arms after sitting for 5 min; at least two measurements were taken, with a 1-min interval between them, and the average of the measurements recorded.

3.3 Metabolic analysis

Biochemical tests including total cholesterol, low-density lipoproteins, high-density lipoproteins, triglycerides, fasting blood glucose (reference values [r.v.] ≤ 100 mg/dl), and insulin were measured after overnight fasting in all cases.

Total cholesterol (r.v. ≤ 200 mg/dl), LDL (r.v. ≤ 100 mg/dl) and triglycerides (r.v. ≤ 150 mg/dl) evaluation was carried out on an automated analyzer (DAX, Bayer, Pittsburgh, PA, USA) using commercial reagents (Randox Laboratories, Co. Antrim, UK). HDL (r. v. ≥ 40 mg/dl) was measured using commercially available reagents (Wako Corp., Osaka, Japan). Insulin resistance was defined by a homeostasis model assessment index [HOMA-IR: plasma glucose (mmol/l) x insulin (mU/l)/22.5] > 2.4.[103]

Metabolic syndrome was defined following the National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATP III) criteria (three of the following: waist circumference ≥ 102 cm for European men and ≥ 88 cm for European women; raised triglyceride level ≥ 150 mg/dl; reduced HDL cholesterol: < 40 mg/ dl in males and < 50 mg/dl in females; raised blood pressure: systolic ≥ 130 or diastolic ≥ 85 mmHg; raised fasting plasma glucose: ≥ 100 mg/dl or previously diagnosed type 2 diabetes).[104]

Plasma levels of interleukin-6 (IL-6) (r.v. < 5.9 ng/l), TNF (r.v. < 8.1 ng/l) and leptin (r.v. 2 - 15 μg/l) were measured by ELISA (R&D Systems, Minneapolis, MN, USA). High-sensitive C reactive

protein (hs-CRP) (r.v. < 3 g/l) was measured by a turbidimetric assay on the Integra 800 analyzer (Roche, Basel, Switzerland). White blood cells and platelet count (r.v. 4.5 - 10.0*10^9/l and 150 - 450 × 10^9/l, respectively) were measured on the Sysmex Counter XE-2100 (Dasit Spa, Milan, Italy).

3.4 Coagulation parameters

Nine ml of venous blood was drawn directly in the laboratory from the antecubital vein with a light tourniquet, using a butterfly device with 21-gauge needle without venostasis. The first few ml were discarded to avoid the contact phase activation. Blood was collected directly into syringes pre-filled with 1 ml of sodium citrate 109 mol/l. Vacuum collection tubes were not used because of possible artificial cellular disruption and formation of MP by this collection method. Platelet-poor plasma (PPP) was prepared within 1 hour of blood collection by double centrifugation (2 × 15 min at 2,500 g) at room temperature. Prothrombin time (PT) (r.v. 70 - 100 %), partial thromboplastin time (PTT) (r.v. 24.4 - 36.5 seconds), fibrinogen activity (r.v. 150 - 450 mg/dl), factor VIII activity (r. v. 60 - 160 %), and antithrombin activity (AT) (r.v. 80 - 120 %) were measured, using fresh unfrozen plasma, in all samples as previously described. [105]

Aliquots (1.5 ml) were immediately frozen and then stored at - 80 °C until use. Samples were thawed by incubation for 5 min in a water bath at 37ºC immediately before assay. Assays were performed at least one month after storage at - 80 °C. Samples were analyzed only after a single freeze-thaw cycle, and repeated freezethaw cycles were avoided. Patient and control samples were all Processed in the same way by the same experienced operators. The investigators who performed the laboratory tests were unaware of the obesity status of the patients. The FVIIa-AT complex was measured by

ELISA (Asserachrom FVIIa-AT, Stago, Asnieres, France). Factor VIIa activity (FVIIa:act r. v. 60–108 mU/ml) was determined in plasma by a clotting assay (STA-Clot® VIIa-rTF, Diagnostica Stago, Asnieres, France) performed on an ACL 3000 Research (IL, Milan, Italy) according to the protocol supplied by the manufacturer.

3.5 MP assessment and characterization

MP were identified by size and annexin V- fluorescein isothiocyanate (FITC) (Bender MedSystems GmbH, Vienna, Austria) labelling. To measure the different populations of MP, the MP were colabelled with antibodies against cell-type specific antigens and annexin V. Thirty µl of freshly thawed PPP were incubated for 15 min at room temperature in the dark with 3 µl of monoclonal antibodies against cell-type specific antigens and 3 µl of annexin V-FITC (AMP). Platelet-derived MP were identified using CD61-PE (phycoerythrin) (PMP) and CD62P-PE (P-selectin+) -both from Beckman Coulter (Miami, FL, USA); Endothelial-derived MP (EMP) using CD62E-PC5 (phycoerythrin-cyanin 5.1) (Beckman Coulter); Leukocyte-derived MP (LMP) using CD45-PC5 (BioLegend Europe, Uithoorn, The Netherlands) and Tissue factor-bearing (TF+ MP) with CD142-PE, (clone HTF-1, BD, Biosciences, Milan, Italy). An antibody against CD36 (clone NL07) conjugated to eFluor 660 was used to determine CD36+MP. The isotype controls used were IgG1-PC5, clone MOPC-21 (BioLegend Europe), IgG1-PE, clone MOPC-21 (BD Biosciences, Milan, Italy); mouse IgG1-FITC, clone MOPC-21 (BioLegend Europe).The final concentration of monoclonal antibodies ranged from 1 to 4 µg/ml. The samples were diluted in 500 µl of annexin-V kit binding buffer (Bender MedSystems GmbH) before analysis. Thirty µl of counting beads with an established concentration (Flow Count TM Fluorospheres, Beckman Coulter) were added to each sample in order to calculate MP as absolute numbers per µl of PPP. The MP gate was established using a blend of mono-dispersed fluorescent beads of three diameters (0.5, 0.9 and 3µm) (Megamix, BioCytex, Diagnostica Stago). All assays performed on a Cytomics FC500 flow cytometer (Beckman Coulter) as previous described.[106]

3.6 MP procoagulant activity

Procoagulant activity of the MP was measured using the STA® Procoag PPL assay (Diagnostica Stago). The assay measures the clotting time in a system dependent on the

procoagulant phospholipid content of the sample. The assay is performed using a phospholipid depleted substrate plasma to eliminate the influence of any coagulation factors upstream. Factor Xa, in the presence of calcium, triggers the coagulation cascade and a shortening clotting time of the sample indicates an increased concentration of procoagulant phospholipids - a shorter clotting time indicating increased PPL activity. This activity linearly correlates with the functional activity of MP present in the sample.[107]

3.7 **Thrombin generation assay (CAT)**

Thrombin generation (TG) was determined in PPP using the CAT method (Diagnostica Stago). [108] Eighty µl of PPP were pipetted into the well of a microtiter plate together with 20 µl of PPP Reagent (Thrombinoscope BV), a mixture of tissue factor (5pM final concentration per assay) and synthetic phospholipids (4 µM final concentration in the assay). The reaction was started with 20 µl of a mixture composed of the fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC, Thrombinoscope BV) and CaCl2 (final concentrations 417 µM fluorogenic substrate and 16 mM CaCl2). Fluorescence was read in a Fluoroscan Ascent® reader (Thermo Labsystems, Helsinki, Finland) and TG curves were calculated using the Thrombinoscope Software (Thrombinoscope BV). A Thrombin Calibrator (Thrombinoscope BV) was used in order to compensate for any quench by the patient PPP. Using the Thrombin calibrator as a reference, the concentration of thrombin was continuously calculated during the time course of measurement. TG curves were plotted and the lag time (min), peak thrombin (nMol) and endogenous thrombin potential (ETP, area under the curve, nM*min) were recorded. Shorter lag time and higher ETP and thrombin peak are indicator of a hypercoagulable state, on the other hand prolonged lag time and reduced ETP and peak are indicator of a hypocoagulable state.[44]

3.8 Thromboelastometry (ROTEM®)

Viscoelastic clotting measures were performed by ROTEM® (Tem International GmbH,Munich, Germany) tests according to the manufacturer's protocol. In particular, whole blood (WB) was incubated at 37°C in a heated cup. Within the cup is suspended a pin connected to an optical detector system. The cup and pin are oscillated relative to each other through an angle of 4°45". As fibrin forms between the cup and pin, the

transmitted impedance of the rotation of the pin is detected at the pin and a trace generated. All investigations were performed within two hours after blood collection and, once initiated, the blood coagulation was allowed to run until 60 min. Extrinsic coagulation cascade was studied with EXTEM test (ex-TEM®; Tem International GmbH) and intrinsic coagulation cascade was studied with INTEM test (in-TEM®; Tem International GmbH). The influence of fibrinogen on clot firmness was estimated with the platelet-inactivating FIBTEM test (fib-TEM®; Tem International GmbH), as previous described.

The following ROTEM® parameters were analyzed: i) Clotting time (CT, sec), ii) Clot Formation Time (CFT, sec), iii) Maximum Velocity (MaxV, mm/min), iii) Maximum Clot Firmness (MCF, mm), iv) Area Under the Curve (AUC, mm*100). "Hypercoagulable profile" was defined as CT or CFT shorter and/or MCF, MaxV or AUC higher than in the healthy controls.

3.9 Impedance aggregometry (MULTIPLATE®)

Aggregometry impedance measure in WB was performed on the Multiplate® function analyser. [88] Test cells of this device incorporate a duplicate sensor for acceptance sampling and the results, given as Area Under the aggregation Curve (AUC, AU*min), are calculated as the mean values of the two curves. The platelets, after activation by different agonists, adhere to the two copper wires of each of the two independent measuring units. During analysis, the instrument continuously measures the changes of the electrical resistance (called "impedance") between the electrodes that is proportional to the amount of platelets adhering to each couple of electrodes. Platelets were stimulated in three different ways: i) using TRAP-6 via the thrombin receptor (TRAP test - Roche Diagnostics GmbH, Mannheim, Germany); ii) via arachidonic acid, checking cyclooxygenase-dependent aggregation (ASPI test - Roche Diagnostics GmbH, Mannheim, Germany). This test is sensitive to acetylsalicylic acid, non-steroidal anti-inflammatory drugs or other inhibitors of platelet cyclooxygenase; iii) using ADP via the ADP receptor (ADP test - Roche Diagnostics GmbH, Mannheim, Germany).

3.10 Statistical analysis

3.10.1 Hypercoagulability detected by MP and thrombin generation

Continuous variables were investigated for normality using the Shapiro-Wilk test. Normally distributed variables were summarized as mean (± SD) and Student's t-test was performed, whereas non normally distributed variables were summarized as medians with interquartile ranges (IQR) and Mann-Whitney U-test was conducted. Bonferroni corrections were used to correct for multiple comparisons. Frequencies were provided for all nominal values and differences were calculated using Chi-square test. Pearson's correlation analysis was used to detect significant correlations between MP numbers, ETP and metabolic, inflammatory and coagulation parameters. Multivariate regression analysis was used to examine the associations of MP subtypes with BMI and components of metabolic syndrome with models that adjusted simultaneously for several variables selected a priori, specifically: age, sex, physical activity and statin/antihypertensive medication use. Intraassay variability was determined by evaluating 20 random plasma samples 10 times within the same assay run (AMP numbers by flow-cytometry, PPL, TG assays) [%CV=(s/mean)*100, where CV= coefficient of variation]. Inter-assay variability was determined by evaluating the 20 random plasma samples in 10 consecutive assay runs [%CV=(s/mean)*100]. Data were analyzed by using PASW Statistics 17.0.2 (SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was considered significant.

3.10.2 Hypercoagulability detected by ROTEM® and MULTIPLATE®

The sample size calculation was based on pilot observations and the following assumptions: i) Expected increase in MCF of ≥ 3 mm; ii) Expected SD of 4.0mm (1); iii) power = 95%; iv) alpha = 0.05. Based on these assumptions we needed a group of at least 42 obese patients. Continuous variables were investigated for normality using the Shapiro-Wilk test. Normally distributed variables were summarized as mean (±SD) and Student's t-test was performed, whereas non normally distributed variables were summarized as medians with interquartile ranges (IQR) and Mann-Whitney U-test was conducted. Bonferroni corrections were used to correct for multiple comparisons. Pearson's correlation analysis was used to detect significant associations between the parameters analyzed. A "p" value 0.05 was considered statistically significant. Statistical analysis was performed using the PASW Statistics 17.0.2 (SPSS Inc.) for Windows.

3.10.3 MP after weight loss

Continuous variables were investigated for normality using the Shapiro–Wilk test.

Normally distributed variables were summarized as mean (±SD) and Student's t test was performed, whereas non normally distributed variables were summarized as medians with interquartile ranges (IQR) and Mann-Whitney U-test or Kruskall-Wallis test for multiple comparisons were conducted. Percentages of variation in variables were calculated as differences in values between after and before intervention and, divided by the values before the intervention. Spearman's correlation analysis was used to detect significant correlations between MP and other parameters. The Bonferroni post hoc test was applied. Statistical significance was accepted at p\0.05, and SPSS ver. 22 was used.

4. RISULTS

4.1. Metabolic and anthropometric parameters

The anthropometric and metabolic characteristics of the study population are shown in Table 20. Patients had significantly higher HOMA-IR than controls. Patients with II and III degree of obesity had significantly lower mean levels of HDL and higher mean levels of triglycerides than controls. A similar worsening effect of the increase in BMI on atherogenic dyslipidaemia was also noted in overweight patients even if it was not statistically significant. Twenty-two obese patients (36%) were taking antihypertensive therapy (7 with I degree, 4 with II degree, and 11 with III degree): 10 patients were taking an angiotensin-converting enzyme (ACE) inhibitor, nine an angiotensin receptor blocker (ARB), and three a beta-blocker. Four overweight patients (20%) (2 ACE-inhibitors and 2 ARBs) and five controls (12.5%) (2 ACE-inhibitors, 2 ARBs and 1 beta-blocker) were taking antihypertensive drugs. Thirteen obese patients (21%), two (10%) overweight subjects and three (7%) controls were taking statins. The dietary habits and physical activity did not differ significantly between cases and controls in the seven days prior to admission to the study. Thirtyfive (44%) obese patients had metabolic syndrome (7 in I degree obesity group, 14 in II and 14 in III degree obesity group). Categorization according to the different components of the metabolic syndrome was reported in Table 1.

	Controls (N = 40)	**Overweight (N = 20)**	**Obesity**		
			I degree (N = 20)	**II degree (N = 20)**	**III degree (N = 20)**
Anthropometric parameters					
Age (years)	49 ± 14	45 ± 13	44 ± 15	41 ± 12	43 ± 12
Male sex (No - %)	20 (50)	10 (50)	10 (50)	10 (50)	10 (50)
BMI (kg/m^2)	22.2 ± 1.55	27.8 ± 1.09*	32.2 ± 1.06**	36.9 ± 1.60***	47.5 ± 6.84***
Waist (cm)	84.2 ± 4.4	98.8 ± 4.8*	105.4 ± 8.0**	120.0 ± 7.4***	138 ± 19.2***
Metabolic parameters					
Systolic blood pressure (mmHg)	118 ± 5	131 ± 8	132 ± 13*	142 ± 17**	141 ± 16**
Diastolic blood pressure (mmHg)	77 ± 5	84 ± 10*	88 ± 11*	90 ± 8*	92 ± 13*
Antihypertensive therapy, (N - %)	5 (12.5)	4 (20)	7 (35)*	4 (20)	11 (55)***

	Controls (N = 40)	Overweight (N = 20)	Obesity		
			I degree (N = 20)	II degree (N = 20)	III degree (N = 20)
HDL cholesterol (mg/dL)	64 ± 20	58 ± 30	48 ± 13	45 ± 9*	44 ± 10*
LDL colesterol (mg/dL)	103 ± 38	116 ± 35	126 ± 31	119 ± 28	120 ± 31
Triglycerides (mg/dL)	79 ± 25	103 ± 58	100 ± 37	122 ± 32*	120 ± 47*
Statins (N - %)	3 (7)	2 (10)	3 (15)*	5 (25)**	5 (25)**
Fasting glucose (mg/dL)	83 [75-101]	86 [83-92]	90 [80-95]	94 [88-103]*	94 [87-100]*
HOMA-IR	0.93 [0.8-1.1]	1.35 [0.9-1.6]*	2.0 [1.02-2.6]**	2.16 [1.0-2.7]**	5.04 [2.4-6.4]***
Physical activity (min/week)	120 [30-190]	100 [30-180]	90 [30-150]	90 [20-120]	90 [20-120]
Metabolic syndrome: n (%)	-	-	7 (35)	14 (70)	14 (70)
Metabolic syndrome criteria: n (%)					
Waist	-	-	7/7 (100)	14/14 (100)	14/14 (100)
Blood pressure	-	-	6/7 (85)	7/14 (50)	11/14 (78)
Fasting glucose	-	-	2/7 (28)	3/14 (21)	4/14 (28)
HDL cholesterol	-	-	5/7 (71)	12/14 (86)	12/14 (86)
Triglycerides	-	-	2/7 (28)	6/14 (42)	7/14 (50)

Data are expressed by mean ± standard deviation [SD] or median (interquartile range [IQR]). The Bonferroni adjusted significance level was 0.025 (0.05/2). p-values are expressed vs controls: *p < 0.025 **p < 0.01 ***p < 0.001.

Table 1: Metabolic parameters.

4.2. Inflammatory and coagulation parameters

Obese patients presented with significantly higher mean levels of leptin and hs-CRP than healthy controls. A significant higher mean plasma level of IL-6 was observed only in III degree obese patients. There was no statistical difference in PT, PTT, platelet and white blood cells between cases and controls. Second and III degree obese patients had significantly higher levels of fibrinogen than controls. In I degree obese individuals the

fibrinogen was raised, compared to controls, but this difference was not statistically significant. No significant increase in FVIIa or FVIIa-AT complex was seen in any patient group compared to controls. All cases had significantly higher levels of FVIII than healthy individuals (Table 2).

	Controls (N = 40)	**Overweight (N = 20)**	**Obesity**		
			I degree (N = 20)	**II degree (N = 20)**	**III degree (N = 20)**
IL-6 (ng/L)	2.0 [1.1-2.3]	2.1 [1.5-2.5]	2.4 [1.4-2.7]	2.4 [1.9-2.7]	2.6 [2.4-3.1]*
Leptin (ug/L)	10 [8.5-12]	13 [8.8-18]	18 [7.7-26]*	24 [11-30]**	43 [32-53]**
TNFα (ng/L)	6.1 [5.5-7.1]	7.6 [5.6-8.5]	7.4 [5.8-8.5]	7.5 [6.6-8.3]	8.9 [7.5-10.3]
hs-CRP (g/L)	0.35 [0.28-0.9]	0.54 [0.53-1.2]	1.60 [1.15-2.9]*	2.61 [1.52-4.45]**	7.43 [5.21-16]***
PT (%)	96 [87-105]	95 [81-109]	95 [75-115]	96 [81-112]	94 [85-103]
PTT (sec)	29 [25-33]	26 [22-30]	28 [25-32]	24 [20-28]	25 [17-33]
White blood cells ($x10^9$/L)	6.1 ± 1.08	5.83 ± 0.96	6.85 ± 1.70	7.11 ± 2.25	7.27 ± 1.68
Plateles ($x10^9$/L)	227 ± 46	237 ± 45	249 ± 57	270 ± 62	265 ± 63
Fibrinogen (mg/dL)	288 [249-360]	283 [271-330]	328 [302-353]	348 [299-444]*	365 [332-464]**
FVIII (%)	78 [64-92]	84 [66-105]	109 [87-137]*	128 [99-162]**	139 [90-173]**
AT (%)	90 [66-121]	92 [80-112]	100 [78-116]	102 [82-127]*	105 [84-129]*
FVIIa (%)	74 [58-102]	86 [62-92]	81 [59-92]	81 [58-101]	93 [60-110]
FVIIa-AT (pM)	81 [80-98]	89 [76-110]	92 [82-113]	92 [81-118]	98 [84-112]

Data are expressed by mean ± standard deviation [SD] or median (interquartile range [IQR]). The Bonferroni adjusted significance level was 0.025 (0.05/2). p-values are expressed vs controls: *$p < 0.025$ **$p < 0.01$ ***$p < 0.001$.

Table 2: Inflammatory and coagulation parameters.

4.3. Microparticles

Table 3 shows the median and interquartile range (IQR) of the circulating MP levels in cases and controls. Significantly increased levels of AMP, LMP, TF+ were seen in overweight patients compared with controls ($p < 0.025$). Overall, significantly increased levels of all types of MP measured were seen in obese patients ($p < 0.01$) compared to controls. AMP, PMP, EMP and P-selectin + were markedly higher in II and III degree obese patients than in I degree ($p < 0.001$) and overweight patients ($p < 0.001$). LMP were similarly higher in overweight ($p < 0.001$ vs controls and $p > 0.05$ vs obese subjects) and obese patients ($p < 0.001$ for all comparisons) as compared with controls, with the highest levels in III degree obese subjects. Moreover, II and III degree obese had significantly higher AMP, PMP, EMP, P-selectin+ and LMP than I degree obesity ($p < 0.001$ for all the comparisons, $p < 0.01$ for EMP). TF+ MP were significantly higher in overweight and obese patients compared with controls ($p < 0.001$ for all comparisons). There was no significant difference in TF+MP levels between overweight and obese individuals and between I degree obese and II-III degree obese subjects. Finally, III degree did not show increased levels of MP compared with II degree obesity. In the intra- and inter-assay analyses the enumeration of AMP by flow-cytometry in the 20 samples tested had CV 8% and 10%, respectively.

PPL clotting time was significantly shorter ($p < 0.01$) in overweight and obese patients compared with controls; the shortest clotting time was found in II and III degree obese subjects. There were no differences in PPL between overweight and obese subjects. In the intra - and inter-assay analyses the 20 samples tested with PPL assay had CV < 10 % for both analyses (Table 3).

	Controls (N = 40)	Overweight (N = 20)	Obesity		
			I degree (N = 20)	II degree (N = 20)	III degree (N = 20)
AMP (N/µl)	1728 [782-2122]	2391 [1937-2733]*	2296 [2157-2635]**	4029 [3061-5241]***	4976 [3242-6202]***
PMP	364 [229-529]	399 [381-430]	715 [602-883]***	3120 [2867-3420]****	3228 [1782-4112]****
P-Selectin+ MP	544 [450-602]	595 [537-632]	1193 [985-2252]***	2014 [1997-2340]***	2134 [1603-3073]***
EMP	97 [81-127]	129 [119-149]	191 [160-220]***	239 [209-291]***	234 [176-290]***
LMP	95 [87-117]	276 [227-299]***	203 [173-313]***	299 [271-334]***	375 [251-448]***
MP-TF+	31 [17-83]	161 [149-188]***	184 [129-206]***	167 [149-234]***	180 [153-222]***
PPL (sec.)	71 [61-75]	64 [39-75]**	57 [49-75]***	44 [40-60]***	46 [40-64]***

Data are expressed by median and interquartile range (IQR). The Bonferroni adjusted significance level was 0.025 (0.05/2). p-values are expressed vs controls: *p < 0.02 **p < 0.01 ***p < 0.001 ****p < 0.0001.

Table 3: Circulating levels of different MP subtype in the study population.

4.4. Thrombin generation

The median and IQR lag-time, peak thrombin and ETP found in overweight and obese patients compared with controls are shown in Table 4. TG test results showed that obese patients had a significantly shorter mean lag time ($p < 0.025$), higher mean peak thrombin ($p < 0.01$) and increased mean ETP ($p < 0.001$) compared to healthy controls. Similar trends were seen in overweight patients though only the increase in ETP reached significance ($p < 0.025$). Additionally, overall obese patients showed significantly higher median ETP than overweight patients ($p = 0.004$), and II and III degree obesity patients had higher ETP than I degree obesity patients, but the difference was not statistically significant, after Bonferroni correction ($p = 0.036$).

	Controls (N = 40)	Overweight (N = 20)	Obesity		
			I degree (N = 20)	II degree (N = 20)	III degree (N = 20)
Lag Time (min)	2.98 [2.17-4.17]	2.83 [2.25-3.17]	2.50 [2.50-3.17]*	2.50 [2.17-2,83]*	2.45 [1.83-2.83]**
Peak thrombin (nM)	276 [178-288]	306 [219-361]	317 [292-372]*	323 [232-394]*	371 [335-404]**
ETP (nM/min)	1437 [1235-1526]	1714 [1448-1829]*	1617 [1452-1938]*	1904 [1507-2078]**	2103 [1972-2400]***

Data are expressed by median and interquartile range (IQR). The Bonferroni adjusted significance level was 0.025 (0.05/2). p-values are expressed vs controls: *p < 0.02 **p < 0.01 ***p < 0.001.

Table 4: Comparison of thrombin generation test parameters between cases and controls.

4.5. Correlation between TG and MP levels with anthropometric, inflammatory and coagulation parameters

AMP, PMP and ETP positively correlated ($p < 0.001$) with BMI ($r = 0.51$, 0.81 and 0.68, respectively) and waist circumference ($r = 0.50$, 0.79 and 0.66, respectively) and with hs-CRP ($p < 0.001$) ($r = 0.46$, 0.73, and 0.57, respectively) and leptin ($p < 0.001$) ($r = 0.35$, 0.64 and 0.52, respectively). AMP and PMP showed a significant correlation with ETP ($p < 0.001$) ($r = 0.39$ and 0.45, respectively). PMP positively correlated with IL-6 ($r = 0.37$, $p = 0.0007$), platelet and white blood cells count ($r = 0.58$, $p = 0.004$ and $r = 0.73$, $p = 0.007$, respectively). In the overall study population, the number of total MP, measured using annexin V (AMP) shows an inverse correlation with PPL ($r = - 0.43$, $p = 0.0002$). PMP inversely correlated with PPL ($r = – 0.35$, $p = 0.003$) and HDL ($r = - 0.39$, $p < 0.001$). P-selectin+ MP showed a similar pattern but also positively correlated with TNFα ($r = 0.35$, $p < 0.001$). EMP showed a similar pattern to P-selectin+, but they also correlated positively with FVIII and Fibrinogen ($r = 0.37$, $p < 0.01$ and $r = 0.40$, $p < 0.001$, respectively). Finally, TF+MP and LMP showed the same pattern as EMP and they also correlated with white blood cells count (Table 5).

	AMP		PMP		P-selectin+ MP		EMP		LMP TF+MP		ETP	
	r	p	*r*	p	*r*	p	*r*	p	*r*	p	*r*	p
BMI	0.51	<0.001	0.81	<0.001	0.80	<0.001	0.69	<0.001	0.71	0.001	0.58	<0.001
Waist	0.50	<0.001	0.79	<0.001	0.70	<0.001	0.71	<0.001	0.65	0.001	0.66	<0.001
hs-CRP	0.46	<0.001	0.73	<0.001	ns		ns		ns		0.57	<0.001
Leptin	0.35	<0.001	0.64	<0.001	ns		ns		ns		0.52	<0.001
IL-6	ns		0.37	0.0007	ns		ns		0.45	0.001	ns	
TNFα	ns		ns		0.35	<0.001	0.42	0.005	0.50	0.002	ns	
WB cells	ns		0.73	0.007	ns		ns		0.64	0.001	ns	
Platelets	ns		0.58	0.004	ns		ns		ns		ns	
ETP	0.39	<0.001	0.45	<0.001	ns		ns		ns		-	
PPL	-0.43	0.0002	-0.35	0.003	-0.41	0.002	ns		ns		ns	
HDL	ns		-0.39	<0.001	-0.40	<0.001	ns		ns		ns	
Fibrinogen	ns		ns		ns		0.40	<0.001	0.49	0.001	ns	
FVIII	ns		ns		ns		0.37	<0.01	0.35	0.001	ns	

r expressed linear correlation (Pearson analysis).

Table 5: Linear correlation between TG and MP levels with anthropometric, inflammatory and coagulation parameters.

4.6 Correlation between MP levels/ETP and component of metabolic syndrome

The 35 obese patients with metabolic syndrome did not show statistically significant higher MP levels and ETP than obese subjects without metabolic syndrome (data not shown). To investigate the impact of metabolic abnormalities associated with metabolic syndrome and BMI on MP subpopulation and ETP, a multivariate analysis, corrected for age, sex, physical activity and medication was performed (Table 6). In particular, in this multivariate model we showed that BMI and waist circumference were significantly correlated with all MP subtypes and with ETP. HDL significantly correlated with AMP

and PMP. The other components of metabolic syndrome did not correlated with MP and ETP.

	AMP		PMP		EMP		LMP		MP-TF+		ETP	
	beta	*p*	*beta*	*p*	*beta*	*p*	*beta*	*p*	*beta*	*p*	*beta*	*p*
Waist	0.28	<0.001	0.45	0.01	0.14	0.04	0.23	0.02	0.19	0.04	0.34	<0.01
Systolic BP	0.098	0.1	0.08	ns	0.07	ns	0.04	ns	0.07	ns	0.02	ns
Diastolic BP	0.060	0.5	0.02	ns	0.03	ns	0.05	ns	0.054	ns	0.02	ns
Glycaemia	0.082	0.20	0.01	ns	0.04	ns	0.03	ns	0.03	ns	0.06	ns
HDL	-0.20	<0.001	-0.29	0.01	-0.02	ns	-0.05	ns	-0.09	ns	-0.01	ns
Trygliceridies	0.027	ns	0.062	ns	0.060	ns	0.07	ns	0.09	ns	0.03	ns
BMI	0.301	<0.001	0.25	0.01	0.26	0.03	0.16	0.02	0.24	<001	0.37	<0.001

For regression multivariate model age, gender, physical activity and medication use were included as independent variables.

Table 6: Multiple linear regression models predicting the number of different types of MP and ETP.

4.7 Thromboelastogram Parameters

CT in EXTEM was significantly shorter in II and III degree obese subjects than in controls. MCF, and AUC, both in INTEM and EXTEM, were significantly higher in II and III degree obese compared with controls. MCF in FIBTEM was significantly increased in I, II and III degree obesity

than in healthy controls (Table 7). A representative figure of the ROTEM® profile from an III degree obese patient and a normal weight control sample is showed in Figure. 1.

A significant linear correlation between MCF in FIBTEM with BMI ($r = 0.62$, $p = 0.019$) and waist circumference ($r = 0.69$, $p = 0.006$) and between MCF in FIBTEM with leptin, hs-CRP and fibrinogen levels ($r = 0.61$, $p < 0.001$, $r = 0.78$, $p < 0.001$ and r 0 0.40, $p < 0.001$, respectively) was observed (Table 9).

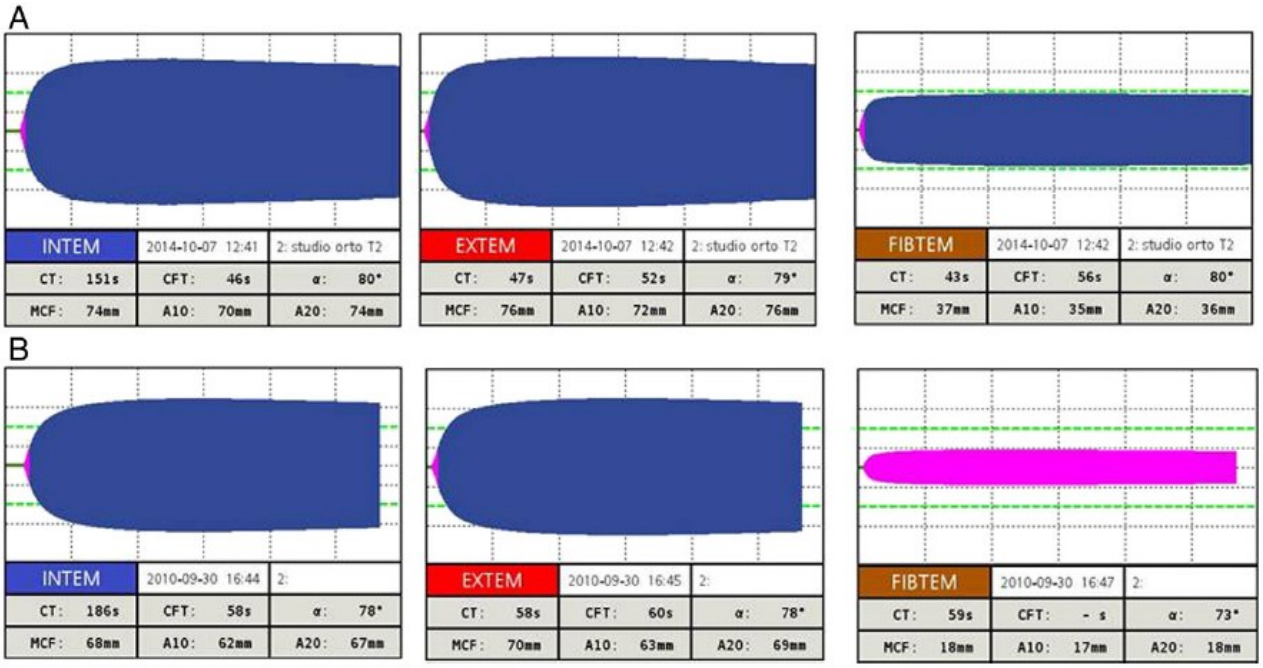


Figure 1. Representative ROTEM® profile from a III degree obese subject and a normal weight control. A. INTEM, EXTEM and FIBTEM of a III degree obese subject. B. INTEM, EXTEM and FIBTEM of a normal weight subject.

	Controls (N = 40)	**Overweight (N = 20)**	**Obesity**		
			I degree (N = 20)	**II degree (N = 20)**	**III degree (N = 20)**
INTEM					
Ct (s)	177 ± 9	180 ± 33	185 ± 23	164 ± 22	177 ± 35
CFT (s)	67 ± 16	61 ± 13	66 ± 15	60 ± 19	55 ± 15
MCF (mm)	64 ± 5	66 ± 7	63 ± 5	67 ± 6	70 ± 4***
MaxV (mm/min)	21 ± 5	22 ± 5	21 ± 6	24 ± 5	26 ± 5
AUC	6333 ± 473	6426 ± 390	3677 ± 406	6797 ± 458***	6954 ± 414***
EXTEM					
Ct (s)	57 ± 11	51 ± 14	60 ± 11	47 ± 8*	49 ± 13*
CFT (s)	74 ± 24	68 ± 15	76 ± 12	64 ± 16	61 ± 16
MCF (mm)	65 ± 5	67 ± 5	66 ± 5	69 ± 4	71± 4*
MaxV (mm/min)	19 ± 5	19 ± 5	19 ± 4	21 ± 5	22 ± 5
AUC	6365 ± 497	6610 ± 460	6530 ± 430	6802 ± 381**	6973 ± 398**
FIBTEM					
AUC	16 ± 5	20 ± 6	22 ± 5*	35 ± 7**	27 ± 10**

Data are expressed by mean ± SD. Bonferroni adjusted significance level was 0.025 (0.05/2). p are expressed vs controls: *p < 0.025 **p < 0.01 ***p < 0.00.

Table 7. ROTEM® parameters.

4.8 Whole Blood Aggregometry Parameters

A statistically significant increase in platelet aggregation was seen between III degree obese subjects and normal weight controls for all the agonists used (Table 8). No difference was found between the other subgroup of cases and controls for the three agonists considered. A significant linear correlation between a) BMI with ADP and ASPI test ($r = 0.79$, $p < 0.0001$ and $r = 0.70$, $p < 0.001$, respectively); b) waist circumference with ADP and ASPI ($r = 0.62$, $p < 0.001$ and $r = 0.57$, $p < 0.0001$, respectively); c) leptin levels with ADP and ASPI ($r = 0.41$, $p = 0.014$ and $r = 0.52$, $p < 0.001$, respectively); d) hs-CRP with ADP and ASPI ($r = 0.45$, $p = 0.001$ and $r = 0.55$, $p = 0.017$, respectively); e) fibrinogen levels with ADP and ASPI ($r = 0.49$, $p = 0.001$ and $r = 0.62$, $p < 0.001$, respectively) was found.

	Controls (N = 40)	**Overweight (N = 20)**	**Obesity**		
			I degree (N = 20)	**II degree (N = 20)**	**III degree (N = 20)**
TRAP (AUC)	83 ± 20	84 ± 12	90 ± 15	90 ± 22	101 ± 23*
ADP (AUC)	55 ± 8	54 ± 16	56 ± 21	63 ± 17	67 ± 18*
ASPI (AUC)	47 ± 15	41 ± 15	49 ±17	58 ± 17	58 ± 15*

Data are expressed by mean ± SD. Bonferroni adjusted significance level was 0.025 (0.05/2). p are expressed vs controls: *$p < 0.025$.

Table 8. Multiplate® parameters in the study population.

	INTEM-MCF		**EXTEM-MCF**		**FIBTEM-MCF**		**ADP**		**ASPI**	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	0.26	0.011	0.22	0.035	0.31	0.011	ns		ns	
BMI	ns		0.44	<0.011	0.62	0.019	0.79	<0.001	0.70	<0.001
Waist	ns		0.51	<0.001	0.69	0.006	0.62	<0.001	0.57	<0.001
Platelet count	ns		ns		ns		0.47	0.001	0.40	0.0011
Leptin	ns		ns		0.61	<0.001	0.41	0.014	0.52	<0.001
Hs-CRP	ns		ns		0.78	<0.001	0.45	0.001	0.55	0.017
Fibrinogen	ns		ns		0.40	<0.001	0.49	0.001	0.62	<0.001

Table 9. Correlations between laboratory and clinical parameters.

4.9 ROTEM® and aggregometry parameters and metabolic syndrome

The 35 obese patients with metabolic syndrome did not show statistically significant difference in ROTEM® and aggregometry parameters compared with the 45 obese subjects without metabolic syndrome. In particular, patients with metabolic syndrome showed higher MCF in INTEM (71 ± 6 mm), EXTEM (68 ± 5 mm) and FIBTEM (23 ± 6 mm) than obese subjects without metabolic syndrome (69 ± 5, 67 ± 3 and 21 ± 5 mm, respectively), but the difference was not statistically different ($p > 0.05$ for all comparisons). Obese with metabolic syndrome had higher TRAP (92 ± 18 AUC), ADP (59 ± 7 AUC) and ASPI (52 ± 12 AUC) than patients without metabolic syndrome (90 ± 20, 56 ± 8 and 49 ± 17 AUC, respectively) without statistically significant difference ($p > 0.05$).

4.10 Clinical characteristics and metabolic, laboratory and inflammatory parameters after weight loss

The anthropometric and metabolic characteristics of the study population are shown in Table 10. Obese patients had a significantly higher mean BMI and waist circumference at baseline (T0) than 3 months (T3) and 12 months (T12) after SLG. Obese patients had a reduction of 18% of BMI at T3 and of 41% at T12; there was a reduction of 29% of the BMI at T12 compared with T3. Moreover, obese patients had a significant reduction of triglycerides, basal insulin, glucose and HOMA, and a significant increase of HDL at T12 compared with T0. There was no statistical difference in platelet and white blood cells counts between obese patients at T0 and T12. Obese patients showed a meaningful reduction of TNFα, leptin and hs-CRP at T12 compared with T0 (Table 11).

	Baseline (N = 20)	**3 months after SLG (N=20)**			**12 months after SLG (N=20)**		
			% of variation	p versus baseline		% of variation	p versus baseline
Anthropometric characteristis							
Age (years)	43 ± 12	-	-	-	-	-	-
Male sex-no (%)	10 (50)	-	-	-	-	-	-
BMI (kg/m^2)	47.5 ± 6.9	40.3 ± 7.4	-18 ±6	0.001	34.8 ± 7.3	-41 ±10	<0.001
Metabolic parameters							
Systolic blood pressure (mmHg)	141 ± 16	130 ± 11	-12 ±3	ns	129 ± 12	-13 ± 9	ns
Diastolic blood pressure (mmHg)	92 ± 13	83 ± 7	-10 ± 6	ns	85 ± 8	-9 ± 5	ns
Total cholesterol (mg/dl)	197 ± 32				176 ±26	-7 ± 3	ns
HDL (mg/dl)	44 ± 10				56 ± 17	25 ± 5.3	0.04
LDL (mg/dl)	120 ± 31				102 ± 23	-12 ± 7.2	ns
Tryglicerides (mg/dl)	120 ± 47				80 ± 28	- 21 ± 10	0.002
Insulin (mU/l)	14 [12-22]				9 [4-11]	-4 ± 3.3	0.019
Fasting glucose (mg/dl)	94 [87-100]				82 [76-87]	-12 ± 7	0.008
HOMA-IR	5.0 [2.4-6.4]				1.78 [0.8-2-1]	-20 ± 5.1	0.007

Data are expressed by mean ± standard deviation (SD) or median and range interquartile (IQR).

Table 10. Anthropometric and metabolic characteristics of obese patients at baseline, 3 and 12 months after sleeve gastrectomy (SLG)

	T0 (N=20)	T12 (N = 20)	% of variation	*p*
White blood cells (x10^9/L)	7.27 ± 1.6	6.23 ± 1.40	-9 ± 2	ns
Platelets (x10^9/L)	265 ± 63	275 ± 68	3 ± 2	ns
TNFα	9 ± 1.6	7.1 ± 2.6	- 18 ± 9	0.027
IL-6	3.2 ± 1.8	2.3 ± 0.7	-14 ± 10	0.056
Leptin	46.4 ± 16.5	21.7 ± 16.3	-53 ± 27	< 0.001
Hs-CRP	10.71 ± 8.40	4.21 ± 3.61	-20 ± 14	0.008

Data are expressed by mean ± standard deviation.

Table 11. Laboratory and inflammatory parameters of obese patients at baseline (T0) and 12 months after sleeve gastrectomy (T12).

4.11 Microparticles plasma levels after weight loss

Figure 12 shows the median and interquartile range (IQR) of the circulating MP levels in III degree obese patients before and after weight loss. Significantly increased levels of Annexin V-MP, platelet-derived MP (both CD61+ and P-Selectin+), leukocyte-derived MP, TF+MP and CD36+MP were detected in obese patients at T0 compared with obese patients 3 months after SLG (T3). On the contrary, no statistically significant difference was detected in endothelial-derived MP at T0 compared with T3. Furthermore, obese patients at T0 showed significantly higher levels of all types of MP measured compared with T12 (see Fig. 2). Finally, subjects at T12 showed higher median levels of all types of MP, except endothelial-derived MP, compared with T3 but the differences were not statistically significant. Levels of endothelial-derived MP were lower at T12 compared with T3, but the difference was not statistically significant. In Table 12 we show the reported percentages of reduction of the different subtypes of MP.

Figure 2 (grey area) also reports the median plasma levels of MP previous found in a healthy lean population. In particular, obese subjects at T3 show significantly higher levels of Annexin V-MP, endothelial-derived MP, platelet-derived MP (CD61+), leukocyte-derived MP and TF+MP than healthy lean controls. They also show significantly higher levels of all MP subtypes at T12 compared with controls.

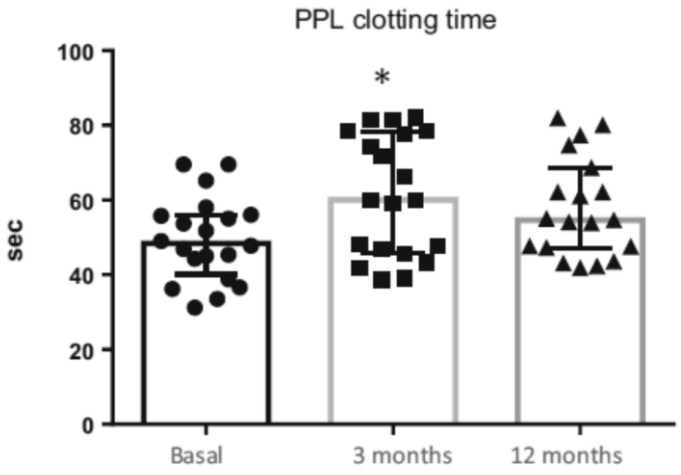
	% of variation					
	T0-T3	***p***	**T0-T12**	***p***	**T3-T12**	***p***
AMP	- 30 ± 15	0.0015	- 16 ± 10	0.035	25 ± 12	ns
EMP	- 9 ± 14	ns	- 30 ± 23	0.009	-10 ± 4	ns
PMP	- 38 ± 20	< 0.0001	- 27 ± 16	0.0062	20 ± 18	ns
P-selectin+MP	- 9 ± 5	0.0036	- 26 ± 12	0.031	53 ± 45	ns
LMP	- 36 ± 16	< 0.0001	- 35 ± 18	0.0011	4 ± 3	ns
TF+MP	- 33 ± 19	< 0.0001	- 32 ± 27	0.0012	10 ± 2	ns
CD36+	- 27 ±12	0.0011	- 33 ± 15	0.0044	24 ± 10	ns

Data are expressed by mean ± standard deviation.

Table 12. Percentage of reduction of all microparticles subtypes 3 and 12 months after sleeve gastrectomy (T3) and (T12).

4.12 MP functional activity

The PPL clotting time is significantly shorter in obese patients at T0–T3. There is no significant disparity in PPL clotting time between morbidly obese patients at T0 and T12, and between individuals at T3 and T12 (Fig.3).



Phospholipid-dependent clotting time in III degree obese patients at baseline, 3 and 12 months after sleeve gastrectomy. Data are expressed by median and interquartile range; p are calculated versus basal. *p < 0.05

Figure 3. PPL clotting time.

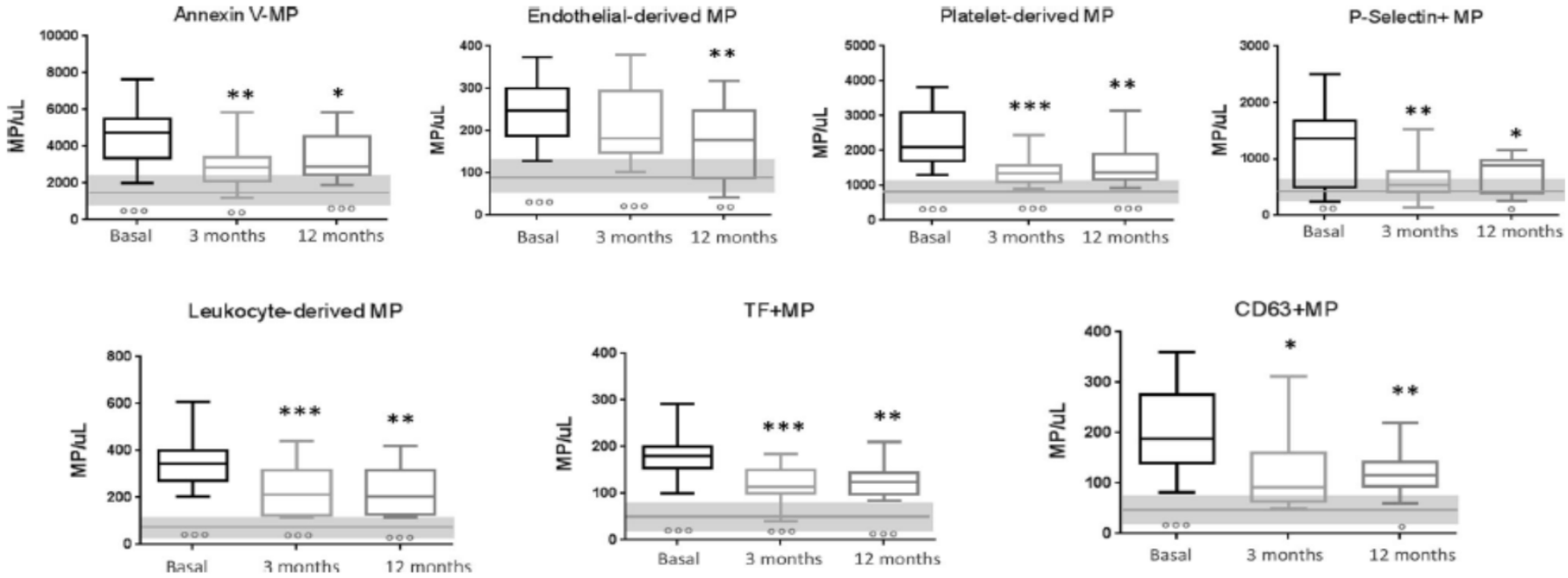


Figure 2

Circulating microparticles (MP) in III degree obese patients at baseline, 3 and 12 months after sleeve gastrectomy. Data are expressed by median and interquartile range; grey area refers to MP levels found in healthy lean controls.

*p calculated versus basal. *p < 0.05; **p < 0.001; ***p < 0.0001.

°p calculated versus lean controls °p < 0.05; °° p< 0.001; °°°p < 0.0001.

4.13 Correlation between weight reduction and microparticles

The correlations among percentages of reduction in MP levels, anthropometric and inflammatory parameters before and 12 months after SLG are shown in Table 13. There is a statistically significant positive correlation between percentages of reduction in all MP considered with percentages of reduction in BMI. A significant positive correlation exists between the reduction of leukocyte-derived MP and leptin reduction, TF+MP reduction and hs-CRP reduction, and between CD36-MP reduction and waist circumference, hs-CRP and leptin reduction.

	AMP		EMP		P-selectin+MP		LMP		MP-TF+		CD36+	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
BMI	0.38	0.027	0.46	0.027	0.42	0.022	0.59	0.002	0.36	0.036	0.71	<0.001
Waist	0.50	0.031	0.08	ns	0.17	ns	0.19	ns	0.04	ns	0.46	0.01
hs-CRP	0.32	ns	0.02	ns	0.15	ns	0.58	ns	0.80	0.0003	0.81	<0.001
TNF α	0.20	ns	0.01	ns	0.04	ns	0.01	ns	0.38	ns	0.01	ns
IL-6	0.41	ns	-0.29	ns	-0.02	ns	-0.16	ns	-0.04	ns	0.25	ns
Leptin	0.26	ns	0.062	ns	0.06	ns	0.54	0.032	0.52	0.041	0.57	0.034

BMI: body mass index, MP: microparticles, IL: interleukin, hs-CRP: high sensitive C reactive protein, TF: tissue factor.

Table 13. Correlations between percentages of reduction of microparticles and percentages of reductions of anthropometric and inflammatory parameters

5 DISCUSSION

Obesity is often associated with alterations in the coagulation factor levels predisposing to hypercoagulability. In this case-control study of 20 overweight and 60 obese subjects presented a prothrombotic state compared with normal weight controls and hypercoagulability was demonstrated with tests that consider hemostasis in a holistic manner.

Different subpopulations of circulating MP (Annexin V+, endothelial-, platelet-, P-selectin+, leukocyte-derived and TF+) were found to be significantly higher compared with normal weight healthy controls. The phospholipid procoagulant activity was increased in overweight and obese patients than in controls. The levels of all subtypes of MP increased progressively with the severity of obesity and this trend was also observed in overweight subjects free of metabolic syndrome for EMP, LMP and TF+MP. Moreover, thrombin generation tests confirmed this prothrombotic profile: obese subjects had significantly shorter lag time and increased peak thrombin and ETP levels compared with lean subjects. A similar trend was seen in overweight patients but this difference only reached significance for ETP. Finally, no difference in the level of FVIIa-AT complex levels could be seen between the patient groups and the normal subjects. Similarly, the whole blood thromboelastometry profiles in subjects with III degree obesity were consistent with a prothrombotic state characterized both by an acceleration of the propagation phase of blood clot formation (i.e., reduction of CT and CFT in EXTEM) and a higher clot strength (i.e., increase in MCF and AUC in INTEM, EXTEM and FIBTEM). Indeed, the shortening of CT in EXTEM detected in II and III degree of obesity could be explained by an enhanced tissue factor-driven thrombin generation capability. As far as the whole blood aggregometry was concerned, III degree obesity subjects showed hyperaggregability in all the three tests (i.e., TRAP, ADP and ASPI) performed. Moreover, a significant correlation between BMI and waist circumference and ADP/ASPI tests was found.

It’s known that PMP levels are significantly elevated in obese non-diabetic subjects in comparison to non-obese subjects, and the values correlated with BMI, waist circumference, fat tissue mass; significant reductions in PMP levels were observed after weight loss. In our study we described the presence of increased concentrations of PMP and P-selectin+ MP in subjects with obesity. P-selectin is a cell-adhesion molecule that translocates from alpha-granules to the platelet surface upon activation. In controls,

overweight and I degree obese patients we observed higher levels of P-selectin+ than PMP. Probably P-selectin+ MP contribute to the development of early stage vascular dysfunction by facilitating the initial recruitment of leukocytes, LMP and platelets to the endothelium. A variety of platelet function abnormalities, including increased adhesiveness and activation in vitro and in vivo and reduced sensitivity to physiological agonists, has been characterized in obese subjects.[109] Moreover, it's known that obese subjects have greater native platelet reactivity measured by dual-channel lumiaggregometer in platelet-reach plasma. [64, 110]

In this study we also described a significant difference in platelet aggregation between III degree obese patients and healthy controls in whole blood multiple impedance aggregometry tests. Indeed, a significant correlation between aggregometry parameters with BMI, waist circumference, leptin levels and hs-CRP was also found. Proinflammatory adipokines have also a role in enhancing platelets aggregating responses. As previously showed by Corsonello et al. [96], leptin produces a dose-dependent enhancement of ADP-induced platelet aggregation in healthy controls. The prothrombotic actions of leptin in vivo include enhanced agonist-induced platelet aggregation and increased stability of arterial thrombi. Leptin induces platelet aggregation by synergizing with subthreshold concentrations of agonists, in particular ADP and thrombin (TRAP test).[95, 109]

Moreover, the increased oxidative stress present in obesity leads to enhanced lipid peroxidation and free radical-catalyzed conversion of arachidonic acid into bioactive forms that are able to affect platelet functions such as adhesive reactions and activation by low concentrations of other agonists via cyclooxygenase-dependent aggregation.[109] These observations could explain our findings of increased platelets responses to Adenosine diphosphate (ADP), Thrombin activating peptide (TRAP) and Arachidonic Acid (ASPI) agonists in III degree obesity compared to healthy controls and the significant correlation between leptin and hs-CRP with ADP and ASPI aggregation capability.

Finally, the presence of platelet activation in obesity is confirmed by the observation that plasmatic levels of circulating PMP and P-selectin+ MP are significantly elevated in obese subjects and by aggregometry tests. These findings suggest how platelet hyper-reactivity/activation in obese patients can play a pivotal role for an accelerated risk of atherothrombosis.

Leukocyte-derived MP have been reported to be key factors in pathways activated by obesity-mediated inflammation. In this study, LMP were significantly higher in overweight and obese patients than in controls. Adipose tissue is a source of adipokines such as TNFα, IL-6 and leptin, which influence systemic and vascular inflammation.[111] We observed a progressive increase in all inflammatory markers (TNFα, IL-6, leptin and hs-CRP) consistent with the levels of obesity. These changes were only significant for IL-6 in III degree obesity and for leptin and hs-CRP according to the degrees of obesity. In our study, the levels of MP positively correlated with those of leptin and hs-CRP. As previously reported [46, 112], we found increased levels of FVIII and fibrinogen which are also likely to confirm the chronic inflammatory condition of the obese patients. LMP positively correlated with fibrinogen, FVIII and white blood cells count. Thus we confirmed that the majority of MP detected in these individuals was derived from platelets (PMP and P-selectin+) and leukocytes, and that they correlated with inflammatory marker.[64, 113] LMP were demonstrated to induce endothelial dysfunction, to favor the secretion of pro-inflammatory cytokines by activated endothelial cells and to promote the expression of TF.[113] It is well known that obese patients exhibit increased TF-mediated coagulation, with raised adipocyte and monocyte TF expression secondary to elevated CRP, leptin, TNFα, TGFβ, angiotensin II and insulin.[111] We detected higher TF+ MP levels in overweight and obese patients than in controls, even in the absence of an increase in TF activation, as assessed by FVIIa-AT levels. TF+MP also positively correlated with TNFα, fibrinogen, FVIII and white blood cells count. Thus, TF+MP may be induced by LMP and play a role in the global prothrombotic state.

Our findings confirm the close relationship between obesity and hypercoagulability. The levels of proinflammatory adipokines increase with increasing adipose tissue, diminish after weight loss and have a procoagulant potential.[113] We also found that MCF in FIBTEM positively correlated with leptin and hs-CRP, thus confirming that prothrombotic and proinflammatory states may be metabolically interconnected. As a result of exposure to an inflammatory stimulus, circulating blood cells express tissue factor and PAI-1, fibrinogen release, and platelet activation leading to intravascular clotting, vessel occlusion, and thrombotic pathology. Fibrinogen, an acute-phase reactant like CRP, rises in response to a high cytokine state.[114] Elevated levels of several

coagulation factors (i.e. FVIII, FVII, and fibrinogen) often detected in obese patients [46] could partly explain the hypercoagulable profile seen by thromboelastometry.
We detected significantly higher both fibrinogen levels and FIBTEM-MCF in II and III degree obese patients compared to normal weight controls, although fibrinogen level remained within the normal range. It has been recently well demonstrated that exist a good correlation between the fibrinogen level and the FIBTEM assay.[115, 116] Importantly, since FIBTEM is designed to measure elasticity of the fibrin clot under platelet inhibition by cytochalasin D.[117] FIBTEM MCF should not only be considered as a measurement of fibrinogen concentration, but also, mainly, a measurement of mechanical properties of the fibrin clot which are only in part related to fibrinogen concentration.[118] Thus we can state that in obese population there was increased fibrin monomer polymerization, as detected by high FIBTEM MCF, more than increase fibrinogen concentration (whose levels remained within the normal range).

Obesity is often associated with metabolic syndrome. Circulating MP have been found to be associated with the cluster of abnormalities characteristic of metabolic syndrome.[119] In particular, obesity can induce the development of type 2 diabetes, a condition characterized by altered MP status. Zhang et al. described the presence of PMP and LMP in patient with type 2 diabetes independently to obesity.[120] Our study population comprises also 35 subjects affected by obesity without metabolic syndrome, therefore our results suggest that the hypercoagulable state linked to obesity starts independently. We found a positive correlation between BMI and waist circumference and circulating MP levels on the one hand and increased TG on the other, but not with the other components of metabolic syndrome, even after adjusting for medication, suggesting a tight association between prothrombotic potential and fat mass. Similarly, the 35 obese patients with metabolic syndrome did not show statistically significant difference in ROTEM® and aggregometry parameters compared with the 45 obese subjects without metabolic syndrome, but a significant correlation between FIBTEM-MCF and aggregometry parameters with BMI, waist circumference, leptin levels and high sensitive-C reactive proteins was also found.
Our data suggest that the degree of hypercoagulability rises progressively as body weight increases from overweight to third degree obesity. EMP, LMP, TF+ MP and thrombin generation test, in particular ETP, are higher in overweight subjects than in healthy lean controls. Thus, the prothrombotic state is present in overweight individuals, even before

the appearance of insulin resistance (measured by HOMA-IR) and the complications of the disease, such as type II diabetes, atherogenic dyslipidemia, or metabolic syndrome. [121] Obesity can be considered a chronic, low-grade inflammatory state [111] in which altered metabolic signals emerging from adipocytes start the inflammatory responses and damage metabolic homeostasis.[122]

A recent study, in a mouse model, showed that leptin and IL-6 levels were already increased in overweight mice and were further elevated in obese mice. Adipocyte size and number of adipose macrophage infiltrations showed a similar trend.[123] This pro-inflammatory state, already present in overweight, may lead to a prothrombotic state indirectly by inducing oxidative stress and endothelial dysfunction. Thrombosis and inflammation are related and mutually reinforcing processes, involving inflammatory mediators (e. g. endotoxin, TNFα, and CD40 ligand), tissue factor expression on monocytes and the activated endothelium, and circulating TF–bearing MP.[77] Notably, our data suggest that the hypercoagulability measured by increased release of EMP, LMP and TF-bearing MP and increased ETP may be the first sign of this chronic low-grade inflammation and microvascular dysfunction and may have a role in the management of patients who could benefit therapeutic interventions to inhibit inflammatory pathways.

In order to evaluate the effect of weight loss on prothrombotic state, in a prospective study of 20 morbidly obese patients, different subpopulations of circulating MP (Annexin V+, endothelial-, platelet-, P-Selectin+, leukocyte-derived, TF+ and CD36+) were measured before, 3 and 12 months after sleeve gastrectomy. We find all MP, except endothelial-derived MP, significantly decreased 3 months after SLG when patients had lost 18% of body weight, and all MP subtypes significantly decreased 12 months after SLG when patients had lost 41% of body weight. All types of MP, except endothelial-derived, show a partial upturn at 12 months compared with the 3 months measurement, even if patients had lost 29% of their initial body weight. Finally, all MP subtypes at T12 remain significantly higher compared to the levels previously found in a healthy lean population.

The levels of proinflammatory adipokines with a procoagulant potential increase in proportion to the amount of adipose tissue and diminish after weight loss.[122] Our results are in line with previous observations, in particular Murakami et al. [124] who show that weight reduction by either calorie restriction with or without exercise reduces

platelet-derived MP production, probably through reduction of adipose tissue. More recently, Morel et al. [113] show that a short-term very low-calorie diet and weight loss results in an overall improvement of the hemostatic balance characterized by the reduction of platelet and leukocyte-derived MP and a reduction in leptin levels in obese individuals. Also Cheng et al. [125] demonstrate that the reduction in microparticles after bariatric surgery in patients with type 2 diabetes reflects an attenuation of inflammation, and this mechanism may contribute to normalization of glycemic control. In our study we confirm an improvement of metabolic parameters (fasting glucose, insulin, HOMA and HDL cholesterol) and inflammatory parameters (TNFα, IL-6, leptin and hs-CRP) 12 months after SLG. We find that percentages of reduction of all MP considered are closely correlated with the percentage of reduction of BMI, confirming a very active role of the adipose tissue in the production of MP. However, only the reduction of leukocyte-derived, TF+ and CD36+MP are significantly correlated with reduction of leptin, and the reduction of leukocyte-derived and CD36+MP is significantly correlated with hs-CRP decrease. The present study further confirms a role for leukocyte-derived MP and TF+MP as key factors in obesity mediated inflammation pathways.

CD36 is a multifunctional signaling molecule with several known ligands, including thrombospondin-1, long chain fatty acids and both native and atherogenic lipoproteins, including oxidized low-density and high-density lipoproteins.[126] CD36 is expressed on a variety of cells and tissues, including platelets, erythrocytes, monocytes, endothelial cells and leucocytes. It is intimately involved in lipid metabolism and homoeostasis, and has been strongly implicated in pathological conditions associated with metabolic dysregulation, including obesity, insulin resistance, diabetes, diabetic nephropathy and atherosclerosis. Previous studies demonstrate that soluble CD36 in plasma is associated with circulating MP, and that CD36+MP levels are significantly higher in obese people with type 2 diabetes compared to controls. Here we also confirm the presence of CD36+MP in obese patients independently of diabetes, and an association with BMI, waist circumference, leptin and hs-CRP reduction. [78, 79] We find an overall slight increase in all MP subtypes (except endothelial-derived) at T12 compared with T3 measurement, despite a further decrease in BMI. This may suggest a persistence of inflammatory responses, in which altered metabolic signals, probably emerging from adipocytes, reignite a low-grade inflammatory state, and slowly impair coagulation homeostasis. This might also be related to the median BMI at T12 (34.8 kg/m^2) namely a

II degree obesity, which is correlated with a low grade chronic inflammation regardless of comorbidities. It would be useful to know the trend of the MP even years after bariatric surgery when the weight reaches stabilization. Finally, we have to comment on the trend of endothelial-derived MP that decrease at a slower rate than other MP subtypes, and keep decreasing from T3 to T12. It seems that the low-grade inflammatory response at T12 involves blood cells (mainly platelets and leukocytes) more than endothelial cells, and that this initial procoagulant-proinflammatory response does not determine any endothelial dysfunction.

The strength of our study lies in the prospective evaluation of a cohort of consecutive patients with III degree obesity who have undergone the same sleeve gastrectomy procedure. Potential confounders were avoided by excluding subjects with conditions known to be associated with high levels of circulating MP. Since MP are an essential part of the physiological and pathological cellular remodelling, they mirror the turnover of different cell types in the circulation. From a clinical point of view, MP can be considered mediators in the cross-talk between inflammation and hemostasis, and they provide new prognostic opportunities and therapeutic approaches to attenuate prothrombotic complications by steering thromboprophylaxis or anti-inflammatory therapy.

Our study suffers from some limitations. Firstly, the size of the study population is limited, mainly because we enrolled obese patients free from other pathological conditions potentially associated with prothrombotic state. We only collected 20 patients in each group. Nonetheless, we were able to detect significant differences between the groups but this needs to be validated by larger studies. Although it was well known that different dietary habits and physical exercise between cases and controls could influence coagulation parameters [127], we enrolled patients and controls with similar dietary habits and exercise in the five days prior to enrollment.

Moreover, due to the cross-sectional design of the study, causality in the associations is not demonstrated and further research is needed in order to determine the mechanisms underlying the prothrombotic tendency detected.

As for methods, it is also important to bear in mind that currently available methods for detection of MP are still suboptimal. Flow cytometric (FCT) assays may not be sensitive enough to detect all sizes of MP, given that many of these fall below the detection threshold, though recommendations by the ISTH SSC Working Group on Vascular Biology [106, 128] were used. It has been shown that the TG was associated with MP

smaller than 0.1 μM below the cut-off threshold of FCT. [129] Although these could be indirectly measured by both TG and PPL we would not have identified them by FCT.

Second, we only considered MP expressing phosphatidylserine, but only a minority of circulating microvescicles expose phosphatidylserine.[130-132] Thus we could only account for a minority percentage of MP, and this may lead to interpretation bias. Despite these limitations, annexin V is considered a valuable marker of MP of different origins, with the advantages of having a relatively high density of the target molecules on MP and excellent reproducibility of measurements.[131]

Third, with our current staining method, we can only account for a single cellular marker to detect the possible origin of the MP, thus inability to show the possible origin of TF+ or CD36+MP. A new model has been recently hypothesized where MP are complex ambivalent structures that express both activators and inhibitors of coagulation, and also convey fibrinolytic properties [133]. In this study, to better characterize the measurement of these MP, we also used a functional MP activity assay. The PPL clotting time assay precisely reflects the flow cytometry results.

We only performed an indirect measure of the active TF levels, by using circulating TF-bearing MP and FVIIa-AT complex, whether direct measurement of TF by ELISA assays mirrors the data obtained by FVIIa-AT complexes assays need to be confirmed. Moreover, the cellular origin of the TF+ MP was not investigated due to our FCT detection limit, so we could only account for TF+MP without qualifying the TF+ source. However, the co-labelling method between anti-TF antibody and others activation markers is not fully standardized. Finally, it has been recently hypothesized

a new vision of MP [133] as complex ambivalent structures that express both activators and inhibitors of coagulation and also convey fibrinolytic properties. In our study we could only explore

the presence of MP with procoagulant properties and no information is available on their potential fibrinolytic role.

Moreover, we did not perform TG assay in the absence of both TF and phospholipids to further characterize the procoagulant potential of plasma and MP of patients with obesity, but this method is not standardized. It should also be noted that all MP and TG testing was performed on frozen samples, frozen and thawed prior to analysis, as is usual with these types of clinical studies. As MP may break down during storage at –80 °C and lose capacity to bind the anti-Annexin V antibody [130], care was taken to ensure that storage time of platelet-free plasmas was similar between groups. We cannot exclude that the

freezethawing cycle could also have had some influence on the TG and PPL assay. To eliminate any cellular contamination of the samples only double-spun plasma samples were used. The samples tested had only been frozen once and no repeat frozen and thawed samples were used.

6 CONCLUSION

In conclusion, this study shows that a hypercoagulable state is already present in overweight patients and progressively increased in relation to the severity of obesity.

Secondly, we found that the high levels of inflammatory markers, associated with an excessive amount of adipose tissue, may induce an overproduction of different types of procoagulant MP and an increase of thrombin generation and thromobelastometric/aggregometry parameters, thus confirming the emerging concept that hypercoagulability and inflammation are related and mutually reinforcing processes in obesity.

In addition, the significant correlation between degree of obesity and multiple global prothrombrotic parameters suggests that hypercoagulability is associated with adipose tissue itself.

Also the decrease of different MP after surgery weight loss is positively correlated with the decrease of BMI and waist rather than the ameliorated metabolic and flogistic state.

Finally, hypercoagulability precedes the onset of metabolic syndrome and seems to be independent of insulin resistance.

Further prospective work is needed to determine how these global hemostasis assays can be useful to identify a group of obese patients at increased arterial and thrombotic risk in driving the better anticoagulant prophylaxis/therapy.

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