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ADIPOSE TISSUE AND INSULIN SECRETION IN THE PATHOPHYSIOLOGY OF OBESITY AND ITS COMPLICATIONS

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ABSTRACT

Type 2 diabetis mellitus (T2DM) and obesity are global health care problems that are closely linked together. The precise mechanisms linking the two conditions remain unclear. Indeed, while the close relationship between T2DM and weight gain is well established, not all obese subjects are diabetic and this paradox is still unexplained. Impaired tissue perfusion has been proposed as one of the common metabolic defects, but little is known about adipose tissue (AT) microangiopathy and its possible role in T2DM. In animal models of obesity and diabetes, expanding AT microvasculature appears structurally altered and the angiogenetic potential of adipose derived stem cells impaired.

Several studies, in humans, suggest that obesity leads to an impaired angiogenesis and AT hypoxia, inducing an inflammatory and a profibrotic response that plays a pivotal role in the pathogenesis of metabolic complications related to weight gain, first of all insulin resistance and diabetes. Moreover, from a pathophysiological point of view it is well established that dysfunctional visceral adipose tissue (VAT) is one of the major determinants of metabolic complications of obesity, while subcutaneous depots has been considered metabolically healthy. Nevertheless it could be hypothesized that in the progress of obesity through the metabolic impairment, SAT could become dysfunctional as VAT.

On the basis of these data, we planned to study both subcutaneous and visceral adipose tissue in terms of adipocytes size, capillary density, adipose tissue stem cells (ASCs), endotelial precursor of AT and adipogenic potential, in obese subjects compared to lean subjects and in obese patients with a different glyceamic profile.

We collected subcutaneous (SAT) and/or visceral (VAT) adipose tissue (AT) from 249 patients divided in 5 different groups: 18 lean normal weight and normoglycemic subjects (18.5 < BMI < 24,9 kg/m2) as control group, 68 normoglycemic obese subjects (ob N), 65 pre-diabetic obese subjects (ob pre-T2DM), 57 diabetic obese subjects (ob T2DM) and 41obese patients after underwent to a relevant weight loss (ob WL), corresponding to at least 10% of body weight. In different representative subgroups of these samples we performed: 1) immunohistochemical analysis to evaluate the morphometry of adipocyte and capillary density; 2) flow cytofluorimetric analysis of stromal vascular fraction (SVF) in order to quantify adipose tissue stem cells (ASCs), defined as CD45-CD34+CD31-, and endothelial precursors cells (EPs) defined as CD45-CD34+CD31+; 3) i*n vitro* culture of ASCs obtained from SVF, in order to estimate the adipogenic potential in the different groups and different depot of AT; 4)

gene expression profile by RT-Real Time PCR of PPRγ, Leptin, VEGFA, VEGF2, HIF1α to correlate their expression with previous findings.

Our study confirm that obese AT is less vascularized than lean AT but T2DM does not represent an aggravating factor to the vascular reduction already present in obesity. On the contrary, T2DM and also prediabetic condition are able to further modify AT architecture, remodeling mature adipocyte size and adipogenic potential mediated by ASCs, importantly reducing AT hyperplastic growth capacity. Moreover our results allow us to assume that *primum movens* in development of T2DM must be searched in AT architecture and that both depots, SAT and VAT, play a pivotal role in the development of this disease.

Furthermore, considering the continuous increase in bariatric procedures to treat both weigh gain and associated co-morbidities, we plan to evaluate the effects of laparoscopic sleeve gastrectomy (LSG) after one year. Indeed, whereas the beneficial effects of this bariatric procedure are well known, side effects are lesser known. In particular, postprandial hypoglycaemia is a well described side effect after RYGB, but few data are available for LSG..

We enrolled a total of 197 consecutive non-diabetic morbidly obese who underwent to LSG in our Center for the Study and the Integrated Treatment of Obesity (Ce.S.I.T.O.). All patients were studied 12 months before and after LSG and, anthropometrics parameters, medical history, clinical examination, complete blood count and complete metabolic panel including a 3- hour OGTT, were collected.

One year after LSG, all patients had a significant reduction in weight and BMI, a significant improvement in glucose and insulin profile, and a significant decrease in inflammatory markers. We found an high incidence of severe hypoglycaemia (32,8%) after a provocative test (OGTT). Patients with hypoglycaemic events had a lower weight and BMI and a greater %EBML after LSG. compared to patients without hypoglycemic events. Hypoglycaemia was more frequent in patients having lower age, lower fasting blood glucose levels and higher triglycerides levels before LSG.

RIASSUNTO

L'obesità e il diabete mellito tipo 2 (T2DM) sono due patologie strettamente correlate tra loro e, insieme, rappresentano una delle maggiori emergenze sanitarie a livello mondiale. I meccanismi fisiopatologici che legano le due patologie, non sono ancora stati completamente spiegati. Infatti, mentre sono abbastanza note le alterazioni che portano dall'aumento del peso corporeo alla comparsa di T2DM, meno noti sono i motivi per cui non tutti i pazienti obesi sviluppano la patologia diabetica. Per spiegare tale paradosso, alcuni studi si sono concentrati sulla possibile diversa capacità di espansione del tessuto adiposo (TA). Come tutti i tessuti, anche il TA, per poter espandersi, necessita di un'adeguata consensuale vascolarizzazione. E' stato ipotizzato che un'alterata angiogenesi durante l'espansione del TA in alcuni soggetti, e la presenza di un danno a livello del microcircolo dello stesso TA, possano influire negativamente sul peggioramento del profilo glicemico. In alcuni modelli di animali, affetti da diabete e obesità, si sono evidenziate alterazioni a carico del microcircolo del TA e a carico del potenziale adipogenico. Consensualmente, alcuni studi sul TA dell'uomo, hanno suggerito che l'obesità porta ad una alterazione dell'angiogenesi a livello del TA con contemporanea comparsa di uno stato ipossico a sua volta responsabile della risposta infiammatoria e profibrotica. Infiammazione e fibrosi, hanno un ruolo fondamentale nello sviluppo dell'insulino-resistenza e quindi del T2DM. Inoltre, è noto che il tessuto adiposo viscerale (VAT) rappresenta il deposito di TA con maggior grado di infiammazione, mentre il tessuto adiposo sottocutaneo (SAT) è considerato un tessuto meno infiammato e in grado di avere un ruolo protettivo nei confronti dello sviluppo delle patologie metaboliche. Nonostante ciò, è possibile ipotizzare che con l'aumento progressivo del peso corporeo anche il SAT acquisisca caratteristiche disfunzionali come il VAT.

Sulla base di questi presupposti, abbiamo deciso di analizzare le possibili variazioni in termini di morfologia, di densità capillare, di quantità di precursori adipogenici, di potenziale adipogenetico sia nel SAT che nel VAT di pazienti obesi e di pazienti normopeso normoglicemici. Inoltre, tra i pazienti obesi, sulla base delle caratteristiche cliniche e biochimiche, abbiamo selezionato coloro che erano normoglicemici (ob N), pre-diabetici (ob pre-T2DM) e diabetici (ob T2DM).

Sono, quindi, stati raccolti campioni di SAT e/o il VAT da 249 pazienti divisi nei 4 gruppi sopra descritti: 18 pazienti normopeso e normoglicemici (18.5 < BMI < 24,9 kg/m2), 68 ob N, 65 ob pre-T2DM e 57 ob T2DM. Abbiamo, inoltre, avuto l'opportunità di analizzare il SAT di 41

pazienti obesi dopo significativo calo ponderale (ob WL). I campioni di TA sono stati studiati (1) mediante analisi immunocitochimica, al fine di valutare la morfologia degli adipociti e la densità capillare, (2) mediante analisi citofluorimetrica della frazione vasculo stromale (FVS) per quantificare la presenza di precursori adipocitari (CD45-CD34+CD31-) e di precursori endoteliali (CD45-CD34+CD31+), (3) attraverso la coltura dei preadipociti estratti dalla FVS, per valutare il potenziale adipogenetico; (4) mediante espressione genica di leptina, PPRγ, VEGFA, VEGF2 e HIF1-α.

L'analisi dei nostri dati ci ha permesso di confermare che il tessuto adiposo dei soggetti obesi è significativamente meno vascolarizzato, sia nel SAT che, dato ad oggi non noto, nel VAT, rispetto al tessuto adiposo dei soggetti magri. Diversamente da quanto ipotizzato, la presenza di un alterato profilo glicemico, come quello presente nel pre-diabete, o la presenza di un diabete franco, non peggiorano ulteriormente la vascolarizzazione del TA, né nel SAT, né nel VAT. Ciò che si modifica in maniera significativa e precoce è l'architettura del TA. Infatti, già nei pazienti ob pre-T2DM e, anche nei pz ob T2DM, abbiamo osservato un progressivo aumento del diametro degli adipociti. Inoltre, nel TA dei pazienti con alterato profilo glicemico abbiamo osservato una significativa riduzione sia nella percentuale dei preadipociti presenti nella FVS sia nella loro capacità di differenziare *in vitro*. Questi dati ci permettono di ipotizzare che il TA dei pazienti con alterato profilo glicemico cresce maggiormente per ipertrofia che per iperplasia e che il "primum movens" nello sviluppo della patologia diabetica è da ricercare nelle modificazioni a carico della cellula adiposa più che nelle modificazioni del microcircolo del tessuto adiposo sia nel VAT ma, anche nel SAT.

Inoltre, considerando il progressivo incremento nell'utilizzo della chirurgia bariatrica per trattare sia l'aumento di peso ma anche le complicanze metaboliche a esso correlate, è stato eseguito uno studio sugli effetti della sleeve gastrectomy per via laparoscopica (LSG) a distanza di un anno dall'intervento. Mentre gli effetti positivi di questa procedura chirurgica sono ormai noti, meno noti sono gli effetti collaterali; in particolare, l'ipoglicemia post prandiale è stata ben descritta dopo intervento di by pass gastrico ma resta ancor poco indagata dopo intervento di LSG. Abbiamo, pertanto, reclutato 197 pazienti obesi non diabetici sottoposti a LSG e li abbiamo studiati prima e a distanza di un anno dall'intervento bariatrico. In tutti i pazienti è stata raccolta la storia clinica, è stato eseguito esame obiettivo e sono stati eseguiti gli esami bioumorali comprensivi di screening endocrino-metabolico completo, incluso OGTT prolungato a 180 minuti, e dosaggio delle citochine infiammatorie. Un anno

dopo l'intervento, tutti i pazienti hanno avuto una significativa riduzione del peso corporeo e del BMI, un significativo miglioramento dei parametri metabolici, compreso il profilo glicemico e insulinemico, e una significativa riduzione delle citochine infiammatorie. Il 32,8% dei pazienti ha sviluppato un'ipoglicemia severa dopo test provocativo (OGTT). I pazienti con ipoglicemie hanno mostrato un peso e un BMI significativamente minore rispetto ai pazienti che non hanno sviluppato ipoglicemia e una percentuale di perdita di BMI significativamente maggiore. L'ipoglicemia si è dimostrata essere più frequente in quei pazienti che, prima dell'intervento, erano più giovani, con un peso e un BMI inferiore e con livelli di trigliceridemia superiori ai pazienti che non avevano sviluppato ipoglicemie dopo LSG.

1. INTRODUCTION

Worldwide obesity is doubled since 1980. In 2014, more than 1.9 billion adults, 18 years and older, were overweight. Of these over 600 million were obese. Most of the world's population live in countries where overweight and obesity kills more people than underweight (WHO, 2016). The WHO world health statistics report in 2015 shows that in the European region the overall obesity rate among adults is 21.5% in males and 24.5% in females. The same report states that the prevalence for overweight among children under the age of 5 is 12.4% (WHO, 2015)

The impact of obesity on morbidity, mortality and health care cost is profound. Overweight and obesity are major risk factors for a number of chronic diseases including diabetes, cardiovascular diseases and cancer and, in Europe, is responsible of 6% of health assistance cost and it causes 1 million deaths per year (WHO, 2006). Obesity is not just a health issue, but a social one as well: it impacts the economical and social system, provoking a decrease in productivity (it must be noticed that in the last 50 years young obeses has widely increased), an increase of direct and indirect healt-assistence costs and finally social isolation especially among young people in Western countries.

In the recent years, given the large number of people suffering from obesity, exciting advances have occurred in all 3 modalities used to treat obesity: lifestyle intervention, pharmacotherapy, and weight-loss procedures including bariatric surgery (Garvey WT, 2013). In particular, bariatric surgery, has continued to increase in recent years, and over 340.000 procedures were performed in the world in 2011 (Buchwald H., 2013). New surgical procedures have been developed and refined and aren't known al the long-term effects.

Moreover, the major disease associated to obesity is type 2 diabetes mellitus (T2DM). It is estimated that about 90% of T2DM is attributable to excess weight (Hossain P, 2007), but the precise mechanisms linking the two conditions remain unclear. Converging data suggest that an impaired function of adipose tissue (AT) could play a pivotal role in T2DM development but lot of mechanisms underlying AT dysfunction remain to be established.

Considering that obesity has become one of the leading causes of disability and death the porpoise of our study is to provide a further contribution to knowledge that allow us to better understand the pathophysiology of obesity and its complication and the effects of weight loss after bariatic surgery.

1.1 Obesity Definition and Classification

Obesity is a chronic disease characterised by an increase of body fat stores. In clinical practice, BMI is the parameter commonly use in order to estimate the amount of adipose tissue in human body and to classify the severity of obesity. BMI is calculated as measured body weight (kg) divided by measured height squared (m^2). On the base of BMI, it's possible to stratify the population in different categories (Tab. I) where obesity is defined by a BMI $\geq$ 30 kg/m^2.

Definition	**Western BMI (Kg/m^2)**	**Class**	**Asiatic BMI (Kg/m^2)**
Underweight	BMI <18,5		
Normoweight	18, 5 ≤ BMI ≤ 24,9		18,5 ≤ BMI ≤ 22,9
Overweight	25 <BMI ≤ 29,9		23≤ BMI ≤ 24,9
Obese	**30,0 ≤ BMI ≤ 34,9**	**I**	**25≤ BMI≤29,9**
	35,0 ≤ BMI ≤ 39,9	**II**	**BMI ≥30**
	BMI ≥ 40,0	**III**	

Table I: Western and Southeast Asian population weight classification based on BMI.

Nevertheless BMI is not useful either to distinguish between Fatty Free Mass, Fatty Mass and liquid or to describe the adipose tissue distribution. For instance with lower BMI values, Asian population has a similar cardiovascular risk (CVR) compared to European population and this is the reason why lower BMI cut-off points are applied for some ethnic groups as shown in table I.

That proves that BMI can not be used as a singular tool in order to define obesity and stratify the risk of developing cardiovascular and metabolic complications. It is likely necessary to define obesity with other parameters and to relate its value with geographical distribution (Yusuf S., 2005).

In 1947 Vague defined a gynoid obesity and an android obesity. In gynoid obesity adipose tissue is mainly subcutaneous and it is localized in the buttock and the thighs, in android

obesity adipose tissue is mainly localized at the level of abdominal organs causing an increase of waist. They have different activities and visceral adipose tissue has a greater correlation with development of obesity cardiovascular and metabolic complications rather then subcutaneous adipose tissue (Zhu S., 2002).

The amount of abdominal fat can be assessed by waist circumference which highly correlates with intra-abdominal fat content. Following WHO recommendations (1995) waist circumference is measured with a tailor-meter at the level of the midpoint of the line which connects the inferior border of the lowest rib and the superior border of ilium crest.

The most recent International Diabetes Federation (IDF) consensus defined central obesity (also known as visceral, android, apple-shaped or upper body obesity) in Euripides as a WC of ≥ 94 cm in men and ≥ 80 cm in non-pregnant women. Lower cut-off points for central obesity are proposed for different ethnic groups.

Therefore, in clinical practice, to assess the risks associated with being overweight or obese, is necessary to considered both BMI and WC as shown in Table II (NICE guidelines, 2006).

BMI classification	**Waist circumference**		
	Low	**Hight**	**Very high**
Overweight	No increased risk	Increased risk	High risk
Obesity	Increased risk	High risk	Very high risk
For men, waist circumference of less than 94 cm is low, 94–102 cm is high and more than 102 cm is very high. For women, waist circumference of less than 80 cm is low, 80–88 cm is high and more than 88 cm is very high			

Table II. Base assessment of the health risks associated with being overweight or obese in adults on BMI and waist circumference

1.2 Obesity Epidemiology

For thousands of years obesity was rarely seen (Haslam D., 2007). It was not until the 20th century that it became common, so much so that in 1997 the World Health Organization (WHO) formally recognized obesity as a global epidemic (Caballero B., 2007). Once considered a problem only of high-income countries, obesity rates are rising worldwide. The transfer of Western eating habits from more developed countries to those in developing increases the prevalence of obesity but with an important difference: while in developing countries the classes with a major risk of obesity are economically privileged because wealth and prestige correspond in more food availability, in rich countries the higher level of obesity is among deprived groups. The only remaining region of the world where obesity is not common is sub-Saharan Africa (Haslam DW., 2005).

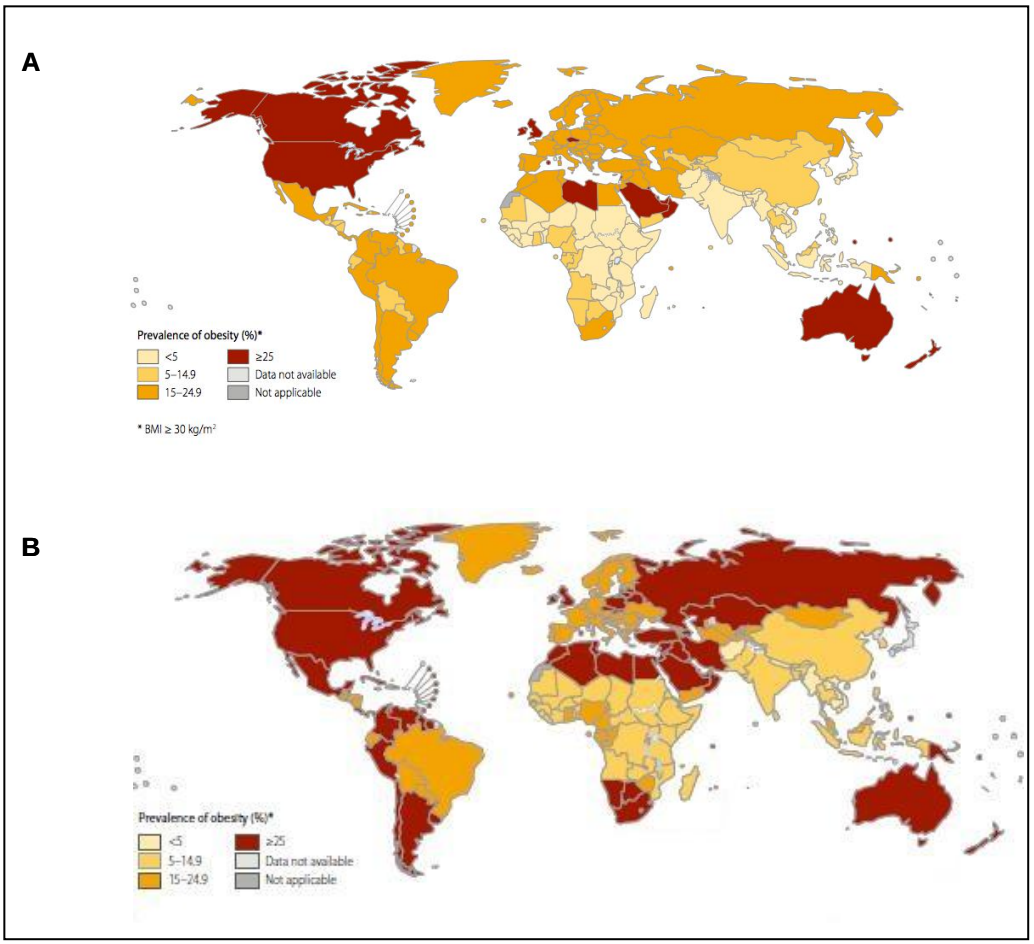


Figure 1: **Age standardized prevalence of obesity in man (A) and woman (B) age 18 years and over, 2014**

According with the last report of WHO, in 2014, 39% of adults aged 18+ were overweight (BMI ≥ 25 kg/m2) (39% of men and 40% of women) and 13% were obese (BMI ≥30 kg/m2) (11% of men and 15% of women).

Thus, nearly 2 billion adults worldwide were overweight and, of these, more than half a billion were obese. The prevalence of overweight and obesity is highest in the Region of the Americas (61% overweight or obese in both sexes, and 27% obese) and lowest in the South-East Asia Region (22% overweight in both sexes, and 5% obese).

In the European and Eastern Mediterranean Regions and Region of the Americas, over 50% of women are overweight, and in all three regions roughly half of overweight women are obese (25% in the European region, 24% in the Eastern Mediterranean Region, 30% in the Region of the Americas).

In all WHO regions, women are more likely to be obese than men. In the African, South-East Asia and Eastern Mediterranean regions,, women have roughly double the obesity prevalence of men (adapted from WHO 2014) (Figure 1).

1.3 Obesity Complications

The development of obesity is correlated with increasing incidence of diseases affecting several organs and systems of human body, which may all be considered as complications of obesity. The increase of body weight and the condition of low grade chronic inflammation, present in the most of obese subjects, lead to alterations of several organs and tissues.

Common alterations and dysfunction in obese individuals are:

- development of a state of insulin-resistance and type 2 diabetes;
- development of hypertension and dyslipidemia;
- promotion of the atherosclerotic process;
- development of a pro-thrombotic state;
- immunological alterations, susceptibility to the development of inflammatory and autoimmune diseases;

In table III all the main diseases and alterations connected to obesity are described.

Organ/System	Complication
Cardiovascular System	- Hypertension - Ischemic Cardiomyopathy - Heart Failure - Pulmonary Heart Disease - Chronic Venous Insufficiency - Pulmonary Tromboembolism - Ictus
Respiratory System	- Dyspean at rest or during exercise - Obstructive sleeping Apnoea - Pickwich Syndrome - Pulmonary Hypertension - Increased post-surgical risk
Metabolic-Endocrine System	- Insulin-resistance, Diabetes Type II - Dyslipidemia - Metabolic Syndrome - PCOS - Alterations in reproductive system - Hyperuricemia and Gout
Central Nervous System	- Mood Alterations - Depression - Eating Disorders
Gastrointestinal System	- GERD - Cholelithiasis - Abdominal Hernia
Muscular-skeletal System	- Osteoarthritis - 2. Discal Hernia
Urinary System	- Urinary Incontinence
Neoplastic Diseases	- Breast - Ovary - Endometrium - Esophagus - Stomach - Pancreas - Bowel - Kidney - 9.Prostate

Table III: Main obesity complications

1.3.1 Diabesity

T2DM and obesity are global health care problems that are closely linked together. The concept of diabesity (obesity-T2DM) emerges by the estimation that about 90% of T2DM is attributable

to weight excess (Hossain P, 2007). As shown in Figure 2, there is a parallel escalation of the two diseases. Both these metabolic disorders are characterized by defects of insulin action.

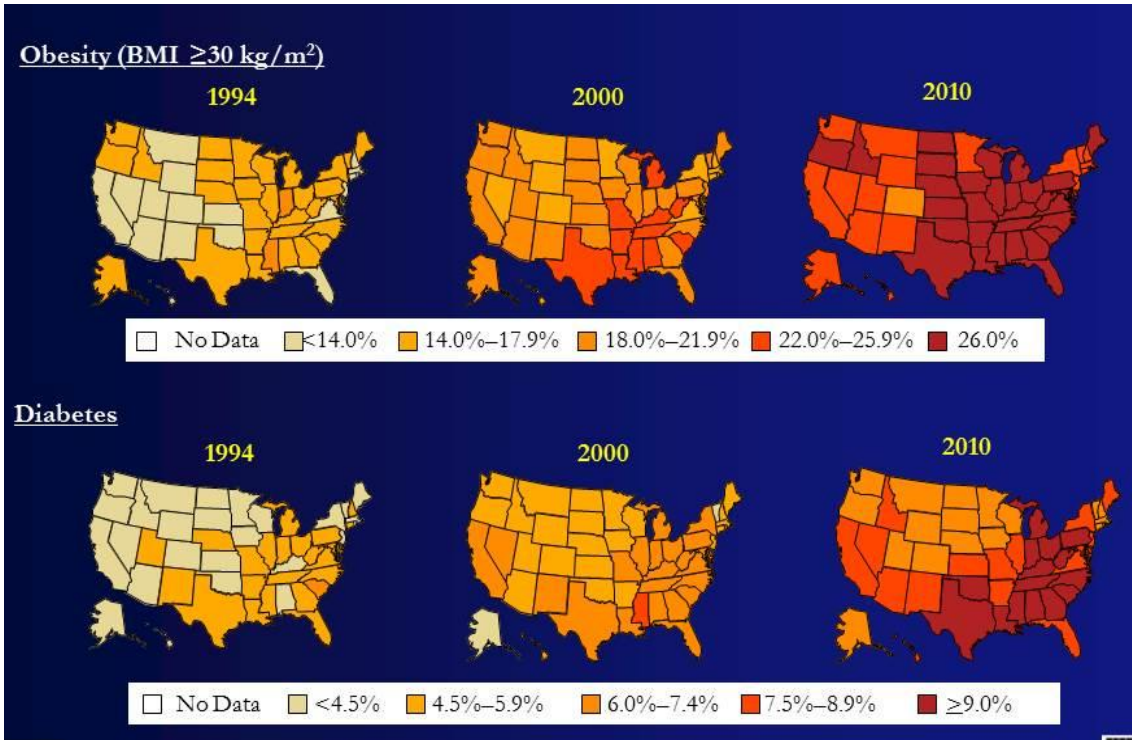


Figure 2: Age adjusted prevalence of obesity and diagnosed diabetes among U.S. Adults 18 years or older

The classical pathways from obesity to diabetes contemplated that the permanent elevation of free fatty acid (FFA) present in obesity, from one side leads to an accumulation of triglycerides in liver and in beta-cell, from the other side, leads to a predominant utilization of lipids by muscles with a concomitant inhibition of glucose transporter activity. These actions induce a diminution glucose uptake by muscles and decreased rates of glycogen synthesis by muscles themselves. Chronic hyperglycaemia, developed from these events, further impairs insulin sensitivity and leads to an increase of insulin secretion. The result is a pathological glycation of circulation proteins and formation of advanced glycation end products that worsen the pancreatic beta-cell insulin secretion and lead to beta cell apoptosis.

These pathways supply a good explanation for the close association between weight gain and heightened risk of T2DM but they do not explain why not all individuals with obesity become diabetic, and certain individuals become diabetic after very minor weight gain. For this reason, in recent years an increasing number of works have studied common metabolic aspects and molecules in T2DM and obesity as sleep disturbances, androgens, vitamin D, gut hormones and

microbiota but the main common flaw between the two diseases probably, must be researched in the large individual variation that exist in the size and expandability of different adipose tissue depot and in the microvasculare dysfunction that occurs during AT growing.. Indeed, the growth and function of AT, depends on its vascularisation. If angiogenesis is inadequate, this may result in dysfunction of adipose tissue and it increases the risk of development T2DM. Evidences in rodents have shown that rapid adipose tissue growth induced by high-fat diet determinates hypoxia in response to expansion of adiposity; these results suggest that the response of adipose tissue may be insufficient to elicit sufficient compensatory angiogenesis. These data are concordant with some results which have shown how adipose tissue hypoxia is associated with fibrosis and inflammation, rather then a compensatory angiogenetic expansion.

Moreover, alterations of adipokines pattern usually present in obese patients, is involved in the development of insulin-resistance up to diabetes type II. Initial condition of insulin-resistance is promoted by the reduction of adiponectin associated with increased levels of pro-inflammatory cytokines, IL-6 and TNF- α. At the same time there is an increased release of free fatty acids (FFA) that contributes with a mechanism of lipotoxicity, actually FFAs act on pancreas inducing apoptosis of β-cells, as described above.

Another interesting field of research is how adipose tissue behaves with overt T2DM disease. A vicious circle of progressive microvascular dysfunction due to AT inflammation and hyperglycaemia could be present. Hyperglycaemia, acting via oxidative stress, inflammation and advanced glycation end products, can worsening microvascular angiogenesis and function in AT but little is known about it.

1.4 Obesity therapy

In recent years, exciting advances have occurred in all 3 modalities used to treat obesity: lifestyle intervention, pharmacotherapy, and weight-loss procedures including bariatric surgery. Clinical trials have established the efficacy of lifestyle and behavioural interventions in obesity; moreover, there are now 5 weight-loss medications approved by the US Food and Drug Administration (FDA) for chronic management of obesity. Bariatric surgical practices have been developed and refined, together with improvements in pre- and postoperative care standards, resulting in better patient outcomes.

Initial treatments are neither pharmacological nor surgical, actually they consist in modifications of life-style, based on a low-caloric intake dietary regime and increasing in

physical activity. Moreover cognitive behaviour therapy and/or psychological support are an integral part of obesity therapy (Europ G L).

Indeed, the treatment of obesity needs a multidisciplinary skill that involved various specialists experienced in obesity management. For these reason on April 2011, at Padua University, was opened the Centre for the integrated treatment for obesity (CeSTIO) which comprise various operative unit and different specialists as physicians, surgeons, anaesthetists, psychologists and psychiatrists, nutritionists and dieticians.

1.4.1 Pharmacological Therapy

As reported on European Guide Lines for Obesity Management in Adult (Rif. Biblio), pharmacological treatment should be considered as part of a comprehensive strategy of disease management. Pharmacotherapy can help patients to maintain compliance, ameliorate obesity-related health risks and improve quality of life. It can also help to prevent the development of obesity co-morbidities (e.g. type 2 diabetes mellitus). The efficacy of pharmacotherapy should be evaluated after the first 3 months. If weight loss achieved is satisfactory (>5% weight loss in non-diabetic and >3% in diabetic patients), treatment should be continued otherwise should be interrupted.

Pharmacological treatment is suggested for those patients who have BMI>30 kg/m2 and do not show any answer to life-style changes or BMI >27 kg/m2 in presence of complications.

Mechanisms of action of weight loss medication focus mainly on appetite control. Except for orlistat, that acts on pancreatic lipases and so it reduces intestinal lipid absorption, medication for obesity acts on arcuate nucleus to stimulate the POMC neurons, which promote satiety.

The 5 weight-loss medications approved by FDA are:

- Locarserin
- Phentermine/Topiramate
- Bupropion/Naltrexone
- Liraglutide
- Orlistat

The first third one are serotoninergic, as locarserin, no selective inhibitor of dopamine and norepinephrine transporters, as bupropione, or dopaminergic and noradrenergic, as phentermine. The combination of phentermine and topiramate, which is a neurostabilizer and antiseizure medication, seems to be additive; however, it is unclear how topiramate enhances

appetite suppression. Naltrexone, an oppioide receptor antagonist, potentiates the effect of bupropione on activation of POMC neurons.

Liraglutide is a long acting GLP-1r agonist that acts in different way on the appetite control both directly on stomach where slows down gastric emptying and on POMC neuron too. GLP-1r agonist is widely use in T2DM therapy so it is heavily indicated in obese patients affected by T2DM.

Despite these 5 therapies approved by FDA, EMA (European Medicine Agency) approved just orlistat, bupropione/nalexone and liraglutide, while AIFA (Agenzia Italiana del Farmaco) approved only orlistat and liraglutide. Due to the unpleasant side effects of orlistat, steatorrhoea and fecal urgency, and to high cost of liraglutide, in Italy, pharmacological therapy for obesity is not so widespread.

1.4.2 Bariatric Surgery

Bariatric surgery has continued to increase in recent years, and over 340.000 procedures were performed in the world in 2011 (Buchwald H., 2013) A recent systematic review concluded that bariatric surgery leads to a greater improvements in weight loss outcomes and weight associated co-morbidities compared with no surgical intervention, regardless of the type of procedure used (Colquitt JL., 2014).

Surgery should be considered for patients aged 18–60 years with a BMI $\geq$ 40.0 kg/m 2 or with BMI between 35.0 and 39.9 kg/m 2 and co-morbidities, in whom surgically induced weight loss is expected to improve the disorder (such as type 2 diabetes and other metabolic disorders, cardiorespiratory disease, severe joint disease and obesity-related severe psychological problems). BMI criterion may be the current BMI or a documented previous BMI of this severity (Buchwald H., 2009).

Recently, the scientific community debating to considered bariatric surgery in patients with a BMI lower than 35 Kg/m^2 affected by T2DM, as there is evidence-based data supporting bariatric surgery benefits in regards to T2DM remission or improvement

Nowadays it is possible to perform bariatric surgical interventions even before 18 years old and over 60 years old, when patients are accurately selected, nevertheless these categories are still a minority of the total amount of patients.

Exclusion criteria for Bariatric Surgery are:

1. Absence of a period of identifiable medical management.
2. Patient who is unable to participate in prolonged medical follow-up.
3. Non-stabilized psychotic disorders, severe depression, personality and eating disorders, unless specifically advised by a psychiatrist experienced in obesity.
4. Alcohol abuse and/or drug dependencies.
5. Diseases threatening life in the short term.
6. Patients who are unable to care for themselves and have no long-term family or social support that will warrant such care.

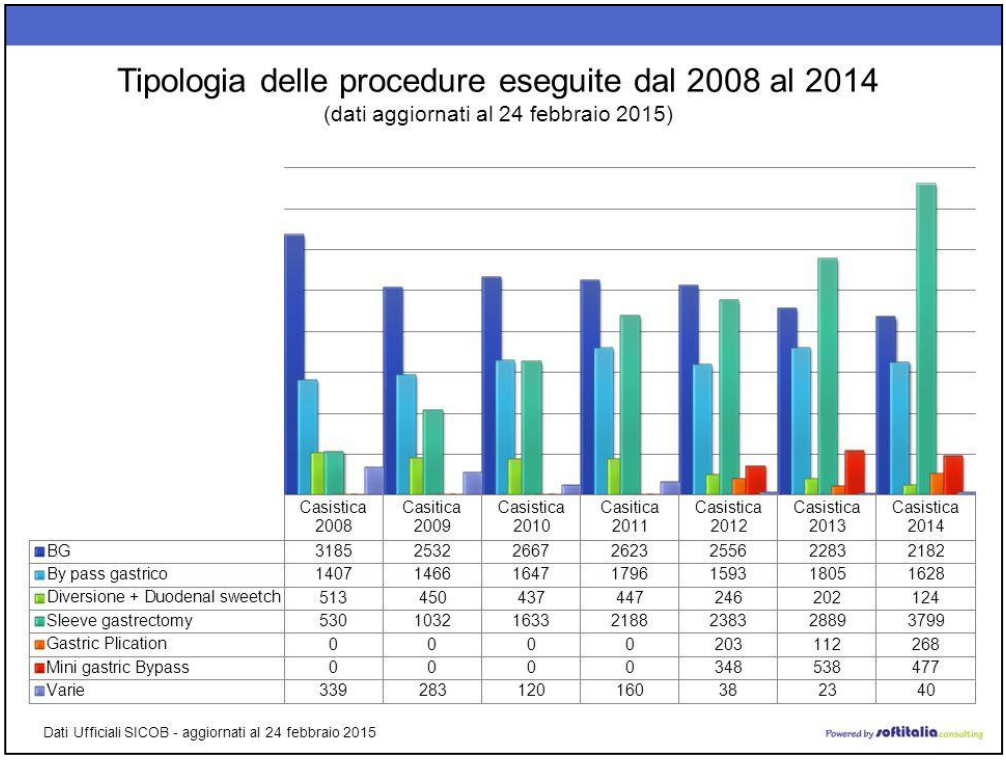
Until a few years ago, bariatric surgery procedures were divided into three groups:

- Restrictive surgery which reduces the amount of ingested food;
- Malabsorptive surgery which reduces the absorption of ingested food;
- Mixed surgery a combination of the two mechanisms described before;

In the first group were included gastric banding, sleeve gastrectomy and vertical gastroplastic, in the malasorbitive group were included duodenal-swith and Scopinaro bilio-pancreatic diversion and in the last group was included Roux en Y Gastric By Pass. Nowadays this classification is still valid but, the knowledges, emerged over time, regarding the effect of bariatric surgery on metabolic effects, have partially changed this classification. This is particularly true for SG that was considered initially a pure restrictive procedure, but it is now considered to act thorough several additional mechanisms as described below.

All the types of bariatric surgery interventions might be performed with laparoscopic procedures, which must be considered as the first option: laparoscopic approach has many advantages compared to the laparotomic one, considering both post-surgical outcome and complications. Up to now there are not sufficient evidence-based data to suggest how to assign a patient to a specific bariatric/metabolic procedure with no evidence in favour of any particular procedure, even if several studies demonstrated that some of the bariatric procedures as SG and RYGB have early weight-independent metabolic effects on HbA1c, LDL cholesterol, blood pressure, prevention and reduced cardiovascular risks and lower surgical risks compared to other bariatric procedures (Lee WJ., 2011; Scopinaro N., 2011). For these reason, in recent years, there was a progressive decreased of gastric banding, duodenal switch and bilio

pancreatic diversion and a progressive increased in gastric by pass and sleeve gastrectomy as shown in Figure 3 (data referring to Italy).



	Casistica 2008	Casitica 2009	Casistica 2010	Casitica 2011	Casistica 2012	Casistica 2013	Casistica 2014
BG	3185	2532	2667	2623	2556	2283	2182
By pass gastrico	1407	1466	1647	1796	1593	1805	1628
Diversione + Duodenal sweetch	513	450	437	447	246	202	124
Sleeve gastrectomy	530	1032	1633	2188	2383	2889	3799
Gastric Plication	0	0	0	0	203	112	268
Mini gastric Bypass	0	0	0	0	348	538	477
Varie	339	283	120	160	38	23	40

Figure 3: Bariatric surgery procedures from 2008 to 2014 in Italy

1.4.2.1 Laparoscopic Adjustable Gastric Banding

Laparoscopic Adjustable Gastric Banding consists of the placement of a silicon ring with a pneumathic chamber in the upper part of the stomach, in order to create a small gastric pouch in the subcardial area. The pneumathic chamber is connect to a silicon tube which ends up with a reservoir located in a subcutaneous position and it allows cutaneous regulation of the banding calibre. The goal is to induce early fullness and reduce the amount of ingested food. It is reversible and it does not lead to many complications. It determinates a decrease of body weight up to 40-50%, nevertheless about 60% of patients regain weight after a while and these is the reason why this bariatric procedure has shown a progressive decreased.

1.4.2.2 Laparoscopic Sleeve Gastrectomy (LSG)

Laparoscopic sleeve gastrectomy has been firstly proposed as the first step of a duodenal switch procedure, but subsequently evolved to an isolated procedure and, since 2008, it has markedly

increase in prevalence, raising from 5% to 27% of all bariatric procedures (Buchwald H., 2013)

It consists of irreversible resection of 2/3 of the stomach and the cut is performed in order to make the stomach gain a tubular shape. The restrict residual volume is around 100-150 ml (Figure 4).

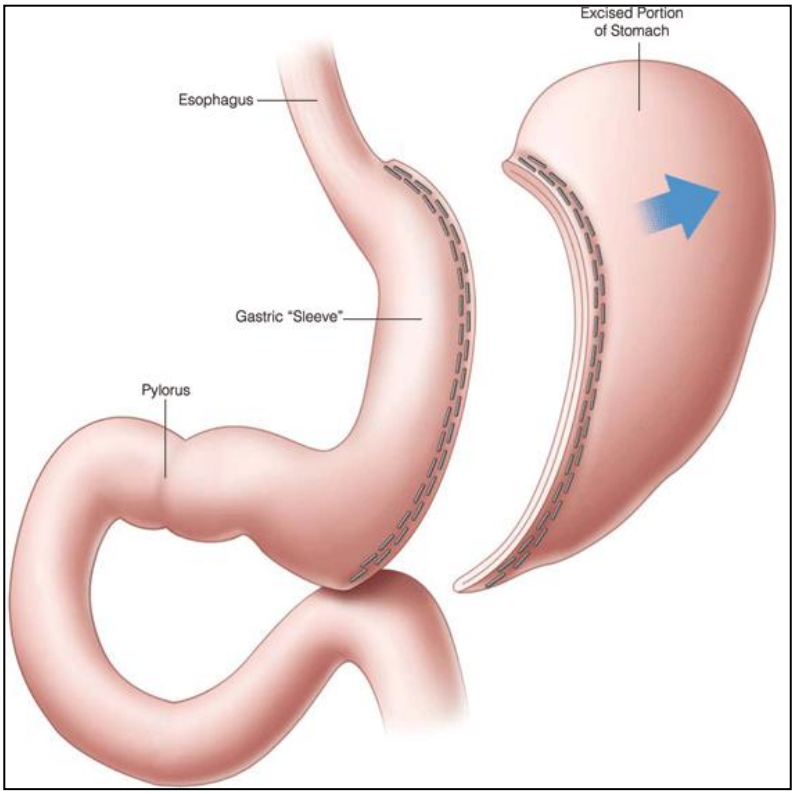


Figure 4: Sleeve Gastrectomy

LSG was considered initially a pure restrictive procedure, but it is now considered to act thorough several additional mechanisms (Melissas J., 2007; Dimitriadis E., 2013). The mechanisms which lead to the loss of weight, are just partially due to the decrease of volume of the stomach, in fact gastric resection determinates alteration of hormonal asset. After LSG there is a decrement of ghrelin levels. Ghrelin is a hormone, mainly secreted by the fundus of the stomach, which have oroxogenic effects via stimulating neuropeptide Y from the hypothalamus and its levels are invertionally proportionate to BMI. Instead after LSG, there is no compensatory increase of ghrelin which should be associated to the weight loss, because of the gastric resection. Moreover, after LSG, increments of PYY and GLP-1 levels are usually observed. These hormones are secreted by the distal part of the intestine, they act on pancreatic cells increasing insulin secretion and they inhibit hypothalamic production of neuropeptide Y. In this way LSG is involved in inducing an improvement of diabetes type II up to resolution of

disease, independently from the weight loss.Weight loss is around 60% of body weight but long-term outcome are still unknown, so further investigations are still needed.

1.4.2.3 Roux en Y Gastric Bypass (RYGB)

Gastric bypass is the most commonly bariatric procedure performed worldwide. It consists of the creation of a small, (15–30 mL/1–2 tbsp) thumb-sized pouch from the upper stomach, accompanied by bypass of the remaining stomach (about 400 mL and variable). This restricts the volume of food which can be eaten. A segment of the small bowel (called the alimentary limb) is brought up to the proximal remains of the stomach. On the basis of the lengths of small intestine used, there are different degrees of food absorption. The most commonly gastric by pass is Roux en Y gastric by pass (Fugure 5) which contemplates the division of the small intestine approximately 45 cm below the lower stomach outlet and the re-arranging into a Y-configuration, enabling outflow of food from the small upper stomach pouch via a "Roux limb". The Roux limb is constructed using 80–150 cm of the small intestine, preserving the rest (and the majority) of it from absorbing nutrients.

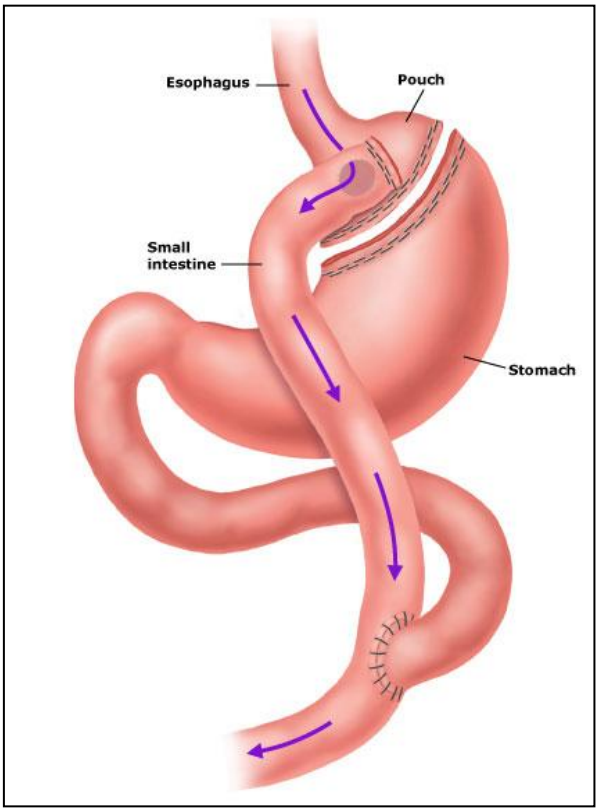


Figure 5: Roux en Y Gastric Bypass

The weight-loss is obtained thanks to the restrictive mechanism and partially to a mild malabsorptive mechanism, actually a decrease of ghrelin levels is usually observed. RYGB induces fast and sustained weight-loss, up to 60% of total body weight in the first year. It reduces global and cardiovascular mortality and obesity-related comorbidities. The mechanisms underlying these beneficial effects are multiple. Improvement of insulin-resistance or even to resolution of diabetes type II, occur within days after the surgical procedure is performed and before any substantial weight-loss, suggesting the hypothesis that the Glycaemic control is restored by mechanisms which are related to gut anatomical rearrangement and modification of flow of nutrients. Levels of GLP-1 increase rapidly after surgery is performed and not after dietary restriction despite similar weight loss, so GLP-1 might play a role in metabolic improvement achieved after RYGB. RYGB and LSG are considered similar for weight loss, improvement of co-morbidities and intra-operative and early postoperative safety.

1.4.2.4 Hypoglycaemia after bariatric surgery

In 2005, Service et al (Service GJ., 2005) reported, for the first time, cases of hypoglycaemic events occurring months to years after RYGB. Since then, an increasing number of publication demonstrated that postprandial hypoglycaemia (PPHG) is not so rare after RYGB and the frequency of asymptomatic hypoglycaemia may be over 30% (Goldfine AB., 2007). Comparable data for laparoscopic sleeve gastrectomy (LSG) are scantier. Recently, Natoudi et al. (Natoudi M., 2014) described the occurrence of severe hypoglycaemic events in 6/29 (37, 5%) normoglycemic patients 12 months after LSG. Moreover, Papamargaritis et al. found that 33% of patients experienced hypoglycaemic events one year after LSG (Papamargaritis D., 2012).

Severe hypoglycaemia can lead to dangerous clinical consequences such as seizure, syncope, and motor vehicle accidents. However, also mild-to-moderate hypoglycaemia (plasma glucose: 2.3-3.9 mmol/L) can have a negative health impact in diabetic and no diabetic subjects. Thus, in diabetic subjects mild-to-moderate hypoglycaemia can be associated with increased risk of cardiac arrhythmias. Furthermore, hypoglycaemia may reduce the amplitude of the blood-oxygenation level dependent (BOLD) responses in primary auditory and visual cortex to simple auditory and visual stimuli (Driesen NR., 2007).

Why some patients develop PPHG while others do not is unclear. After bariatric surgery, alterations of gastrointestinal anatomy and of gastric innervation likely have a profound effect

on gastric emptying (Ukleja A., 2005). Thus, meals are more rapidly transferred from the stomach to the small intestine, so that the distal intestine is exposed to higher loads of undigested carbohydrates, whereas absorption of glucose into the bloodstream is accelerated. The resulting hyperglycaemia stimulates a rapid and excessive secretion of insulin, which can in turn trigger late hypoglycaemia. Other authors (Salehi M., 2011; Ukleja A., 2005) suggested the idea that excessive insulin secretion could be in part consequent upon increased incretin hormone release, but the role of these hormones in the development of hypoglycaemia remains controversial. An increased secretion of GLP-1 and GIP has been observed after an oral glucose challenge in patients after gastric resection, esophagectomy, and RYGB (Romero L., 2012), and these exaggerated responses have been suggested to induce ß-cell expansion via increased expression of islet transcription factors. By contrast, in a recent paper it has been suggested that GLP1 analogs might provide a new treatment option in patients with late PPHG (Abrahamsson N., 2013). Furthermore, it has been postulated that, after RYGB hypoglycaemic counterregulation may be dysfunctional, due to lack of inhibition of insulin secretion, subnormal response of the anti-insulin hormones, changes in neuronal/ sympathetic activity, and/or low glycogen stores (Salehi M., 2015).

Therefore, recurrent hypoglycaemia of any degree can have clinical relevance and to identify predictive risk factors of this late complication of bariatric surgery becomes important. Moreover, very is known about PPHG after RYGB but little is known about PPHG after LSG.

2. Adipose Tissue (AT)

Adipose tissue was originally viewed as a connective tissue with a storage capacity, presently AT is considered an organized endocrine organ, both vascularised and innervated, with a clear anatomy and a high degree of plasticity responding to both corporeal and environmental changes.

It was initially found surrounding the viscera in the peritoneal cavity, but later in evolution, major depots of adipose tissue were located in subcutaneous sites (Gesta S., 2007). Subcutaneous adipose tissue (SAT) is different in thickness and distribution according to sex: in women has a typical distribution involving the buttocks, the thighs, the abdomen under the bell, as a kind of "fat reserve" to protect the pregnancy; instead, in men, SAT follows a typical pattern of distribution which involves the face, the neck, the shoulders and, in particular, the abdomen over the bell with an increased risk of cardiovascular diseases (Mathieu P., 2009).

In the human body, AT is the only tissue that can expand, in adulthood, many times going from 5% to 60% of total body weight. The increase of AT may be both hypertrophic and hyperplastic (especially but not only in the youth). The ability of adipose tissue to expand has clear evolutionary advantages, enabling survival in times of nutrient scarcity; however, concomitant with adipose tissue expansion are metabolic alterations that enhance risk of metabolic disease (Wajchenberg BL., 2000; Corvera S., 2014).

In addition to a different distribution of AT, there are also different types of AT. In fact, AT, is distinguished in white (WAT), brown (BAT) and beige AT. The white adipocytes are spherical cells, large, uniloculate, with relatively low content of mitochondria and they contribute to forming deposits of subcutaneous and visceral adipose tissue. The BAT is formed by cells multi-vacuolated characterized by a high content of mitochondria with characteristic morphology (large size and ridges very developed) and it is well represented in new born baby while, in adult, small residual island of BAT spreaded in WAT (Giordano A., 2014). At least, recently, a new cell type, the beige/brite adipocyte, has been described in the white depots of adipose organ, whose morphological, molecular and functional properties partly overlap brown cells (Wu, 2012).

Taken as a whole, AT represents a complex organ with several functions. It represents the main deposit of triglycerides in human body and it is responsible, also, of triglycerides synthesis and release in form of glycerol plus fatty acids. In addition to storing excess triglycerides and releasing free fatty acids, AT in the form of white AT or brown/beige AT (BAT) is crucial for immune responses, thermo genesis, fertility and lactation (Harms M., 2013; Giordano A., 2014).

Then, as described above, the classical functions of adipose tissue are:

- energetic reserve;
- protection against mechanic trauma and mechanic support to different organs;
- body shaping;
- thermal isolation (white adipose tissue), increase body temperature and elimination of nutritional excess throughout thermal energy (brown adipose tissue);

Furthermore, AT has metabolic and endocrine activities and the substances released may be divide into:

- Energetic compounds (free fatty acids, cheton bodies, glycerol);
- Steroidal hormones (both androgens and estrogens);
- Adipocytokines: Adiponectin, leptine,resistine, PAI-I, TNFα, IL-6

2.1 Subcutaneous and Visceral Adipose Tissue

In the 1950s, Vague was the first to suggest that the regulation of the endocrine and metabolic functions of abdominal AT were controlled, in part, by the anatomical distribution of fat with "android or male-type" obesity associated with T2DM and atherosclerosis (Vague J., 1956). From anatomically point of view, visceral AT (VAT) is centrally located and enclosed by the peritoneum, while the subcutaneous AT (SAT) is located directly below the skin. Both subcutaneous and visceral depots retain extraordinary growth potential throughout adult life (Utzschneider KM., 2004) but there is significant, heritable variation in the relative size of these depots (Fox CS, 2007). SAT and VAT differ in their cellular composition, their molecular properties, and their role in regulation of the whole body metabolism.

There are few studies comparing human SAT and VAT, and they are increased in the recent years thanking to bariatric surgery. Due to the limitation of reproducible isolation methods and standardized cell size measurements, interand intra-individual data regarding depot-specific cellular characteristics are few. Adipocytes in general are smaller in VAT as opposed to the SAT of obese subjects even if, in some works, this trend is confirmed without a significant difference (O. Gealekman, 2011). Considering AT capillarization in different depot, works in literature are even less than those concerning adipocyte size. To our knowledge, only Corvera S. group has analyzed differences between SAT and VAT capillary density in 7 obese patients finding that subcutaneous adipose tissue capillary density is significantly higher compared with visceral adipose tissue (O. Gealekman, 2011). Due to the difficult to collect AT from lean subjects, especially visceral adipose tissue little is known about VAT and SAT of these patients. In literature, there are some more works compare obese's SAT with lean's SAT and they are all agree that adipocytes are smaller in lean/overweight as opposed obese (Goossens GH., 2011; O. Gealekman, 2011; Pasarica M. 2009). Regarding SAT capillary density between obese and lean, is confirm, in different works, that obese's SAT has a significantly lower capillary density compared to lean's SAT (Goossens GH., 2011; O. Gealekman, 2011; Pasarica M. 2009). None is known about capillary density of lean patients' VAT. From a functional point of view, classically, visceral adipose tissue is considered closely connected with the development of

insulin-resistance, dyslipidemia and increased cardiovascular risk (Cinti S., 1999). This concept is partially true; in the recent years several works have begun to demonstrate how even SAT plays an important role in the development of AT inflammation and so in the increased risk of insulin-resistance, dyslipidemia and cardiovascular disease. In fact, if on one side it has been demonstrated that an increased in abdominal AT is a risk factor for the development of metabolic disease, it has to be noted that the association reported between VAT and diseases in numerous epidemiological studies, used surrogate indexes of VAT, such as BMI, waist circumference, waist-to-hip ratio or waist-to-height ratio. In recent years, it has been shown that large subcutaneous fat cells are associated with insulin resistance and with a high risk of developing T2DM, and, studies following Roux-en-Y gastric bypass in women, have demonstrated that a reduction in SAT fat cell size correlates to improved insulin sensitivity (Andersson DP., 2014). Furthermore, in the first mentioned S. Corvera's work, have founded that the capacity of SAT to expand its capillary network decreases with morbid obesity and this decrease correlates with insulin resistance, suggesting that impairment of subcutaneous adipose tissue angiogenesis may contribute to metabolic disease pathogenesis (O. Gealekman, 2011). Besides, previous study of Snijder MB, has reported a different role for SAT dependent on its localization: SAT in the abdomen is directly associated with the metabolic syndrome in normal-weight and obese men; while larger gluteo-femoral SAT is inversely associated with metabolic syndrome in both obese men and women (Snijder MB, 2005).

Considering these indicated studies, it can be said that the classical distinction between SAT, as the safe AT, and the VAT, as the unhealthy AT it's no longer possible. Certainly, VAT has demonstrated to strongly influence the development of obesity-related disease but it is necessary, in the future, confirm these results using more specific methods of fat depot measurements and, it is necessary to consider also the excess of abdominal SAT as a negative event for health. Changing focus from visceral adipose tissue mass as a sole contributor to metabolic disease to functional heterogeneity in adipose tissue depots can help better understand relationship of adiposity and obesity-related disease.

2.2 Adipose Tissue Endocrine Function

The adipose tissue produces differents proteic substances, all together called adipocytokines, which are responsible for the interactions between the adipose tissue and other organs and systems, for instance the immunological one, the central nervous one and the endocrine one, in

order to regulate the human body metabolism. Some of these substances are not specific for the adipose tissue, for instance estrogens, PAI-1 and angiotensinogen. Some others are more specific like IL-1β, IL-6, TNF-α, MCP-1, resistine, and witch are involved in the development of insulin-resistance and chronic inflammatory state. Some factors specifically produced by adipose tissue, have a positive effect on metabolism, like adiponectin which increases insulin-sensibility and has a protective role against the development of glucose intolerance and diabetes. In our study we measured, in particular, leptin, IL-6 and TNF-α.

2.2.1 Leptin

In 1949, a non-obese mouse colony being studied at the Jackson Laboratory produced a strain of obese offspring, suggesting that a mutation had occurred in a hormone regulating hunger and energy expenditure. In the following years, several studies, lead to discover a new obese-gene encoded a novel hormone that circulated in blood and that could suppress food intake: leptin. Leptin was the first fat cell-derived hormone (adipokine) to be discovered (Conde J., 2011).

Leptin is a 167-aminoacids glycoprotein, codified by the gene ob located on chromosome 6. It is produced by white adipose tissue, in smaller amount by the muscular and neuronal tissue and by the gastric epithelium and mammarian epithelium. It is primarily involved in the regulation of food intake and energy expenditure. Although leptin reduces appetite as a circulating signal, obese individuals generally exhibit a higher circulating concentration of leptin than normal weight individuals due to their leptin-resistance. This leptin-resistance results from different mechanisms involving leptin receptor, leptin transport through blood brain barrier and post leptin receptor deficit. Normal sieric levels are between 5-21μg/L and they are increased in female subjects and in obeses, where leptin levels are directly proportional to the total amount of adipose tissue (linked to BMI) and the dimensions of adipocytes.

Leptin receptors are located in several tissues (CNS, hepatocytes, myocytes, pancreatic cells, spleen cells, pulmonary tissue, ovarian tissue) and also on immunological and endothelial cells; it can therefore talk about a pleiotropic role of this molecule (Ahima RS., 2000). One of the first leptin function to be discovered was the action on hypothalamic centres, in particular in the hypothalamic arcuate nucleus, where leptin acts to decrease food intake and increase energy expenditure (Friedman JM., 1998) through the release of inhibitor neurotransmitter of appetite as α-MSH (α-melanocyte-stimulating hormone) and CART (cocaine and amphetamine-regulated transcript respectively) and through the inhibition of neurotransmitter increased food

intake as NPY and with increasing thermo genesis (Vettor R., 2002). However it has been confirmed that leptin has multiple functions besides inducing the sensation of fullness, as reported in Figure 6. In particular, leptin may contribute to the development of classical risk factors of atherosclerosis such as arterial hypertension and diabetes mellitus, it may increase inflammation and it promotes platelets aggregation. It is known that leptin secretion by adipocytes is stimulated by insulin, so leptin plasmatic levels are closely correlated with insulin plasmatic levels. Recent works, have demonstrated that the effect of hypoxia on leptin secretion by human adipocytes is particularly marked and may indicate that a reduction in pO_2 AT could be the primary mechanism for the rise in the production of leptin with obesity (Trayhurn P., 2008).

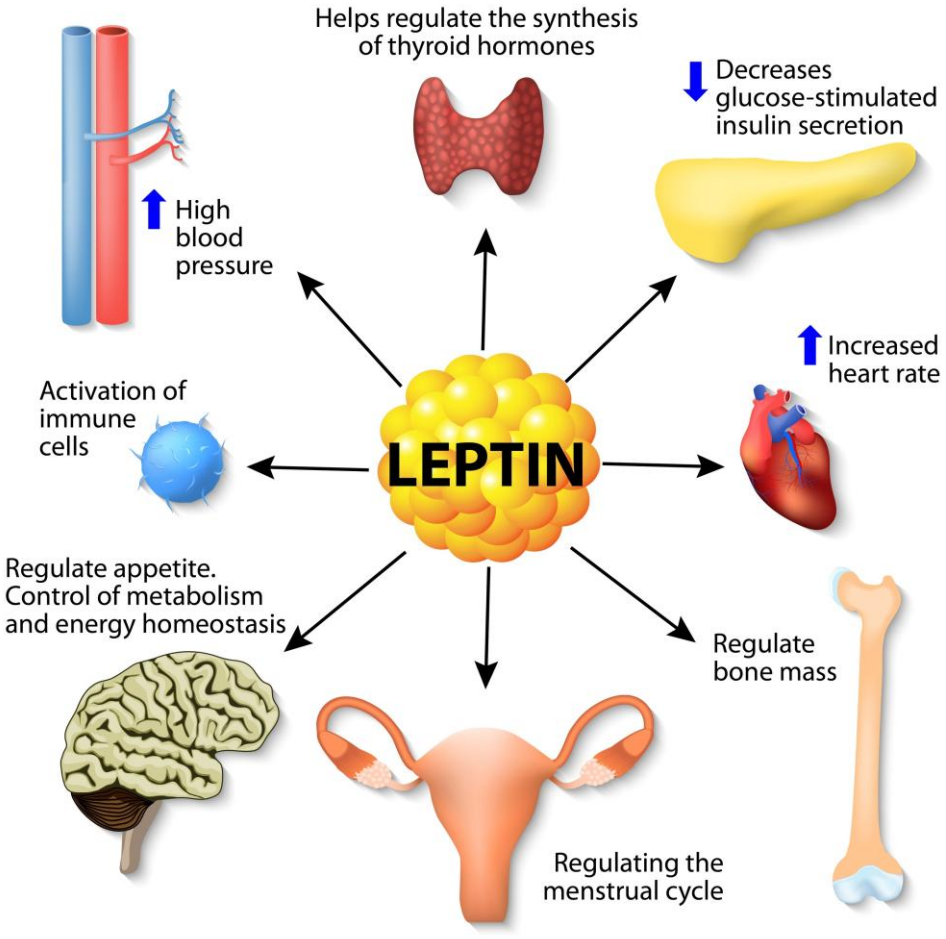


Figure 6: Leptin's multiple functions.

2.2.2 IL-6

Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. It is encoded by the IL6 gene. It has a pleyotropic functions as shown on Figure 7 and it is produced by several cellular types (monocytes, macrophages, endothelial cells, lynphocytes B and T, neutrophils, eosinophils, smooth muscle cells, skeletal muscle cells). The 20-30% of its production comes from adipocytes and this percentage considerably increased proportionally with increase of BMI and waist circumference (Park HS., 2005).

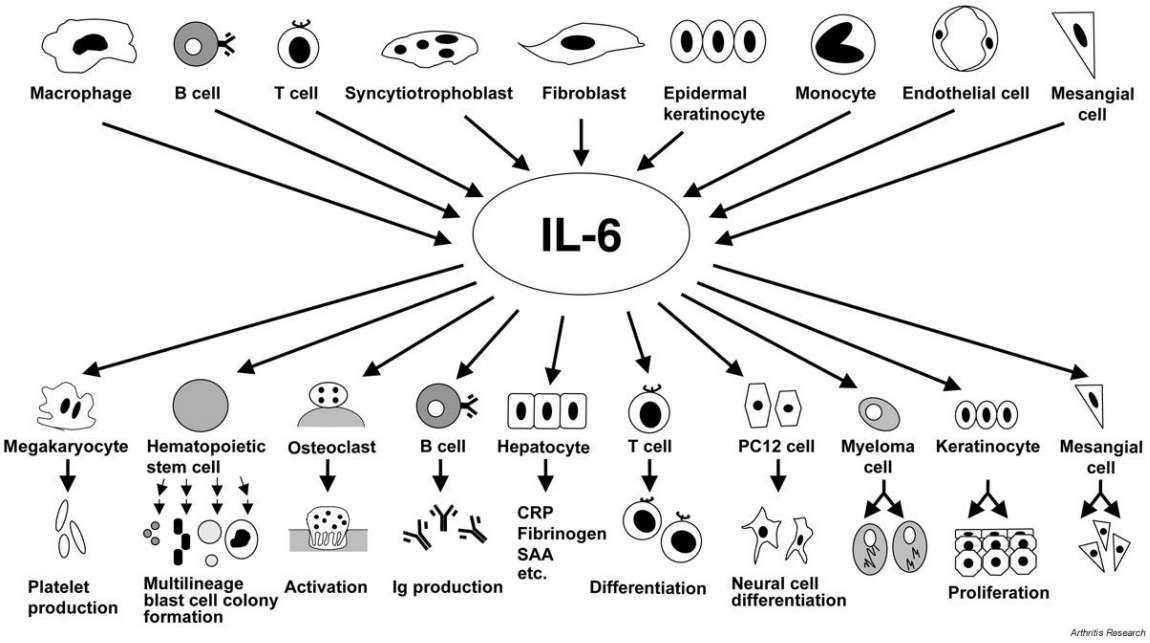


Figure 7: Cells secreting IL-6 and cells undergoing its actions.

IL-6 plays a crucial role in acute phase inflammatory response. It stimulates hepatic production of acute phase proteins like CRP and fibrinogen and its production is mainly induced by TNF-α and IL-1, two of the most important proinflammatory cytokines (Heinrich PC., 1999). It has been demonstrated that IL-6 is also involved in chronic inflammation, because its levels stay high in this phase as well (Gabay C., 2006).

The increasing in inflammation may be the mechanism through IL-6 is involved in the pathogenetic process causing atherosclerotic disease, T2DM and every metabolic disease obesity-related. For T2DM, for example, IL-6, in addition to its immunoregulatory actions, has been supposed to affect glucose homeostasis and metabolism directly and indirectly by action on skeletal muscle cells, adipocytes, hepatocytes, pancreatic β-cells, and neuroendocrine cells.

Recent studies show that while there is evidence that circulating levels of IL-6 are elevated years before onset of type 2 diabetes, the role of IL-6 in precipitating T2DM is still an open question.

In fact, there is no evidence for an independent role of IL-6 in impaired β-cell function and progressive β-cell apoptosis; no evidence in vivo demonstrating the role of IL-6 in causing impaired insulin-signalling in adipocytes and long-term IL-6 stimulation per se does not seem to cause insulin resistance in skeletal muscle. Taken together these results show that IL-6 may contribute to, but is probably neither necessary nor sufficient for development of type 2 diabetes (Kristiansen OP., 2005).

As leptin, IL-6 secretion by adipocytes is partially stimulated by insulin but some works by Trayhurn and also other authors, demonstrated in vitro, that the secretion of IL-6 by adipocytes may be partly a direct result of hypoxia within AT (Trayhurn P., 2008)

2.2.3 TNF-α

TNF-α is one of the most important proinflammatory cytokines, it is produced mainly from the macrophages, included macrophages which are located in the adipose tissue and by adipocytes themselves. TNF-α has several functions: it induces apoptosis and necrosis throughout inducing lyses of the cells, it promotes inflammation stimulating hepatic productions of proteins like PCR and it stimulates macrophages with an autocrine mechanism to produce different proinflammatory cytokines.

TNF-α induces insulin-resistance, promotes the production of hormones (epinephrine, glucagon, cortisol) which increase gluconeogenesis and decrease glucose reabsorbition from skeletal muscle and adipose tissue, in addiction it decreases the production of adiponectin from adipocytes and inhibits the production and activation of the proteins which are involved in the insulin signalling pathway.

In obese patients plasmatic levels of TNF-α are higher then in normal-weight subjects, at the same time TNF-α levels decrease together with the loss of weight. Plasmatic levels also correlate with insulinemia and insulin resistance.

In vitro hypoxic adipocytes secrete TNF- α while Sun et al. has demonstrated that an enforced expression of VEGF in AT significantly down-regulated TNF-alfa expression. Taking together, these data shows once again that AT secretion of chemokines could be heavily affected by hypoxia.

2.3 Adipose Tissue Stem Cells (ASCs)

Adipose tissue shows an extraordinary ability to change rapidly its dimensions, as otherwise just neoplastic tissues do, by hypertrophy (cell size enlargement) and hyperplasia (cell number increase), both in animals and in adult humans (Spalding KL., 2008).

The concept of Mesenchymal Stem Cell was first introduced in the late sixties by isolation from bone marrow (BM-MSC) and just in 2000 from adipose tissue (ASC) (Zuk PA., 2001). In 2001 the team of Zuk firstly described ASCs residing in adipose tissue depots and, actually, we fully recognize their real and potential value but their identity still remains elusive.

Human ASCs are obtained from SVF, a heterogeneous cell population isolated from adipose tissue by the enzymatic digestion, centrifugation and removal of the differentiated adipocytes layer. Cultured cells show important differences compared to freshly isolated stromal cells, the latter one showing different percentages of CD34 positive (CD34+) cells depending on the source: adipose tissue specifically expresses higher levels of CD34 compared to bone marrow (Sidney LE., 2014). A recent comparative study by Pachon-Pena demonstrated how the surface antigens expressed by hASC and BM-MSC are for the major part similar in the immunophenotypic profile, differing fundamentally in the expression percentage of each of them (Pachon-Pena G., 2011). This observation sets these cellular entities more as a continuum rather than two distinct cellular precursors, their distinguishing features arising more from experimental settings (ex vivo or in vitro), number of culture cycles, cell proliferation grade, origin depot rather than from cellular intrinsic properties.

ASCs were shown to express CD34 and their in vitro culture expansion leads to its rapid down-regulation with a concomitant increase in four mesenchymal markers expression, CD13 (APN), CD73 (L-VAP-2), CD90 (Thy-1), CD105 (endoglin). The CD44 is another surface marker detectable on adipogenic progenitors, both in humans and in mice (Sousa BR., 2013). ASCs lie in close contact with other cell types among which pericytes and endothelial cells: pericytes belong to the mural cell compartment and carry out important functions especially in vascular development and maintenance (Gokcinar-Yagci B., 2015; Geevarghese A., 2014), endothelial cells differentiate from endothelial progenitor cells (EPCs), characterized by stem cell features, able to promote vascular regeneration by de novo capillary structures formation (Balaji S, 2013). The close interactions between adipose progenitors, pericytes and endothelial cells and the open controversies about their exact origin still leave many questions open. Zimmerlin et al described three populations in the SVF: the luminal endothelial progenitor cells (CD45-

:CD31+:CD34+), the adventitial pericytes (CD45-:CD31-:CD146+) and the supra-adventitial adipose stromal cells (CD45-:CD31-:CD146-:CD34+); a fourth subset, identified as CD146+:CD34+, showed an intermediate phenotype, being both highly proliferative and with an uniform mesenchymal marker profile (Zimmerlin, 2013) (Tallone T., 2011).

Regarding ASCs and the different AT depot there are always more studies demonstrating how preadipocytes exhibit a site-specific gene pattern expression which is able to condition their behaviour also after isolation and several *in vitro* culture passages (Macotela Y., 2012), A four-way study, in which subcutaneous and visceral adipose tissue were transplanted in SAT or VAT respectively, showed that just subcutaneous tissue transplantation into a subcutaneous or visceral site lead to beneficial effects, including body weight decrease with total adipose mass reduction and whole-body metabolic improvement. Placement of visceral tissue into the donor site did not determine relevant effects in terms of metabolic improvement.

This observation implies the concept of a kind of "origin site cell memory" that can be kept despite the settings changes (Tran T.T, 2010). Again comparing human ASCs from abdominal SAT and VAT isolated and cultured in vitro, subcutaneous ASCs showed a much greater growth rate ability and adipogenic potential compared to visceral ones, by maintaining a high expression level of the polycomb gene BMI-1. Through electrophysiological properties analysis, the stem cell nature of both V-ASC and S-ASC was confirmed, suggesting that the differences between the two depots are probably determined at a stem cell level and "memorized" during later expansion (Baglioni S., 2012).

A greater replicative potential of human subcutaneous preadipocytes compared to omental was already demonstrated: the number of subcutaneous precursors and the rate of clonal replication were higher with increased adipogenesis ability and lower apoptosis susceptibility (Tchkonia T., 2006). Despite this finding, some studies on human obese subcutaneous and visceral adipose tissue, demonstrated that also among V-ASCs there is a predominant hyperplasic grow instead of hypertrophic grow, this observation could be the response to the increased apoptosis rate detectable in this depot; visceral fat is much less malleable than the subcutaneous one, just a slight increase in cell size leading to huge changes in terms of metabolic implications. Moreover we could hypothesize that hyperplasia in VAT correlates with an abnormal and dysfunctional proliferation, nullifying the potential beneficial effects associated with this cell growth mechanism. Probably the past concept of hyperplasia as beneficial and hypertrophy as detrimental is applicable just in a pure and elementary model, in which "confounding factors"

such as location, innervations, vascularisation, epigenetic modulations and others do not need to be taken into consideration.

Moreover, even though white and brown adipose tissue (WAT and BAT) seem to have a common mesodermal origin, recent studies shed light on the different adipogenic precursor. Indeed brown fat seems to share a common Myf-5 progenitor with skeletal muscle; white adipocytes originate from a non-Myf-5 precursor. The commitment through the adipogenic non-Myf-5 line or the myogenic Myf-5 one takes probably place at the mesenchymal stem cell stage (Wu J., 2013). Also regarding the beige AT, some studies suggest an independent origin of white and beige precursors in humans (Di Franco, Guasti et al. 2014).

Another important notion concerning wit ASCs, is that the concept of stemness has been extensively reconsidered, being not just an intrinsic cell property, but rather a feature strictly conditioned by microenvironment.

As for the best known hematopoietic niche, the adipose niche works as a specialized microenvironment that contributes to ASCs quiescence, maintaining their stemness, regulates their proliferation and differentiation. Extracellular matrix (ECM) forms the physiological and plastic scaffold, crucial for determining stem cell behaviour (quiescence versus proliferation and differentiation) and promoting proper contacts between ASCs and the other cellular niche components.

Leptin, for example, secreted from mature adipocytes, regulates ECM composition, modulating the ASCs gene expression profile and therefore conditioning their behaviour in the adipose niche (McCulloch L.J., 2015); this observation provides evidence for a paracrine mechanism by which full differentiated cells regulate their own progenitors. The importance of microenvironment for the ASCs is well demonstrated in studies of obese AT where, as better described below, there is a hypoxia condition due to excessive AT expansion (Cao Y 2013; Corvera S., 2014) which further fires up the inflammatory response in a vicious loop. Many putative mechanisms seem to play a role in obese adipo-niche hypoxia establishment; although many questions still remain unanswered and controversial data are present in literature (Trayhurn P. 2013).

In vitro studies using human ASCs demonstrated an enhanced proliferation in hypoxia (1% of pO_2) conditions, suggesting that altered oxygen homeostasis could affect the quiescent-activated ASCs balance (Kakudo N., 2015; Choi JR., 2014). Interestingly, hypoxia induces a pro-fibrotic program in ASCs, promoting the HIF1α up regulation on one side and the down

regulation of proteins involved in adipogenesis on the other (Rosenow A., 2013), thus perpetuating the pathological circle. Contrasting reports have demonstrated that human obesity does not show metabolic signs of AT hypoxia (Hodson L., 2013), even reporting data of an increased O_2 tension (Goossens GH, 2011); however a normal O_2 tension cut-off for adipose tissue has not been established yet.

Beyond absolute oxygen tension, these data underlie the relevance of an altered oxygen flux in a pathological niche setting. Metabolic signals as well take probably part to the adipo-niche alterations. The often obesity-related diabetes development and thereby hyperglycaemia, implies advanced glycation products formation (AGEs) both in the extracellular and intracellular settings. AGEs are mostly represented by long life proteins, such as albumin, but ECM components could be a target of glycosilation too.

In adipo-niche AGEs negatively affect ASCs pool by inducing cell apoptosis through AGE-receptor (RAGE), as demonstrated in human ASCs when exposed *in vitro* to AGE-serum albumin (Wang Z., 2015).

At least, an emerging field of research is represented by weight loss induced by the mean of bariatric surgery or long-term caloric restriction. Indeed, the analysis of ASCs properties before and after weight loss helps to better understand the role of cell and microenvironment contribution in metabolic disease development.

S-ASCs from ex-obese individuals show a marked adipogenic commitment, probably as consequence of a precursors enrichment. This last observation has raised the hypothesis that hypertrophic adipocytes, after lipid mobilization in massive weight loss, could dedifferentiate again into preadipocytes (Baptista L., 2015), probably through a pericytes involvement in recruitment and generation from mature cells; indeed a fourfold increase in SAT supra-adventitial cells was described in ex-obese patients compared to obese together with an increase in pericytes number in both groups compared to lean subjects (Silva K.R., 2015).

2.4 Adipose Tissue Angiogenesis and Hypoxia

One of the most remarkable features of adipose tissue is its capacity to expand in a nonneoplastic manner. While most of the growth of organs and tissues occurs during development, and their final size remains relatively constant through adulthood, adipose tissue is the unique tissue that can expand many folds also in adulthood.

The cellular and molecular mechanisms by which adipose tissue growth are coordinated with the expansion of its capillary network are unknown. These mechanisms may underlie the basis for adipose tissue dysfunction in metabolic disease.

In each organ or tissue the angiogenesis comprise the proliferation of endothelial cells, their directed migration through the extracellular matrix, the establishment of intercellular junctions, the formation of a lumen, the organization of perivascular supporting cells, the anastomosis with existing vessels, and the establishment of circulation.

The main event of angiogenesis is the stimulation of endothelial cells proliferation by Vascular Endothelial Grow Factor (VEGF). VEGF-A, acting through its receptor, known with the name of VEGF Receptor 2 or KDR, is the most powerful signal for migration and mitogenesis of endothelial cells. In response to the increase of the concentration of VEGF-A, the endothelial cells are able to divide and to acquire a specific phenotype characterized by the formation of branches and numerous filipodi that extend toward the direction where the endothelial cells must migrate.

But, even if the basic steps of angiogenesis are well known, the microenvironment deeply influence the angiogenesis and little is known about the mechanism operating in adipose tissue.

As reported in S. Corvera review (Corvera S., 2014), there are two possible models for the stimulation of angiogenesis during adipose tissue growth. The first one contemplates that an increasing calorie consumption results in adipocyte hypertrophy and hyperplasia, which can generate areas of tissue hypoxia.

Hypoxia, and/or other factors released from the tissue are able to stimulate angiogenesis. Angiogenesis could result in mitigation of hypoxia with an appropriate tissue architecture and function. The second one contemplates that the increasing calorie consumption results in systemic changes in trophic factors such as insulin, which directly stimulate angiogenesis within adipose tissue. Increased angiogenesis facilitates lipid storage in adipocytes and adipocytes' hyperplasia.

The simultaneous expansion of adipocytes and vasculature could prevent development of hypoxia and metabolic stress (Figure 8) Which of the two mechanisms is involved, or if they are both involved, in AT angiogenesis is not yet known but several studies both on cellular cultures, animal models and human adipose tissue, reported that an impaired angiogenesis is involved in AT dysfunction and obesity-related diseases.

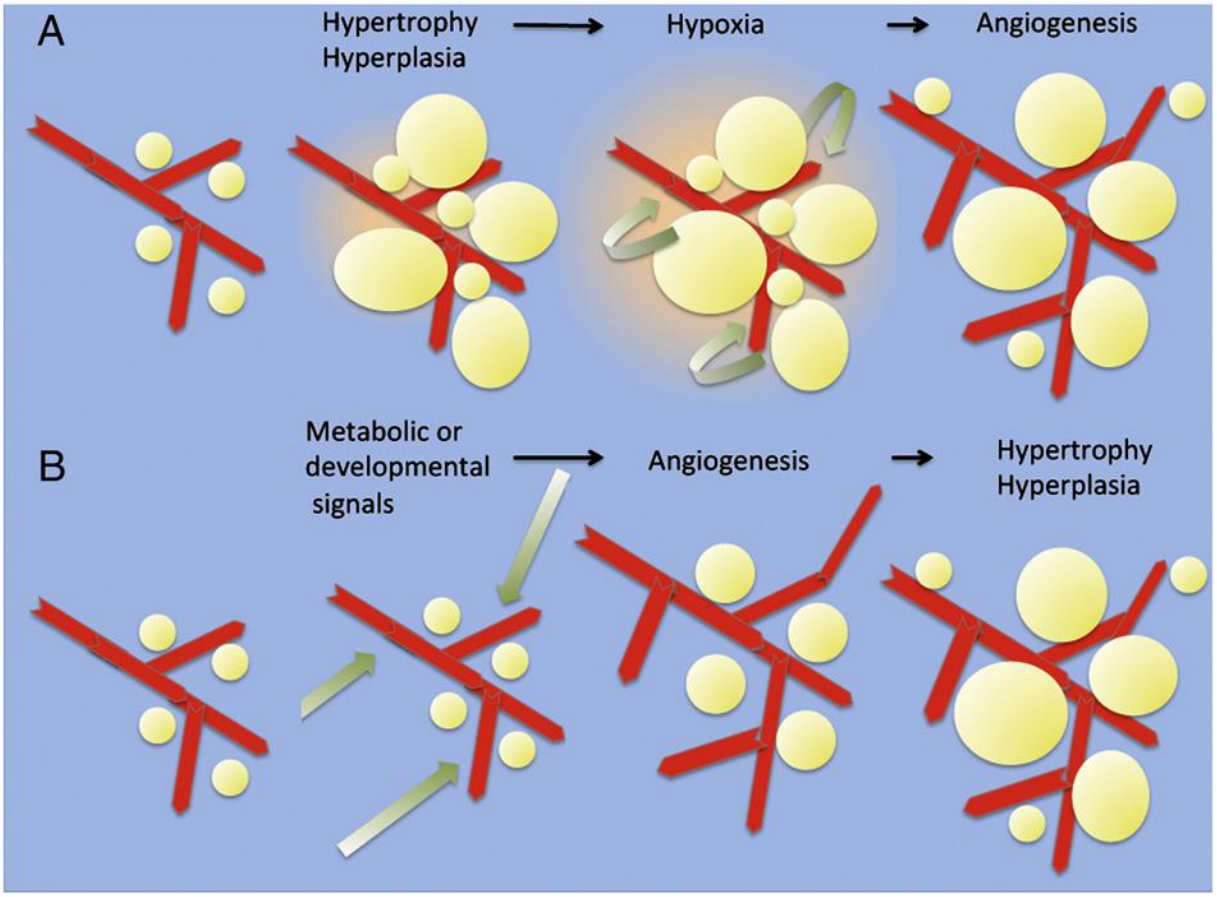


Figure 8: two possible mechanisms of adipose tissue angiogenesis (adapted from S. Corvera review)

Pasarica, Goosens and Gealekman's works have demonstrated a significantly reduction in both capillary density and VEGF m-RNA in obese AT compared to lean AT (Pasarica M., 2009, Goossens GH., 2011, Gealkmann O., 2001).

The impaired AT angiogenesis could be the *primum movens* of AT dysfunction with particular regard to insulin-resistance and diabetes. Indeed, microvasculature plays a central role in glucose homeostasis: an impaired capillary recruitment and capillary rarefaction may reduce glucose uptake and contribute to insulin resistance (Levy BI, 2008).

Even if in human AT, contrasting results regarding AT pO_2 are reported, several studies showed that AT is hypoxic but this hypoxia is inadequate to sustain the neoagiogenetic response however triggers AT fibrosis contributing to a worse metabolic profile (Sung HK, 2013). Indeed, structural and functional abnormalities, such as lower capillary density (Pasarica M, 2009; Gealekman O, 2011), increased adipocyte-capillary distance due to adipocyte hypertrophy (Halberg N, 2009) and increased cell O2-consumption (Lee YS, 2014), have been

postulated as mechanisms leading to AT hypoxia. Furthermore low oxygen tension induces hypoxia-inducible factor 1 alpha (HIF1α) pathway that, in obese AT, was found to be higher than in lean AT. HIF1α is a transcriptional regulator of more than 100 different genes. Encoding for proteins involved in a multiplicity of cellular process, including glucose utilization, inflammation, ECM metabolism and apoptosis.

In AT, HIF1α is unable to induce an angiogenic program but, the presence of HIF1α induces an alternative transcriptional program, mainly entailing enhanced synthesis of ECM components, leading eventually to the development of fibrosis. As it has been well explained by Sun in its review published on Cell Metabolism (Figure 9), hypoxia and then, HIF1α, unregulated a whole set of ‘‘fibrotic response’’ gene. This regulation results in the abnormal development of ECM, leading to local fibrosis, which triggers necrosis of adipocytes. The dead adipocytes then attract classically activated proinflammatory M1 macrophages, which ultimately lead to inflammation and metabolic dysfunction. Moreover HIF1a may also directly induce proinflammatory factors, such as IL6 and MIF, which in turn causes M1 macrophage infiltration.

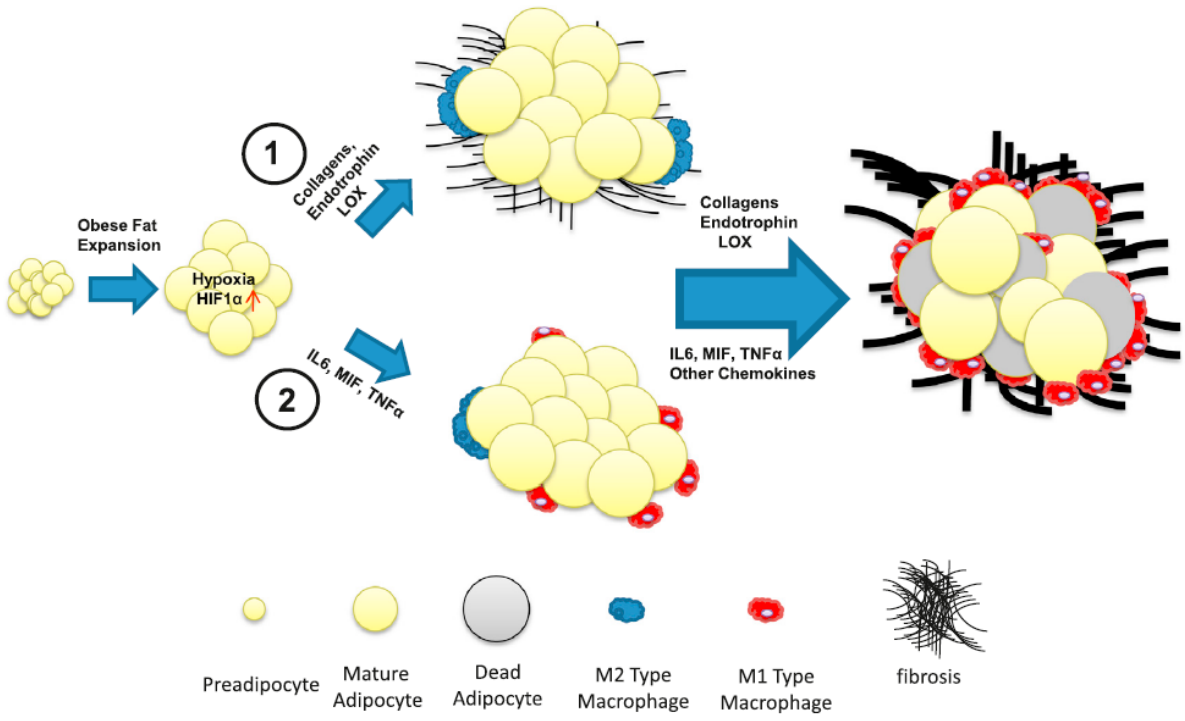


Figure 9: Proposed Models for the Sequential Steps Leading to Adipose Tissue Fibrosis and Metabolic Dysfunction

The preadipocytes, macrophages, and interactions between these cell types ultimately produce fibrotic components, which eventually cause pathological expansion of fat pads (Sun K. 2013). These two mechanisms, able to explain the linking between obese fat expansion, hypoxia and metabolic disease, are well demonstrated in cultured cells and in mice-models but not thoroughly in human.

3. AIMS

3.1 AIMS (1)

Type 2 diabetis mellitus (T2DM) and obesity are global health care problems that are closely linked together. The precise mechanisms linking the two conditions remain unclear. Indeed, while the close relationship between T2DM and weight gain is well established, not all obese subjects are diabetic and this paradox is still unexplained.

We hypothesized that adipose tissue (AT) and its microvascularization could play a pivotal role in this paradox.

Therefore, our project primary aims to characterize AT, through ex vivo/in vitro studies, in terms of adipocytes size, capillary density, adipose tissue stem cells (ASCs), endotelial precursor cells (EPs) of AT and adipogenic potential, in obese subjects compared to lean subjects; secondary, aims to find the possible differences between the two distinct adipose tissue depot, subcutaneus and visceral adipose tissue (SAT and VAT) both in obese and in lean subjects; finally, aims to discover if there are any differences, in the same terms describe above, in SAT and VAT of obese patients with different glycaemic profile. Moreover, in a small number of patients, we aim to characterize SAT after a significant weight loss in terms of ASCs and EPs compared to obese patients SAT and lean patients SAT.

3.2 AIMS (2)

Currently, bariatric surgery is the most effective therapy for weight loss and and weight associated co-morbidities.

Among bariatric surgery procedures, laparoscopic sleeve gastrectomy (LSG) is markedly increased in prevalence, raising from 5% to 27% of all bariatric procedures, in the recent years. The beneficial effects of this bariatric procedure are well known, while are lesser known side effects. In particular, postprandial hypoglycaemia, is a well described side effect after RYGB but few data are available for LSG.

Primary aims of this study are: verify the prevalence of provocative hypoglycaemia after LSG in a large number of non diabetic obese patients and identify any predictors of these events before surgery.

Secondary aims are: evaluate the effects of LSG, after one year, in the same population and if there are any differences in anthropometric and metabolic parameters after LSG between patients with and without hypoglycaemic events .

4. MATERIALS and METHODS

4.1 MATERIALS and METHODS (1)

4.1.1 Ethic Statements

The Padua Ethical Committee for Clinical Research approved the study involving patients and each subject gave informed written consent for adipose tissue biopsy (2892P).

4.1.2 Human Subjects

We collected subcutaneous (SAT) and/or visceral (VAT) adipose tissue (AT) from 249 patients divided in 5 different groups: 18 lean normal weight and normoglycemic subjects (18.5 < BMI < 24,9 kg/m2) as control group, 68 normoglycemic obese subjects (ob N), 65 pre-diabetic obese subjects (ob pre-T2DM), 57 diabetic obese subjects (ob T2DM) and 41obese patients underwent to a relevant weight loss (ob WL), corresponding to at least 10% of body weight.

All obese patients were enrolled in our (Ce.S.I.T.O.) from January 2014 to June 2016. Every obese subject received a baseline clinical evaluation which included collection of clinical history, physical examination, anthropometry, blood pressure measurement and blood samplings (complete blood count, liver, kidney and thyroid function markers, TNF-α, IL-6, hs-CRP, leptin and lipid profile). Moreover a 3-hour 75 g OGTT for blood glucose (BG), insulin plasma levels, c-peptide was performed. In diabetic patients fasted blood glucose, insulin (for non-insulin treated subjects) and HbA1c was determined. Insulin sensitivity was estimated by HOMA IR. All biochemical analysis were performed as described below (Materials and Methods 2, Patients). On the basis of the glycaemic profile according to ADA criteria (ADA, 2016) obese patients were classified in 3 groups: ob N, ob pre-T2DM and ob T2DM. AT samples from obese patients were collected during bariatric surgery by the same chirurgical team (Bernate P., 2006).

Lean subjects were enrolled, as control group, in the General Surgery Unit or in Clinical Surgery I of Padua Hospital, and SAT and VAT samples were harvested during abdominal surgery as laparoscopic cholecystectomy or fundoplication surgery, or SAT alone during plastic surgery for minor abdominal wall defects. For each lean subject we collected clinical history, anthropometry, blood pressure measurement and blood samplings (complete blood count, liver and kidney function markers and fasting glucose). The exclusion criteria for the selection of

lean subjects were: a history of malignancy (the patients included in the study must be free of the disease for at least 5 years), chronic inflammatory diseases, infectious diseases in progress and T2DM.

After sampling, tissues were partly immediately frozen in liquid nitrogen and stored at -80°C before further assays and partly formalin fixed for immunohistochemical analysis (HIC). Moreover, when sufficient tissue was available, it was used to isolate stromal vascular fraction (SVF) cells to set up adipocytes primary cultures and/or to flow cytometry analysis.

4.1.3 Histological and Immunohistochemical Analysis

SAT and VAT samples of 5 ob N, 5 ob pre-T2DM, 5 ob T2DM and 6 lean subjects were formalin fixed, paraffin embedded, sectioned 5 μm thick and floated onto charged slides.

4.1.3.1 *Adipose Tissue Capillarization*

Paraffin-embedded AT sections were stained with a specific endothelial marker (Monoclonal Mouse Anti-Human CD31, Clone JC70A, Dako) and observed under Leica DM LB2 microscope. Digital images were captured with the use of a Leica DFC450C digital camera, in at least 10 random fields at 20X magnification (at least 200 random adipocytes per tissue biopsy were counted). The number of capillaries for every adipocyte were manually counted and the capillary density was expressed as number of capillaries per mm^2 and as average adipocyte area (μm^2) sprayed by one capillary (adipocyte area/capillary).

4.1.3.2 *Adipocyte Size*

Adipocyte area was measured on the same digital images captured with the use of a Leica DFC450C digital camera, in the same samples and fields used for the analysis of AT capillarization. Area of each adipocyte was manually counted using LAS software (Leica Microsystems). Approximately 200 adipocytes per sample were measured.

4.1.4 Human Adipocytes Primary Cultures and Flow Cytometry Analysis

4.1.4.1 *Stromal Vascular Fraction Extraction*

SAT and VAT were minced, digested in collagenase type II solution (1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) on a shaking water bath at 37°C for 1 h, centrifuged 10 minutes at 350 xg, and red blood cells were lysed using lysis buffer (NH_4Cl 1.545 M, $KHCO_3$ 100 mM, EDTA 1.27 mM) as previously described (Sanna M., 2009).

4.1.4.2 *Flow Cytometry Analysis*

SAT samples of 24 ob N, 18 ob pre-T2DM, 23 ob T2DM, 5 lean subjects and 17 ob WL and VAT samples of 26 ob N, 25 ob pre-T2DM, 30 ob T2DM and of 10 lean subjects were used to isolate SVF and to perform multiparameter flow cytometry.

10^5 SVF cells freshly isolated from SAT and VAT of the indicated patients were washed whit cold FACS buffer (2% BSA in PBS 1X), collected by centrifugation at 350 xg for 8 minutes, and simultaneously incubated in the dark for 10 minutes at room temperature with the following monoclonal primary antibodies, as indicated in Figure 10: CD31-FITC and -PE, CD45-FITC, CD34-PerCP-Cy5.5, CD44-PE, CD73-APC, CD90-PE, CD105-APC, CD271-APC, CD146-PE (BD Biosciences) specific for membrane markers used to quantify and characterize Adipose Stem Cells (ASCs), endothelial precursor and mature cells and pericytes/mural cells (Zimmerlin L, 2010; Tallone T, 2011).

	Tube	FITC	PE	PerCP-Cy5.5	APC
FVSc	1				
	2	ms IgG1 isotypic control	ms IgG1 isotypic control	ms IgG1 isotypic control	ms IgG1 isotypic control
	3	CD45	CD31	CD34	
	4	CD31	CD13	CD34	CD29
	5	CD31	CD90	CD34	CD73
	6	CD31	CD146	CD34	CD271

Figure 10 . Schematic protocol for antibody staining for multiparametric FACS analysis.

After washing with FACS buffer (2% BSA in PBS 1X), cells were collected by centrifugation at 350 xg and resuspended in 200 µL of FACS buffer. As negative control, the fluorescence signals were detected using isotype-matched PE-IgG1, FITC-IgG1, APC-IgG1 and PerCp-Cy5.5-IgG1 monoclonal antibodies. Cells were analyzed by FACS Canto Flow Cytometer (BD Biosciences, San Jose, CA, USA).

This method allows to determine *ex vivo* the percentage of cells in the SVF that co-express the different markers in the absence of expression alterations due to culture conditions.

4.1.4.3 *Human Adipocyte Primary Cultures*

SAT samples of 18 ob N, 15 ob pre-T2DM, 14 ob T2DM and VAT samples of 20 ob N, 25 ob pre-T2DM, 23 ob T2DM were used to isolate SVF to perform human adipocytes primary culture in order to evaluate the *in vitro* adipogenic potential.

$1x10^5$ SVF cells/well were seeded in duplicate in 96-well plate (BD Biosciences) in human-standard medium (h-SdM): 10% FBS DMEM F12 supplemented with 150 U/ml streptomycin,

200 U/ml penicillin, 2 mM glutamine, 1 mM HEPES (Thermo Fisher Scientific). At cell confluence (1-2 days after seeding), medium was replaced with human-adipogenic medium (h-AdM): DMEM F12 (with 150 U/ml streptomycin, 200 U/ml penicillin, 2 mM glutamine, 1 mM HEPES) containing 66 nM insulin, 100 nM dexamethasone, 1 nM T3, 10 µg/ml transferrin, 33 µM biotin, 17 µM pantothenate, 0.25 mM IBMX, 10 µM rosiglitazone. IBMX and rosiglitazone were removed after 3 days of culture, cells were further differentiated in h-AdM until day 9 when the percentage of mature adipocytes per well was estimated by optical microscopy using a Leica DM IL LED Microscope equipped with camera.

4.1.5 RNA Extraction and RT-Real time PCR

Total RNA from matched SAT and VAT samples of 20 ob N, 22 ob pre-T2DM, 22 ob T2DM, 13 ob WL and of 6 lean subjects was extracted using RNeasy Mini Kits (QIAGEN, GmbH, Hilden, Germany) according to supplier instructions. RNA content was quantified using NanoDrop technology (Fisher Scientific SAS, Illkirch Cedex, France) and quality-checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, PaloAlto, USA).

RNA samples were then treated with DNase Treatment & Removal Reagents (Ambion, Inc, Austin, TX, USA) and reverse-transcribed for 1 h at 37° C with 150 ng random primers, 0.5 mM dNTPs, 20 units of RNAsin Ribonuclease Inhibitor and 200 units of M-MLV RT (Promega, Madison, WI, USA). Real Time PCR was carried out with SYBR Select MasterMix (Thermo Fisher) on an Applied Biosystems 7900HT Fast Real-Time PCR System. Duplicate samples (5 ng of cDNA) were normalized by the indicate reference gene and reported as arbitrary units ratio.

Primers sequences and reaction conditions were reported in Table IV.

GENE	FORWARD (5'-3')	REVERSE (5'-3')	ANNEALING (°C)	PRIMER (F/R nM)	AMPLICON (bp)
RPLP0	GCAGCATCTACAACCCTGAA	CAGACAGACACTGGGAACAT	60	300/300	95
PPARg2	ACCCAGAAAGCGATTCCTTCA	AGTGGTCTTCCATTACGGAGAGATC	60	900/900	87
LEPTIN	GTGCGGATTCTTGTGGCTTT	GGAATGAAGTCCAAACCGGTG	63	100/100	174
VEGFA	TCACCATGCAGATTATGCGGA	TGTTGTGCTGTAGGAAGCTCA	58	300/300	75
VEGFR1 (FLT1)	CGCCGGAAGTTGTATGGTTAAAA	AGCCACGAGTCAAATAGCGAG	58	300/300	72
VEGFR2 (KDR)	CCGTTAAGCGGGCCAATGGA	TTCAGCCGGTCTCTGGGGAA	60	300/300	142
HIF1A	TTACCATGCCCCAGATTCAG	GGTTCTTTGCTTCTGTGTCTTC	58	300/300	180

Table IV. Primes sequences and real time PCR reaction conditions used.

4.1.6 Statistical Analysis

In dependence of their distribution according to Shapiro Wilk normality test data, are presented as mean ± SD or median, minimum and maximum values. Statistical significance was determined using test t for normal distributed variables and Mann-Whitney non parametric test for skewed data. Differences were considered significant with $p < 0.05$.Correlation analysis was performed using both linear correlation and Spearman's rank correlation coefficient calculation by STATISTICs Software (StatSoft 7.1)

4.2 MATERIALS and METHODS (2)

4.2.1 Ethic Statements

The Padua Ethical Committee for Clinical Research approved the study involving patients and each subject gave informed written consent for adipose tissue biops y (2892P).

4.2.2 Patients

We analysed a total of 197 consecutive non-diabetic morbidly obese patients (140 women and 57 men, BMI 47.4 ± 7.3 Kg/m^2, mean ± SD) who underwent to laparoscopic sleeve gastrectomy (LSG) in our Center for the Study and the Integrated Treatment of Obesity (Ce.S.I.T.O.).

All patients were studied 12 months before and after LSG. LSG was performed by the same surgical team with the same procedures (Bernate P., 2006) in patients with a BMI greater than 35 Kg/m^2 in the presence of co-morbidities or with a BMI greater than 40 Kg/m^2, according to the NIH consensus criteria for bariatric surgery (NIH, 1991).

Pre-operative evaluations included anthropometrics parameters, medical history, clinical examination, dietary counselling, complete blood count, complete metabolic panel including a 3- hour OGTT for blood glucose (BG), insulin plasma levels and c-peptide, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and highly sensitive C-reactive protein (hs-CRP). Abdominal ultrasound, upper gastrointestinal endoscopy and upper gastro-intestinal barium x-ray were also performed before surgery.

Patients with a history of T2DM according to ADA criteria (ADA 2016) were excluded from the present analysis. One year after surgery all patients were evaluated with the same blood analysis, anthropometrics parameters and clinical examination. All blood tests were done after 8 hours fasting. Plasma glucose, insulin and C peptide were collected at basal time and after

30, 90, 120, 150, 180 minutes after glucose load (180 ml of syrup with 82.5 g glucose monohydrate equal to 75 g of glucose). Hypoglycaemia was defined as the detection of a BG level ≤ 2.7 mmol/l at any time during OGTT.

Blood samples were used for the biochemical determinations, performed with standard diagnostic kit: glucose (Glucose HK Gen.3 Cobas C System, Roche Diagnostic, USA), insulin, IL6, TNFα (IMMULITE 2000 Insulin, IMMULITE 1000 Immunoassay System, IL-6 and TNFα, Siemens Healthcare GmbH, Germany), hsCRP (Cardiophase Flex reagent Cartridge, Dimension Vista, Siemens) and Leptin (RIA – CT, Mediagnost, Germany) was standardized according to WHO First International Reference Standard.

Glucose and insulin areas under the curve (AUC) were calculated. The homeostasis model assessment (HOMA) was calculated and used as insulin resistance index (Bonora A, 2000). Ideal body weight was calculated as weight which give patients an hypothetical BMI of 25 $Kg/m^2$and it was used to calculate the percentage of the excess BMI (%EBMI) and the percentage of excess BMI loss (%ELBMI).

4.2.3 Statistical Analysis

Data are expressed as mean ± standard deviation, except as otherwise indicated. The frequencies of hypoglycaemic events and severe hypoglycaemic events are expressed in percentage. Differences in the frequencies of hypoglycaemic events observed before and after LSG were tested by Chi-square test.

Numerical data at baseline and after 12 months were compared with the use of a paired Student's *t*- test. Patients with and without hypoglycaemic events were compared with unpaired Student's *t*- test. Predictors of hypoglycaemic events were investigated with the use of a multiple logistic regression analysis model.

In this model, the occurrence of hypoglycaemia (BG <3.3 mmol/l) at any time during the test was used as the dependent variable. Sex (male =0; female =1), age, BMI before surgery, and the baseline variables found to have a significant difference between patients with and without hypoglycaemic events after surgery were entered as independent variables in the multiple regression analysis. In all statistical analysis, a p-value <0.05 was considered to be significant. Statistical analysis was performed by using the SSPS statistical package, version 21.0 (SSPS, Chicago, IL).

5. RESULTS

5.1 RESULTS (1)

5.1.1 Patients

5.1.1.1 *Clinical Evaluation of Obese Patients*

We analyzed SAT and/or VAT samples from 190 consecutive obese patients underwent to bariatric surgery, whose main demographic, anthropometrics and metabolic parameters are reported in Table V. On the basis of their glycaemic profile, patients were divided in three different groups, 68 patients were normoglycemic (ob N), 65 were pre-diabetics (ob pre-T2DM) and 57 were diabetics (ob T2DM). The three groups did not statistically differed in terms of BMI (46 ± 8; 47 ± 7; 48 ± 8 kg/m^2) and Leptin plasma levels (40 ± 15; 40 ± 16; 35 ± 16 μg/l), while ob T2DM showed a waist circumference (WC) larger than ob N and ob pre-T2DM (137 ± 16; 127 ± 13; 128 ± 20 cm; $p < 0,001$ and $p < 0,05$ respectively). Moreover, ob T2DM were older than ob N and ob pre-T2DM and also ob pre-T2DM were older than ob N (52 ± 9; 47 ± 11; 40 ± 10 yrs; ob T2DM vs ob N $p < 0,00001$; ob pre- T2DM vs ob N $p < 0,001$; ob pre-T2DM vs ob T2DM $p < 0,01$).

The three co-morbidities considered, blood hypertension, dyslipidemia and Obstruction Sleep Apnea Syndrome (OSAS), have seen a progressive increase in terms of percentage of patients, from ob N to ob T2DM groups. Indeed, hypertension was present in the 37% of ob N, in the 54% of ob pre-T2DM and in the 91% of ob T2DM; half of ob N and ob pre-T2DM patients were affected by dyslipidemia which was present in the 77% of ob T2DM. Finally, OSAS was present in only 5% of patients in ob N group, increasing to 28% in the ob pre-T2DM and 42% in the ob T2DM groups.

As expected by the selection criteria used, the 3 groups differed significantly in fasting blood glucose level, mmol/l (5 ± 0,4; 5,9 ± 0,5; 9,4 ± 4; $p < 0,000001$), and ob N compared to ob pre-T2DM showed higher levels of basal insulin mU/l (16,5 ± 9,7; 25,8±19,4; $p < 0,001$) and lower levels of HOMA -IR (3,7 ± 2,3; 6,8 ± 5,7: $p < 0,0001$). We did not evaluate insulin levels and HOMA -IR in ob T2DM patients because 20 out of 57 patients were on therapy with insulin. The others ob T2DM were treated with metformin (46/57) alone or associated to insulin or other hypoglycemic drug treatment as sulfonylureas (3/57), thiazolidinedione (1/57), GLP-1r agonist (4/57); 4 ob T2DM patients were not on therapy at the time of blood tests and 3 were started on metformin therapy, while just 1 was following diet therapy till the surgery. Among

ob T2DM 24 out of 57 patients had a history of T2DM longer of 5 years, 13 out of 57 had a history of T2DM lesser than 5 years, 20 out of 57 had a history of T2DM lesser than 1 year. HbA1c levels in ob T2DM was 66 ± 21 mmol/mol (range 38-122 mmol/mol).

	ob N (n = 68)	**ob pre-T2DM (n = 65)**	**ob T2DM (n = 57)**	**p ob N vs ob pre-T2DM**	**p ob N vs ob T2DM**	**p ob pre-T2DM vs ob T2DM**
Sex (F/M)	55/13	50/15	32/25			
Age (years)	40 ±10	47 ± 11	52 ± 9	<0,001	<0,00001	<0,01
HYP (n; %)	25 (37%)	35 (54%)	52 (91%)			
DLP (n; %)	34 (50%)	34 (52%)	44 (77%)			
OSAS (n; %)	5 (7%)	18 (28%)	24 (42%)			
BMI (Kg/m^2)	46 ± 8	47 ± 7	48 ± 8	ns	ns	ns
Waist(cm)	127 ± 13	128 ± 20	137 ± 16	ns	<0,001	<0,05
Blood Glucose (mmol/l)	5 ± 0,4	5,9 ± 0,5	9,4 ± 4	<0,0000	<0,0000	<0,0000
Insulin (mU/l)	16,5 ± 9,7	25,8±19,4	-	<0,001	-	-
HOMA-IR	3,7 ± 2,3	6,8 ± 5,7	-	<0,0001	-	-
HbA1c (mmol/mol)	-	-	66 ± 21	-	-	-
T-CHL (mg/dl)	186 ± 29	193 ± 37	184 ± 43	ns	ns	ns
HDL (mg/dl)	48 ± 12	46 ± 12	43 ± 11	ns	<0,05	ns
LDL (mg/dl)	116 ± 25	121 ± 36	110 ± 37	ns	ns	ns
TGL (mg/dl)	115 ± 57	139 ± 77	166 ± 89	ns	<0,05	ns
hsPCR (mg/l)	7,8 ± 15,9	7,6 ± 4,2	7,6 ± 3,2	ns	ns	ns
TNF-α (ng/l)	7,7 ± 2,3	8,7 ± 7,4	9,9 ± 6,7	ns	<0,05	ns
IL-6 (ng/l)	3,4 ± 2,4	3,3 ± 1,3	4,4 ± 2,4	ns	<0,05	<0,01
Leptin (ug/l)	40 ± 15	40 ± 16	35 ± 16	ns	ns	ns

Table V. Demographic, anthropometrics and metabolic parameters of obese patients analyzed by AT biopsies collection. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. T-CHL: total cholesterol. HDL: High Density Lipoproteins. LDL: Low Density Lipoproteins. TGL: triglycerides. hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

In regard to systemic inflammation markers statistical differences were obtained between ob N compared to ob T2DM in term of TNF-α (7,7 ± 2,3; 9,9 ± 6,7 ng/l; $p < 0,05$) and between ob N compared to ob T2DM (3,4 ± 2,4; 4,4 ± 2,4 ng/l; $p < 0,05$) and ob pre-T2DM compared to ob T2DM (3,3 ± 1,3; 4,4 ± 2,4 ng/l; $p < 0,001$) in term of IL-6. Blood level of hsPCR was not significantly different among the 3 groups of patients (7,8 ± 15,9; 7,6 ± 4,2; 7,6 ± 3,2 mg/l).

Finally, concerning the lipid profile, significant differences were observed only in diabetic patients (ob T2DM) which displayed lower levels of HDL-cholesterol (43 ± 11 mg/dl; 48 ± 12; $p < 0,05$) and higher level of triglycerides (166 ± 89; 115 ± 57 mg/dl; $p < 0,05$) when compared with normoglycemic obese subjects (ob N). We have to notice that there was a progressive worsening in the lipid profile from ob N, ob preT2DM to ob T2DM groups taking into account

that ob N patients were not treated and, on the contrary, 3 out of 65 ob pre-T2DM patients and 20 out of 57 ob T2DM patients underwent to statin therapy.

5.1.1.2 ***Lean Subjects and Obese Patients After Weight Loss.***

We collected SAT and/or VAT samples from 18 non-diabetic lean subjects and 41 obese patients after weight loss (ob WL), whose main demographic, anthropometrics and metabolic parameters are reported in Table VI.

Nine SAT and/or VAT biopsies of lean subjects were collected during cholecystectomy, 3 SAT and/or VAT biopsies were collected during colonic resection for diverticular disease, 3 SAT and /or VAT biopsies during laparoscopic fundoplication, 2 SAT and /or VAT biopsies during umbilical hernia repair and 1 SAT and /or VAT biopsies during rectal prolapse surgery. Lean subjects displayed a mean value of 48 ± 12 years old (range 21-64 years), BMI of 24 ± 2 Kg/m^2 (range 20-26), fasting blood glucose of 5 ± 0,7 mmol/l (range 4,7-6,6). Among lean subjects 1 was affected by hypertension and 1 by dyslipidemia. When compared with the 3 groups of obese patients, BMI resulted, significantly lower in lean subjects ($p < 0,00001$); mean value of age was not different between lean subjects and ob pre-T2DM and ob T2DM, while it was higher in lean when compared to ob N ($p < 0,02$); mean value of fasting blood glucose of lean subjects was comparable to that of ob N, while it was lower in respect to ob pre-T2DM ($p < 0,01$) and ob T2DM groups ($p < 0,00001$) (data not show).

	lean **(n =18)**	**ob WL** **(n = 41)**
Sex (F/M)	11/7	33/8
Age (years)	48 ± 12	47 ± 12
HYP (n; %)	1 (5%)	5 (12%)
DLP (n; %)	1 (5%)	7 (17%)
OSAS (n; %)	0 (0%)	0 (0%)
BMI (Kg/m^2)	24 ± 2	31 ± 6
Blood Glucose (mmol/l)	5 ± 0,7	5 ± 0,7
%EBMIL	-	74 ± 25

Table VI. Demographic, anthropometrics and metabolic parameters of lean patients and obese patients after weight loss (ob WL) analyzed by AT biopsies collection. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. EBMIL: excess BMI loss. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

Nine SAT and VAT biopsies of obese patients after weight loss were collected during cholecystectomy, while the others 32 SAT biopsies were collected during plastic surgery. Weight loss (WL) was obtained in 23 patients by laparoscopic sleeve gastrectomy, in 9 patients by gastric banding, in 1 patient by duodenal switch, in 1 patient by intragastric balloon and in 7 patients by caloric restriction and physical activity. Ob WL patients displayed a mean value of

47 ± 12 years old (range 21-69), BMI of 31 ± 6 Kg/m^2 (range 22,5-48), percentage of excess BMI loss (% EBMIL) of 74 ± 25 % (range 23-119), fasting blood glucose of 5 ± 0,7 mmol/l (range 3,4-6,4). Among ob WL patients, 5 were affected by hypertension and 7 by dyslipidemia. Before WL 6 patients were diabetics and after WL 5 have experienced a remission of T2DM and only 1 remained diabetic. When compared with the 3 groups of obese patients, BMI resulted significantly lower in ob WL patients ($p < 0,00001$); mean value of age was not different between ob WL patients and ob pre-T2DM, while was higher in ob WL when compared to ob N ($p < 0,01$) and lower in ob WL when compared to ob T2DM ($p < 0,05$); mean value of fasting blood glucose of ob WL patients was comparable to mean value of fasting blood glucose of ob N, while it was lower than that of ob pre-T2DM ($p < 0,05$) and ob T2DM ($p < 0,00001$) (data not show).

5.1.2 Histology and Immunohistochemistry

We performed immunohistochemical analysis (IHC) of SAT and VAT samples from 6 lean subjects and from 15 obese patients (5 ob N, 5 ob pre-T2DM, 5 ob T2DM) whose main demographic, anthropometrics and metabolic parameters are reported in Table VII.

	Lean (n = 6)	**ob N (n = 5)**	**ob pre-T2DM (n = 5)**	**ob T2DM (n = 5)**	**p lean vs ob N**	**p ob N vs ob pre-T2DM**	**p ob N vs ob T2DM**	**p ob pre-T2DM vs ob T2DM**
Sex (F/M)	2/4	4/1	4/1	2/3	-	-	-	-
Age (years)	55±6	42 ±9	56 ± 9	44 ± 13	<0,05	<0,05	ns	<0,01
HYP (n/tot goup)	2/6	1/5	4/5	4/5	-	-	-	-
DLP (n/tot goup)	0/6	3/5	4/5	5/5	-	-	-	-
OSAS (n/tot goup)	0/6	0/5	3/5	1/5	-	-	-	-
BMI (Kg/m^2)	23,7±1,3	54 ± 19	47 ± 6	57 ± 9	<0,01	ns	ns	0,05
Waist(cm)	-	127 ± 19	129 ± 14	154 ± 16	-	ns	0,06	<0,05
Blood Glucose (mmol/l)	5,2±1,02	4,8±0,18	5,6 ± 0,9	10 ± 4,3	ns	ns	<0,05	<0,05
Insulin (mU/l)	-	15 ± 9	23 ± 18	-	-	ns	-	-
HOMA-IR	-	3,3 ± 1,9	5,9 ± 5,7	-	-	ns	-	-
HbA1c (mmol/mol)	-	-	-	73 ± 26	-	-	-	-
T-CHL (mg/dl)	-	193 ± 27	202 ± 47	225 ± 47	-	ns	ns	ns
HDL (mg/dl)	-	51 ± 9	50 ± 13	41 ± 8	-	ns	ns	ns
LDL (mg/dl)	-	123± 17	128 ± 35	154 ± 38	-	ns	ns	ns
TGL (mg/dl)	-	93 ± 26	120 ± 62	223 ± 77	-	ns	<0,01	<0,05
hsPCR (mg/l)	-	4,3 ±2,6	9,1 ± 8,4	9,3 ± 1,8	-	ns	<0,01	ns
TNF-α (ng/l)	-	8,4± 2,9	10 ± 4,5	10,7±2,9	-	ns	ns	ns
IL-6 (ng/l)	-	3,2 ±1,5	3,1 ± 1,6	4,5 ± 2,3	-	ns	ns	ns
Leptin (ug/l)	-	33 ± 17	43 ± 8	38 ± 18	-	ns	ns	ns

Table VII. Demographic, anthropometrics and metabolic parameters of lean patients and obese patients analyzed by IHC. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. T-CHL: total cholesterol. HDL: High Density Lipoproteins. LDL: Low Density Lipoproteins. TGL: triglycerides. hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

5.1.2.1 *Adipocyte Size*

In SAT and VAT samples, as showed in Figure 11, median adipocyte area (μm^2) was lower in lean patients compared to obese patients, regardless of group division ($p < 0,001$).

In SAT, adipocyte area of ob N was significantly smaller than that of ob pre-T2DM ($p < 0,01$) and of ob T2DM ($p < 0,001$) while no significant differences were present between adipocyte area of ob pre-T2DM and ob T2DM. In VAT, adipocyte area of ob N was significantly smaller than that of ob T2DM ($p < 0,001$) and adipocyte area of ob pre-T2DM was significantly smaller than that of ob T2DM ($p < 0,01$), while no significant differences were between adipocyte area of ob N and ob pre-T2DM. Taken together these data show that among obese patients, there is an increasing trend in adipocytes area from ob N to ob T2DM both in SAT and in VAT showing that adipocytes coming from ob T2DM patients are largest in both depots.

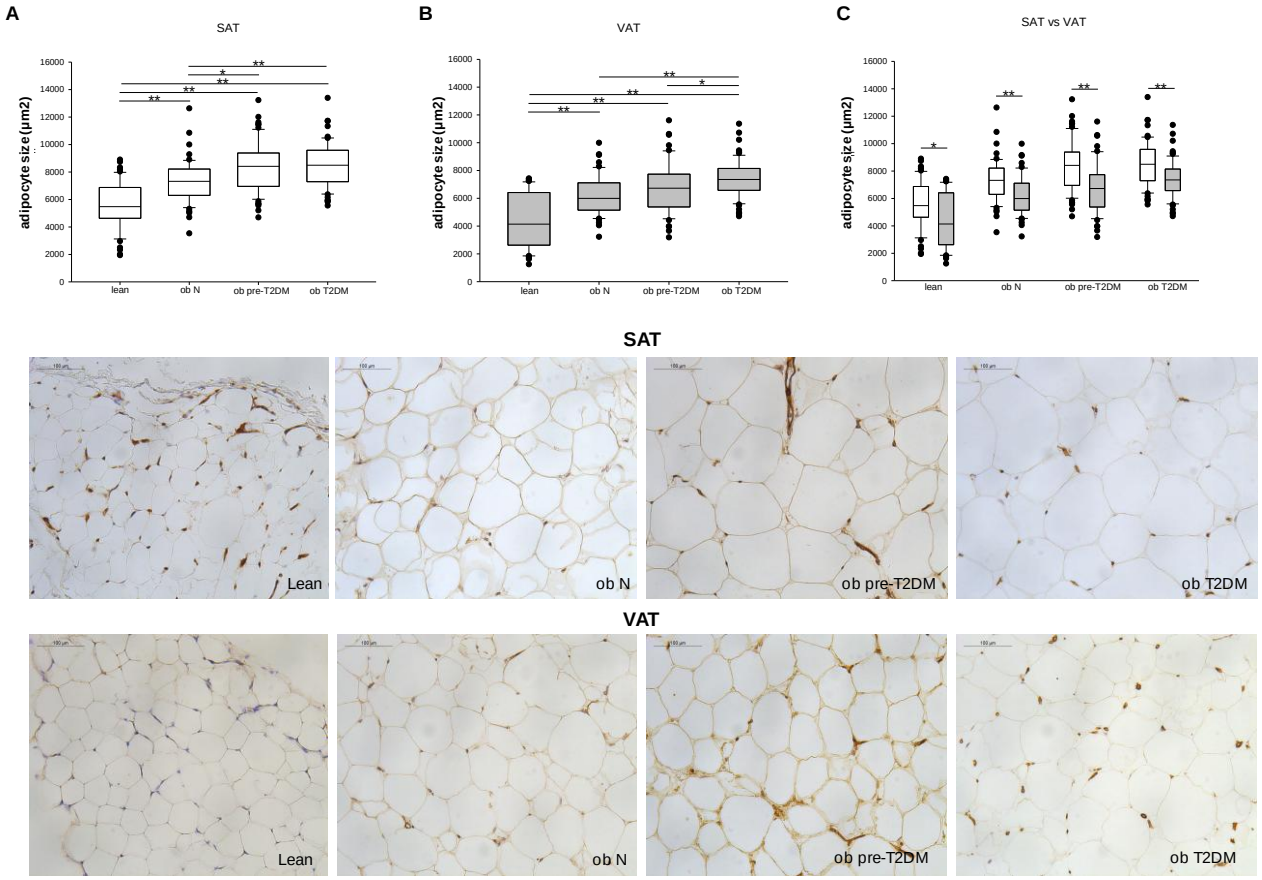


Figure 11. Adipocyte size (μm^2) in subcutaneous, A, (SAT) and visceral, B, (VAT) adipose tissue in the 4 groups of patients: lean (n=6), obese normoglycemic (ob N=5), obese pre-diabetic (ob pre-T2DM= 5) and obese diabetic (ob T2DM=5) (*p <0,01; **p<0,001). C, adypocite size in SAT (indicated by white box) vs VAT (indicated by grey box) in the 4 groups of patients (*p <0, 01; **p<0,001). Representative SAT (D) and VAT (E) sections from lean, ob N, ob pre-T2DM and ob T2DM subjects stained with Monoclonal Mouse Anti-Human CD31, Clone JC70A. Adipocyte size was measured in at least 10 random fields at 20X magnification (at least 200 random adipocytes per tissue biopsy were counted). Median adipocyte area was calculated in each group. Results are reported as box plot graph with median, minimum and maximum values; statistical analysis was performed by Mann-Whitney test.

When we compared SAT and VAT we observed that in all 4 groups considered adipocyte area is larger in SAT than in VAT ($p < 0,01$ for lean subjects, $p < 0,001$ for obese patients) according to what previously described in the literature, considering obese patients regardless the presence of metabolic complications.

5.1.2.2 *Capillary Density*

Regarding to the capillary density quantification (Figure 12), we observed that lean subjects displayed an higher number of vessels per mm^2 both in SAT and in VAT compared to obese patients ($p < 0,001$) despite an higher degree of variability observed in the VAT capillaries' number. On the contrary we did not showed any significant variation in SAT and VAT vascularization between the 3 obese patients group (ob N, ob pre-T2DM and ob T2DM).

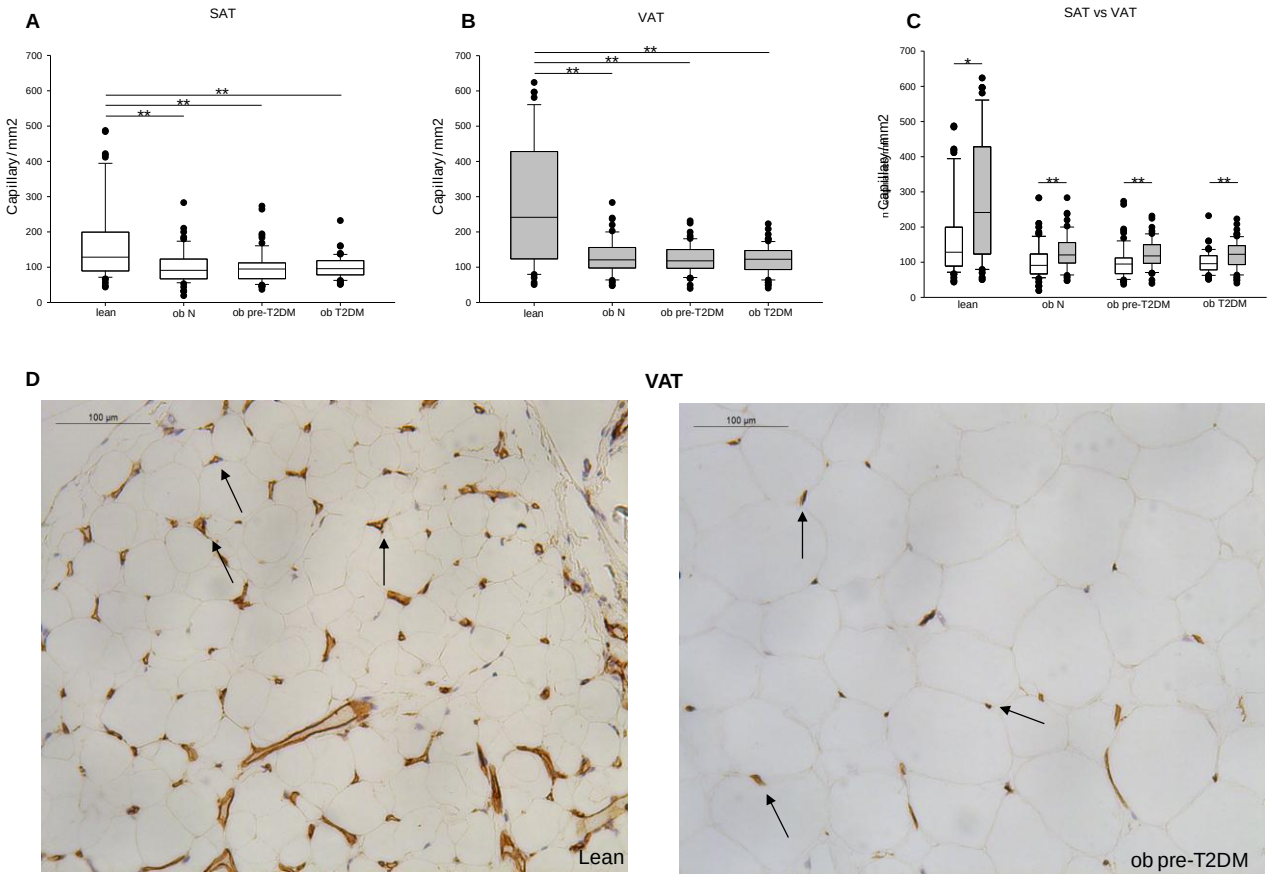


Figure 12. Capillary density (n° capillaries/mm^2) in subcutaneous, A, (SAT) and visceral, B, (VAT) adipose tissue in the 4 groups of patients: lean (n=6), obese normoglycemic (ob N=5), obese pre-diabetic (ob pre-T2DM= 5) and obese diabetic (ob T2DM=5) (**p<0,001). C, capillary density in SAT (indicated by white box) vs VAT (indicated by grey box) in the 4 groups of patients (*p <0, 01; **p<0,001). Representative VAT (D) sections from lean and ob pre-T2DM subjects stained with Monoclonal Mouse Anti-Human CD31, Clone JC70A. Capillary density was measured in at least 10 random fields at 20X magnification (at least 200 random adipocytes per tissue biopsy were counted). The value for each patient was the average number of lumens per field. Results are reported as box plot graph with median, minimum and maximum values; statistical analysis was performed by Mann-Whitney test.

Moreover, we found that VAT is more vascularized than SAT both in lean and in obese patients ($p < 0, 01$ for lean, $p < 0,001$ for obese). An interesting data to be emphasized is that, while the number of vessels per mm^2 decreased of 40% between lean and obese SAT, the parallel reduction resulted higher in VAT, amounting to the 54, 5%.

To further explore TA vascularization we calculated the number of capillaries per adipocyte and we observed that both in SAT than in VAT lean group displayed a ratio slightly higher that obese groups; moreover there was an increasing trend in this ratio in obese patients with increasing metabolic complications. Indeed, in SAT the number of vessels per adipocyte was significantly higher in ob pre-T2DM and in ob T2DM compared to ob N ($p< 0, 0001$), while no differences were noted between ob pre-T2DM and ob T2DM. Similarly in VAT there was a higher number of vessels per adipocyte in ob pre-T2DM ($p<0, 05$) and in ob T2DM ($p<0, 0001$) compared to ob N. Moreover, there was a significant difference also between ob pre-T2DM an ob T2DM ($p<0, 05$).

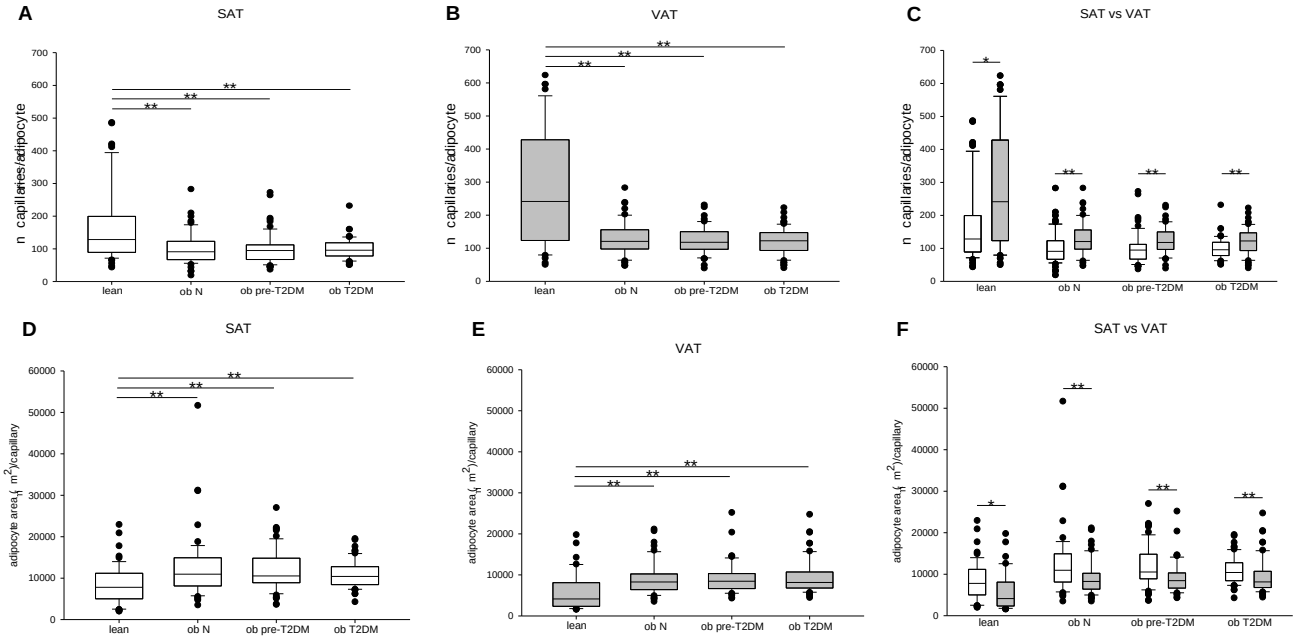


Figure 13. Number of capillaries per adipocyte in subcutaneous, A, (SAT) and visceral, B, (VAT) adipose tissue in the 4 groups of patients: lean (n=6), obese normoglycemic (ob N=5), obese pre-diabetic (ob pre-T2DM= 5) and obese diabetic (ob T2DM=5) (*p<0, 05; **p<0, 01; ***p<0,001). C, number of vessels per adipocyte in SAT (indicated by white box) vs VAT (indicated by grey box) in the 4 groups of patients. **Median adipocyte area supplied by a single capillary** in SAT, D, and VAT, E, in the 4 groups of patients: lean (n=6), ob N (n=5), ob pre-T2DM (n= 5) and ob T2DM (n=5) (**p<0,001). F, number of vessels per adipocyte in SAT (indicated by white box) vs VAT (indicated by grey box) in the 4 groups of patients (*p<0, 01; **p<0,001). Results are reported as box plot graph with median, minimum and maximum values; statistical analysis was performed by Mann-Whitney test.

Lean patients showed an elevated ratio (vessels /adipocyte) both in SAT and in VAT in comparison with ob N group (p < 0, 01) whereas no differences were found with other obese groups. No significantly differences appeared comparing SAT and VAT depots. Finally, we calculated the median adipocyte area supplied by a single capillary and we did not find significantly differences in the 3 obese groups, whereas both in SAT and in VAT of lean patients a single capillary vessel supplies a smaller adipocyte area compared to obese patients (p < 0,001) (Figure 13). When SAT and VAT samples were compare in each group, we observed that a single capillary vessel supplies a higher adipocyte area in SAT than in VAT (p < 0, 01 for lean and p < 0,001 for obese). Median adipocyte size was positive correlated with BMI (*R* = 0,7 in SAT; *R* = 0,5 in VAT; p<0,05) and IL-6 (*R* = 0,5 in SAT; *R* = 0,6 in VAT; p<0,05) both in SAT and in VAT; with waist in SAT (*R* = 0,7; p<0,05) and in VAT, median adipocyte size, negatively correlated with capillary density (*R* = -0,6; p<0,05) as showed in Figure 14.

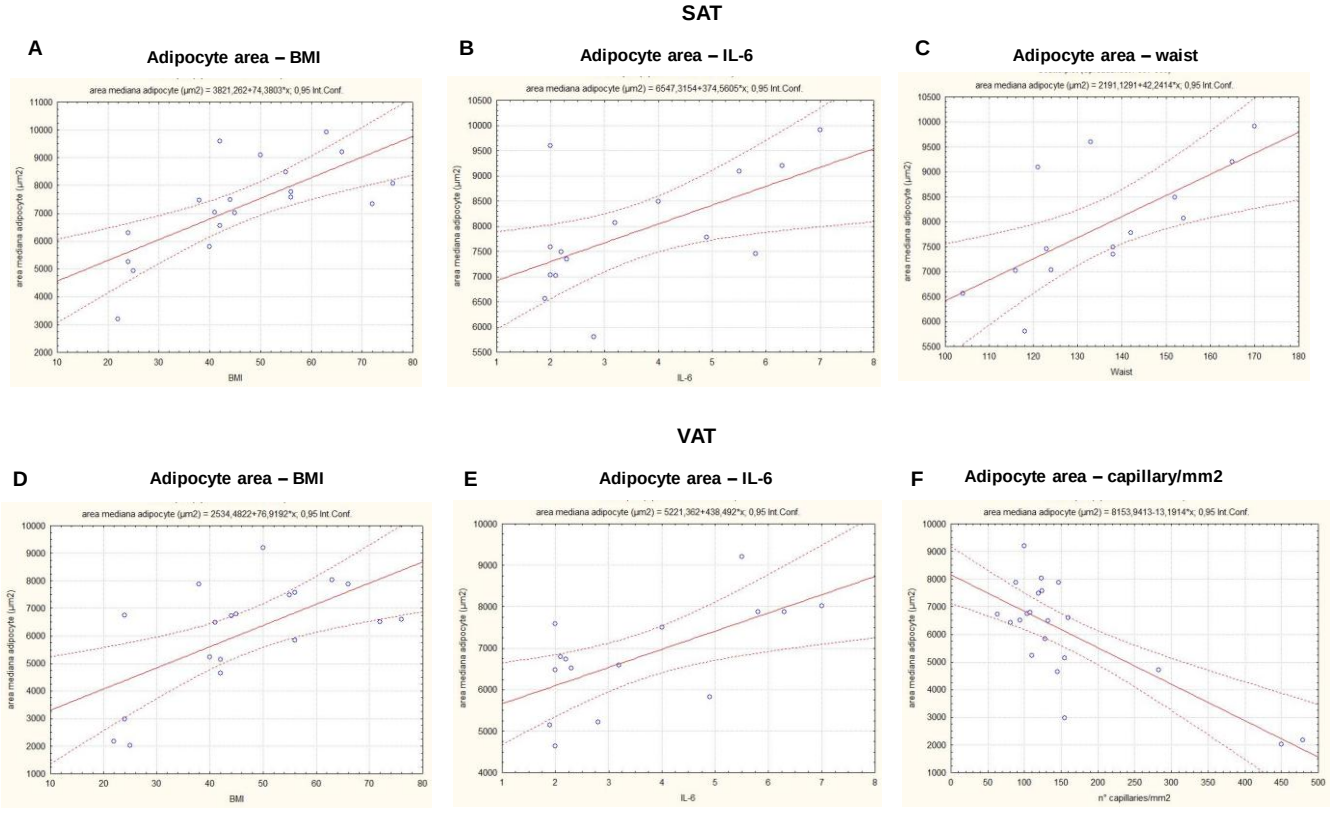


Figure 14. Correlation between adipocyte area and clinical parameters. Median adipocytes area positively correlated with BMI and IL-6 both in subcutaneous (SAT) (*R=0, 7* and *R= 0, 5* respectively), A-B, and in visceral (VAT) (*R=0, 5* and *R=0, 6* respectively), D-E, adipose tissue. In SAT median adipocyte area positively correlated with waist, C, (*R=0, 7*). In VAT median adipocyte area was inversely correlated with capillary density, E, (*R=-0, 6*). Spearman's rank correlation was performed and a value of p<0,05 was considered significant.

In conclusion, we showed that adipocytes area is smaller in lean than in obese patients both in SAT than in VAT. In obese patients, there is an increasing adipocytes area that parallel the worsening of metabolic conditions. Moreover, VAT resulted more vascularized, quantifying the number of capillaries, compared to SAT. Both SAT and VAT of lean subjects appeared more vascularized than AT depots from obese subjects. In obese patients the capillary density and the median adipocytes area supply by a single vessel did not differ if we considered T2DM progression among groups. In these 3 groups the only difference, regarding vascularization, consists in the ratio number of capillary per adipocyte that increases with T2DM progression in both depots.

5.1.3 Flow Cytofluorimetric Analysis of Stromal Vascular Fraction

SAT samples of 24 ob N, 18 ob pre-T2DM, 23 ob T2DM, 5 lean normal weight and normoglycemic subjects (18.5 < BMI < 24,9 kg/m2) and 17 obese patients underwent to a relevant weight loss (ob WL), corresponding to at least 10% of body weight, were used to isolate SVF and to perform multiparameter flow cytometry.

	ob N (n = 24)	**ob pre-T2DM (n = 18)**	**ob T2DM (n = 23)**	**p ob N vs ob pre-T2DM**	**p ob N vs ob T2DM**	**p ob pre-T2DM vs ob T2DM**
Sex (F/M)	19/6	15/3	15/9			
Age (years)	40 ±11	47 ± 11	52 ± 11	<0,05	<0,001	ns
HYP (n/tot goup)	13/24	9/18	21/23			
DLP (n/tot goup)	13/24	11/18	20/23			
OSAS (n/tot goup)	3/24	7/18	11/23			
BMI (Kg/m^2)	48 ± 10	48 ± 8	49 ± 9	ns	ns	ns
Waist(cm)	131 ± 15	135 ± 12	138 ± 16	ns	ns	ns
Blood Glucose (mmol/l)	4,9 ± 0,4	5,9 ± 0,7	8,8 ± 2,8	<0,00001	<0,0000	<0,0001
Insulin (mU/l)	17 ± 12	32 ± 27	-	<0,05	-	-
HOMA-IR	3,7 ± 2,8	8,7 ± 7,8	-	<0,01	-	-
HbA1c (mmol/mol)	-	-	63 ± 17	-	-	-
T-CHL (mg/dl)	188 ± 28	201 ± 34	193 ± 51	ns	ns	ns
HDL (mg/dl)	45 ± 12	48 ± 11	42 ± 12	ns	ns	ns
LDL (mg/dl)	118 ± 28	124 ± 33	121 ± 42	ns	ns	ns
TGL (mg/dl)	135 ± 69	154 ± 87	175 ± 82	ns	ns	ns
hsPCR (mg/l)	5,7 ± 3,7	8,1 ± 4,9	8,2 ± 2,5	ns	<0,05	ns
TNF-α (ng/l)	8,5 ± 2,3	8,5 ± 2,7	10,3 ± 3	ns	<0,05	ns
IL-6 (ng/l)	3,3 ± 1,5	3,7 ± 1,5	4 ± 2,5	ns	ns	ns
Leptin (ug/l)	41 ± 16	42 ± 13	39 ± 17	ns	ns	ns

Table VIII. Demographic, anthropometrics and metabolic parameters of obese patients analyzed by flow cytometry of SAT biopsies. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. T-CHL: total cholesterol. HDL: High Density Lipoproteins. LDL: Low Density Lipoproteins. TGL: triglycerides. hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

VAT samples of 25 ob N, 25 ob pre-T2DM, 30 ob T2DM and of 10 lean normal weight and normoglycemic subjects (18.5 < BMI < 24, 9 kg/m2) subjects were used to isolate SVF and to perform multiparameter flow cytometry. Main demographic, anthropometrics and metabolic parameters of obese groups are reported in Tables VIII and IX.

	ob N **(n = 25)**	**ob pre-T2DM** **(n = 25)**	**ob T2DM** **(n = 30)**	**p** **ob N vs ob pre-T2DM**	**p** **ob N vs ob T2DM**	**p** **ob pre-T2DM vs ob T2DM**
Sex (F/M)	19/6	15/3	15/9			
Age (years)	38 ±11	47 ± 11	52 ± 10	<0,05	<0,00001	ns
HYP (n/tot goup)	13/25	9/25	21/30			
DLP (n/tot goup)	13/25	11/25	20/30			
OSAS (n/tot goup)	3/25	7/25	11/30			
BMI (Kg/m^2)	48 ± 10	49 ± 9	49 ± 9	ns	ns	ns
Waist(cm)	130 ± 14	135 ± 11	136 ± 16	ns	ns	ns
Blood Glucose (mmol/l)	4,9 ± 0,4	5,8 ± 0,6	9,8 ± 4,3	<0,00000	<0,0000	<0,00001
Insulin (mU/l)	17 ± 11	30 ± 24	-	<0,05	-	-
HOMA-IR	3,7 ± 2,5	7,9 ± 6,9	-	<0,005	-	-
HbA1c (mmol/mol)	-	-	66 ± 20	-	-	-
T-CHL (mg/dl)	185 ± 28	196 ± 40	194 ± 46	ns	ns	ns
HDL (mg/dl)	45 ± 11	48 ± 12	42 ± 13	ns	ns	ns
LDL (mg/dl)	115 ± 28	122 ± 43	122 ± 39	ns	ns	ns
TGL (mg/dl)	130 ± 66	153 ± 97	176 ± 81	ns	<0,05	ns
hsPCR (mg/l)	10 ± 21	8,2 ± 5,3	7,9 ± 2,8	ns	ns	ns
TNF-α (ng/l)	8,6 ± 3,3	8,6 ± 3,3	9,8 ± 3	ns	ns	ns
IL-6 (ng/l)	3,7 ± 2,8	3,5 ± 1,3	4,2 ± 2,6	ns	ns	ns
Leptin (ug/l)	41 ± 15	44 ± 16	37 ± 16	ns	ns	ns

Table IX. Demographic, anthropometrics and metabolic parameters of obese patients analyzed by flow cytometry of VAT biopsies. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. T-CHL: total cholesterol. HDL: High Density Lipoproteins. LDL: Low Density Lipoproteins. TGL: triglycerides. hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

5.1.3.1 *Glucose Impairment Early Affects Adipose Stem Cells in Obesity.*

SAT and VAT derived adipose stem cells (ASCs) were quantified *ex vivo* as CD34+CD31-CD45- cells by FACS analysis in normal weight lean subjects, ob N, ob pre-T2DM and ob T2DM patients. Freshly extracted SVFs obtained from AT specimens were analyzed and on the basis of forward/side scatter morphological gate was considered in order to exclude cellular fragments, aggregates and immune cells, and cell positive for CD45 staining (Figure 15).

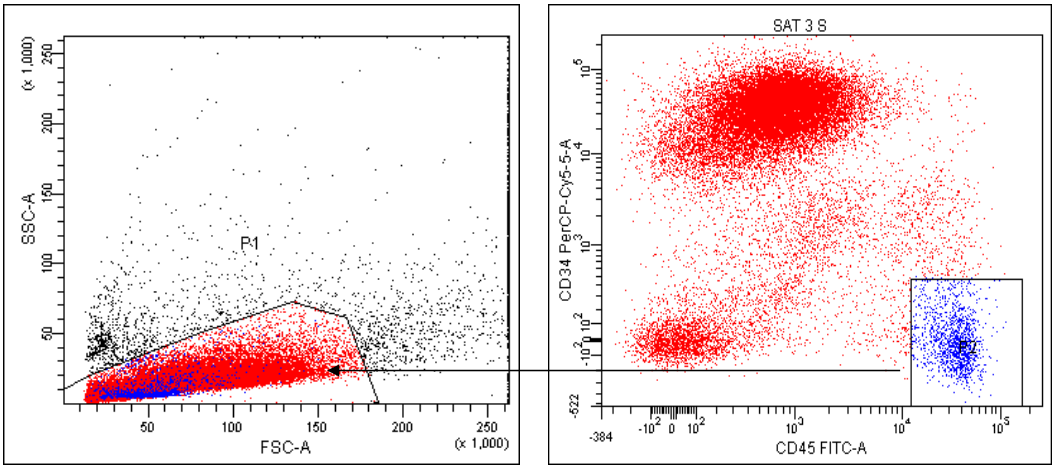


Figure 15. Dot plot of FSC (forward scatter) against SSC (side scatter) on a linear scale. Morphologic localization of CD34 + and CD45 + cells.

As shown in Figure 16, SAT of ob N patients appears to be enriched of ASCs in comparison with lean subjects, although the high variability of data from normal weight controls. SAT ASCs obtained from ob pre-T2DM and ob T2DM patients were lower than those obtained from ob N patients (respectively 28.9 % (10.1-43.9%), 31.2% (10.5-44.7%) vs 40.6% (19-78.7%), p<0.05). Interestingly, after significant weight loss SAT further increases its content of adipogenic precursors (62.4% 46.7-87.6%) in comparison with both lean subjects (11.5% 0.3-47.3%; p<0, 05) and obese patients (p<0,001). Consensually in VAT a clear decreasing trend of ASCs number in ob pre-T2DM and ob T2DM in respect to ob N patients was observed (p<0,001). In all obese patients VAT displays a significantly higher number of ASCs in comparison with control (Ob N 47.4% (19.1-64%), Ob preT2DM 42% (21.6-58.9%) vs lean 29.4% (9.6-40.7%); p<0,001; Ob T2DM 39.6% (25.1-59.2%) vs lean 29.4% (9.6-40.7%); p<0.05); it is worth noting that control group has a high sample number (n=10) and a relatively low variability: VAT collection is easier in normal weight patients and provides a higher amount of tissue in comparison with SAT. Comparing the two deposits, VAT contains a higher number of ASCs in respect of SAT in all obese groups while controls do not present differences; moreover it is to underlie the major decrease of ASCs number of ob pre-T2DM and ob T2DM in respect to ob N patients in SAT (ob pre T2DM vs ob N reduction of 28.8%; ob T2DM vs ob N reduction of 23.2%) than in VAT (ob pre T2DM vs ob N reduction of 11.4%; ob T2DM vs ob N reduction of 16.5%).

Even if ob T2DM and ob pre T2DM were older than ob N, we didn't found any correlation between ASCs and age nor in SAT neither in VAT (data not show). Moreover, adipogenic

precursors were not correlated with anyone of anthropometric and metabolic parameters considered in our population.

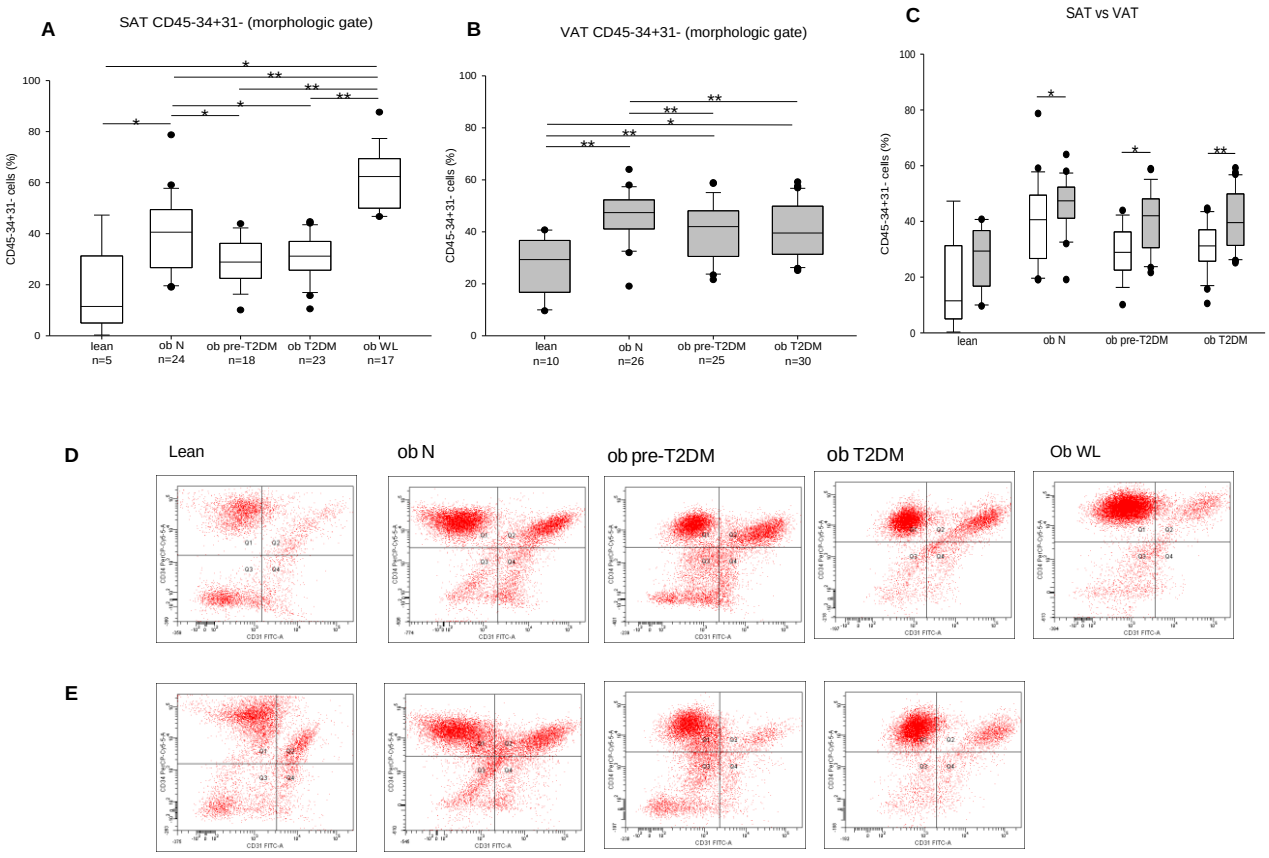


Figure 16. Median percentage of CD45-CD34+CD31- in subcutaneous, A, (SAT) and visceral, B, (VAT) adipose tissue in the 5 groups of patients: lean, obese normoglycemic (ob N), obese pre-diabetic (ob pre-T2DM), obese diabetic (ob T2DM) and, only in SAT, obese after weight loss (ob WL), (*p<0, 05; **p<0,001). C, Median percentage of CD45-CD34+CD31- in SAT (indicated by white box) vs VAT (indicated by grey box) in the first 4 groups of patients. Representative SAT (D) and VAT (E) dot plots of analysis of CD34-31-, CD34-31+, CD34+31-, CD34+31+ immunologic subpopulations from lean, ob N, ob pre-T2DM, ob T2DM and ob WL subjects. Numbers of AT samples analyzed per each group are reported under every box. Results are reported as box plot graph with median, minimum and maximum values; statistical analysis was performed by Mann-Whitney test.

ASCs phenotype was also analyzed in the CD34+ CD31- immunological gate. CD90, CD73 and CD44 result similarly expressed by ASCs of obese patients (see Table X). A greater percentage of SAT derived ASCs express CD90 than VAT in all groups.

CD105 is a mesenchymal marker that is believed to be always expressed by mesenchymal stem cells (MSCs). Differently, only 30-70% of ASCs both in lean and obese subjects expresses CD105 without differences among groups (Figure 17, A-B-C). In obese but not in lean subjects VAT ASCs display a significantly higher expression of CD105 in comparison with SAT.

CD271 represents an additional marker used to characterize a subpopulation of MSC in bone marrow; it was evaluated only in obese patients due to the limited material available in lean subjects. ASCs from obese patients showed a high expression of CD271 (Table XI) with the major percentage in VAT (Figure 18, D-E-F).

CD34+CD31-	CD90+ % (min-max)		CD73+ % (min-max)		CD44+ % (min-max)	
	SAT	VAT	SAT	VAT	SAT	VAT
Ob N	97.7 (82.3-99.7)	95.5 (70-98.8)	97.8 (87.9-99.5)	94.3 (83.7-98.6)	94.1 (90-98.2)	93.6 (90.2-99.3)
Ob preT2D	99 (87.7-99.7)	95 (59-99.5)	96.3 (88.7-97.8)	93.9 (82.8-99.2)	94 (92.3-96.8)	88.9 (61.1-97)
Ob T2DM	91.2 (74.8-100)	95.7 (65.7-99.7)	92.9 (85.3-96.2)	93.9 (81.8-97.8)	99.1 (94.5-99.9)	93.6 (67.8-98.4)

Table X. Percentage of CD34+CD31- cells expressing the mesenchymal markers CD90, CD73 and CD44 in SAT and VAT from normoglycemic (Ob N), prediabetic (Ob preT2D) and diabetic obese patients. Data are reported as median and minimum-maximum values.

CD34+CD31-	CD105+ % (min-max)		CD271+ % (min-max)	
	SAT	VAT	SAT	VAT
Lean	42.2 (24.9-59.4)	48.9 (29.7-87.2)	-	-
Ob N	29.3 (1.8-71.9)	62.6 (6.5-88.8)	37.3 (11.6-54.3)	70.5 (47.8-92.7)
Ob preT2D	42 (4.2-76.8)	63.4 (32.3-88.4)	42.3 (22.7-45.5)	68.2 (38.7-96.7)
Ob T2DM	29.7 (2.7-75.9)	66.8 (7.9-79.2)	28.9 (8.5-60.1)	55.1 (23.1-80.4)
Ob WL	20.9 (4.5-60.1)	-	28.3 (7.5-55.9)	-

Table XI. Percentage of CD34+CD31- cells expressing the mesenchymal markers CD105 and CD271 in SAT and VAT from lean, normoglycemic (ob N), prediabetic (ob preT2D) and diabetic obese patients (ob T2DM). SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue. Data are reported as median and minimum-maximum values.

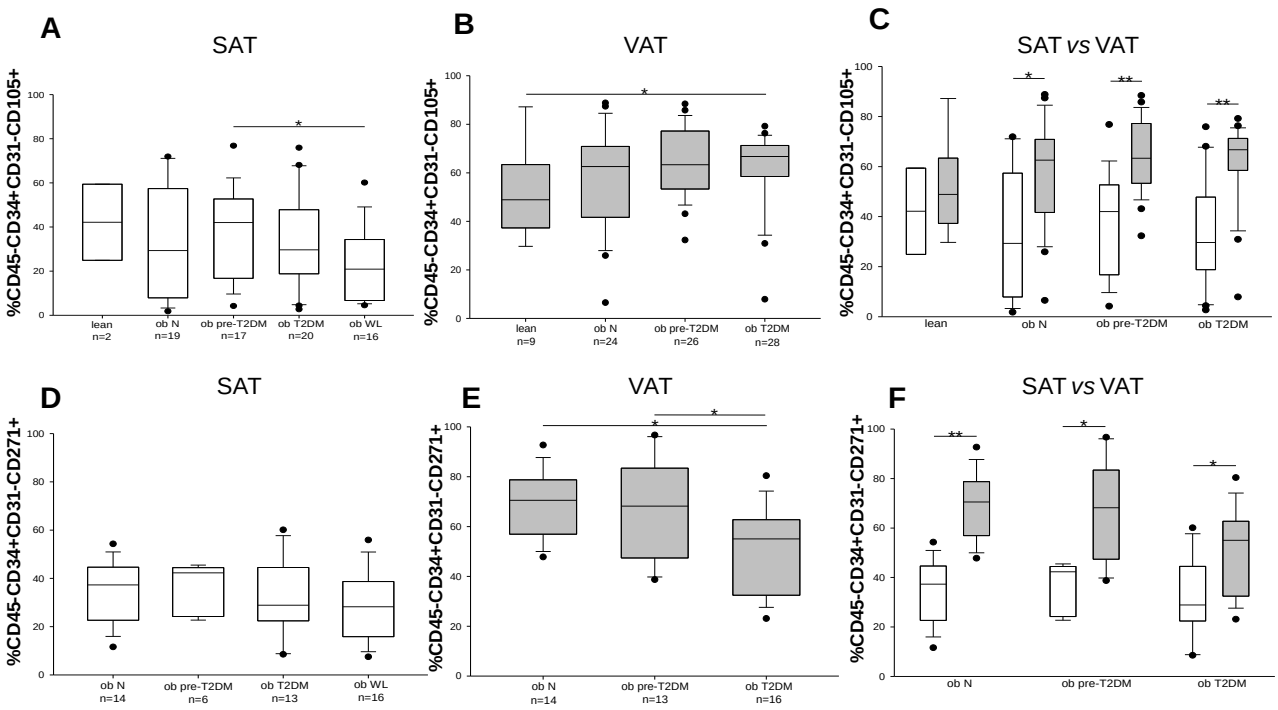


Figure 17. Median percentage of CD34+CD31- cells expressing CD105+ in subcutaneous, A, (SAT) and visceral, B, (VAT) adipose tissue in the 5 groups of patients: lean, obese normoglycemic (ob N), obese pre-diabetic (ob pre-T2DM), obese diabetic (ob T2DM) and, only in SAT, obese after weight loss (ob WL), (*p<0, 05). C, Median percentage of CD34+CD31- cells expressing CD105+ in SAT (indicated by white box) vs VAT (indicated by grey box) in the first 4 groups of patients (*p<0,05; **p<0,001). **Median percentage of CD34+CD31- cells expressing CD271**+ in SAT, D, and VAT, E in the 4 groups of patients: ob N, ob pre-T2DM, ob T2DM and, only in SAT, ob WL (*p<0,05). F, Median percentage of CD34+CD31- cells expressing CD271+ in SAT (indicated by white box) vs VAT (indicated by grey box) in the first 3 groups of patients (*p<0,05; **p<0,001). Numbers of AT samples analyzed per each group are reported under every box. Results are reported as box plot graph with median, minimum and maximum values; Mann-Whitney test was performed.

A deep debate exists about the relationship between ASCs and pericytes in term of a common or distinct origin (Cai, 2011; Zimmerlin, 2012. For this reason CD146, commonly used as pericyte marker, has been also evaluated *ex vivo* in SFVs. This subpopulation represents a relatively high fraction of the AT SVF and the majority of the cells positive for CD146 stains negatively for CD34 and CD31. In VAT CD146+ cells display a similar behavior of ASCs being significantly higher in all obese patients with a decreasing trend within the 3 groups (Lean 1% (0.4-3.5); ob N 5.2% (1.7-13.7%); ob pre-T2DM 3.05% (1.3-7.6); ob T2DM 1.7% (0.8-8.2)). In SAT CD146+ cells have been quantified in a smaller number of obese subjects and not in control due to the low sample amount. In this depot we do not show differences among obese groups and in weight loss group (ob N 6.6% (19.3-2.6%); ob pre-T2DM 10.8% (3.2-11.8); ob T2DM 7.2% (2.6-11.5); Ob WL 6.5% (1-11.5)). Comparing the two AT depots,

SAT is enriched of CD146+ cells in respect of VAT in all obese subjects, similarly to endothelial precursors (see below) (Figure 18).

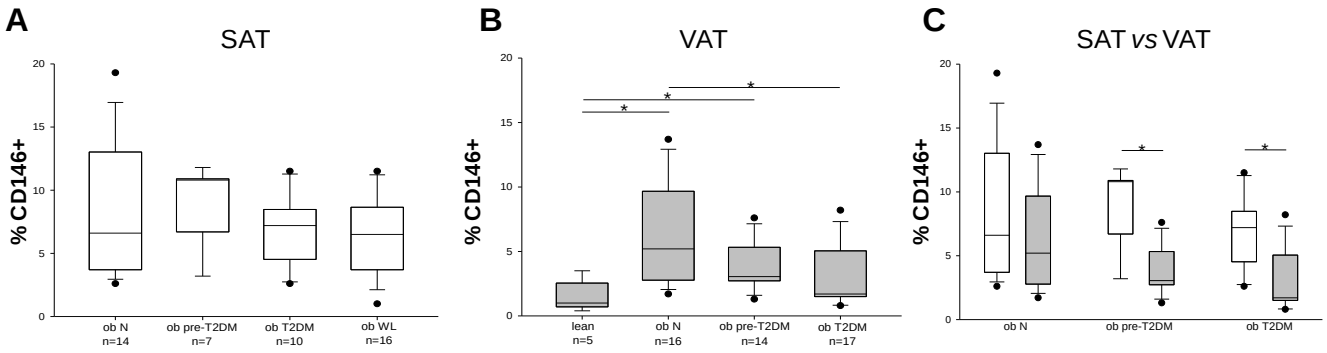


Figure 18. Median percentage of CD146+ in subcutaneous adipose tissue (SAT), A, in obese normoglycemic (ob N), obese pre-diabetic (ob pre-T2DM), obese diabetic (ob T2DM) and obese after weight loss (ob WL) patients, and visceral adipose tissue(VAT), B, in lean, ob N, ob pre-T2DM and ob T2DM (*p<0,05). C, Median percentage of CD146+ in SAT (indicated by white box) vs VAT (indicated by grey box) in the 3 groups of obese patients. Numbers of AT samples analyzed per each group are reported under every box. Results are reported as box plot graph with median, minimum and maximum values; Mann-Whitney test was performed.

5.1.3.2 *Endothelial Progenitors are progressively altered by Obesity and Overt Diabetes*.

Endothelial precursors cells (EPs) were defined as CD34+ CD31+ CD45- cells. In SAT, ob N EPs number increased in respect of lean subjects and was similar in ob pre-T2DM (Lean: 5.8% (0.6-15.5%); Ob N 17.4% (3-55.8%); Ob preT2DM 24% (4.3-41.1%); Ob T2DM 15.9% (0.8-29.6%); Ob WL 9.5% (3.8-28.5%)), differently from what seen with ASCs percentage.

With overt diabetes (patients from ob T2DM group) EPs decreased significantly in comparison with ob pre-T2DM. Interestingly after weight loss EPs revert to lean control level. In VAT EPs displayed the same trend of SAT-extracted precursors, although for VAT we do not have the possibility to analyze weight loss effect.

A part from lean subjects, in all obese patients SAT shows a significantly higher angiogenetic potential than VAT as indicated by EPs quantification (Figure 19).

EPs percentage inversely correlated with age, both in SAT than in VAT (R = -0,2; R= - 0,3 respectively; $p \leq 0,05$) and positively correlated with BMI only in SAT (R = 0,3 R= - 0,3 respectively; $p \leq 0,05$). No other statistically significant correlation was found between EPs percentage and anthropometric and metabolic parameters considered.

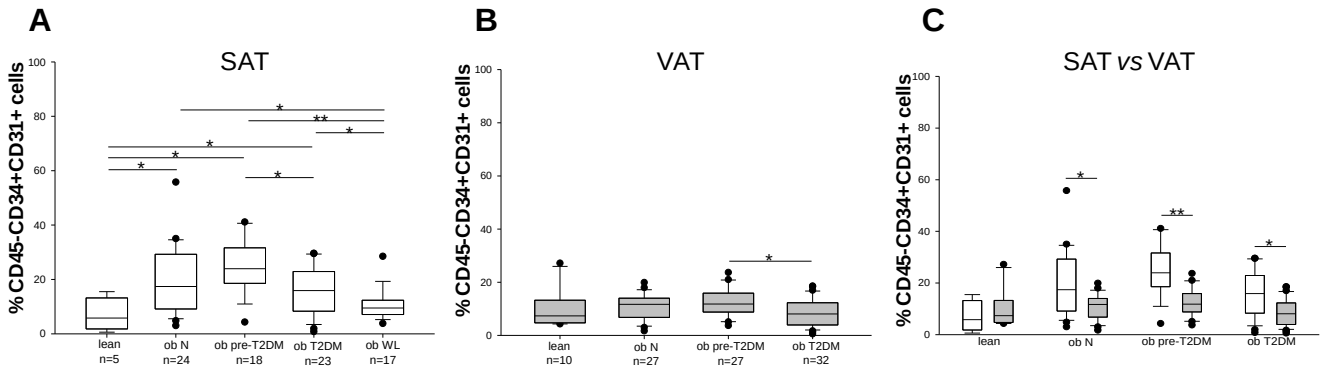


Figure 19. Median percentage of CD45-34+31+ in subcutaneus, A, (SAT) and visceral, B, (VAT) adipose tissue in the 5 groups of patients: lean, obese normoglycemic (ob N), obese pre-diabetic (ob pre-T2DM), obese diabetic (ob T2DM) and, only in SAT, obese after weight loss (ob WL), (*p<0, 05; **p<0,001). C, Median percentage of CD45-CD34+CD31+ in SAT (indicated by white box) vs VAT (indicated by grey box) in the first 4 groups of patients (*p<0, 05; **p<0,001). Numbers of AT samples analyzed per each group are reported under every box. Results are reported as box plot graph with median, minimum and maximum values; statistical analysis was performed by Mann-Whitney test.

5.1.4 *In vitro* Evaluation of Adipogenic Potential of Stromal Vascular Fraction Obtained from Adipose Tissue depots.

In vitro capacity to differentiate towards the adipogenic lineage was analyzed in the 3 obese groups studied (in SAT: ob N=18; ob preT2DM=15; ob T2DM=14. In VAT: ob N=20; ob preT2DM=25; ob T2DM=23). Main demographic, anthropometrics and metabolic parameters of obese groups are reported in Tables XII and XIII.

Freshly isolated SVFs were cultured in adipogenic medium and after 9 days of culture the percentage of mature adipocytes cells containing large lipid droplets was measured by optical microscope analysis.

SVFs obtained from SAT of ob preT2DM and, unless in a minor extent, ob T2DM derived ASCs display a lower adipogenic potential in comparison to ob N group (median value: 66%, range 25-99% in ob N; 40%, range 12-80% in ob pre-T2DM; 55%, range 12-98% in ob T2DM) (Figure 20).

SVFs isolated from VAT display a lower adipogenic potential in culture conditions used which were not able to disclose relevant differences among the obese groups, despite the high number of obese patients studied (median value: 15%, range 5-70% in lean; 21%, range 5-70% in ob N; 22%, range 2-85% in ob pre-T2DM; 22%, range 1-85% in ob T2DM) .

	ob N (n = 18)	ob pre-T2DM (n = 15)	ob T2DM (n = 14)	p ob N vs ob pre-T2DM	p ob N vs ob T2DM	p ob pre-T2DM vs ob T2DM
Sex (F/M)	15/5	13/2	9/5			
Age (years)	38 ±11	46 ± 10	48 ± 10	<0,05	<0,01	ns
HYP (n/tot goup)	6/18	6/15	13/14			
DLP (n/tot goup)	11/18	8/15	13/14			
OSAS (n/tot goup)	1/18	4/15	5/14			
BMI (Kg/m^2)	49 ± 12	50 ± 9	52 ± 7	ns	ns	ns
Waist(cm)	129 ± 15	135 ± 14	144 ± 14	ns	<0,05	ns
Blood Glucose (mmol/l)	4,9 ± 0,4	5,8 ± 0,7	11,6 ± 5,5	<0,00001	<0,00001	<0,005
Insulin (mU/l)	18 ± 12	36 ± 26	-	<0,01	-	-
HOMA-IR	4,1 ± 2,9	9,6 ± 7,7	-	<0,01	-	-
HbA1c (mmol/mol)	-	-	71 ± 24	-	-	-
T-CHL (mg/dl)	187 ± 30	197 ± 27	193 ± 49	ns	ns	ns
HDL (mg/dl)	45 ± 13	48 ± 11	41 ± 14	ns	ns	ns
LDL (mg/dl)	119 ± 30	121 ± 31	124 ± 43	ns	ns	ns
TGL (mg/dl)	138 ± 77	155 ± 80	190 ± 79	ns	<0,01	ns
hsPCR (mg/l)	11 ± 25	7 ± 3,4	8,3 ± 3	ns	<0,01	ns
TNF-α (ng/l)	8,3 ± 2,7	8,9 ± 3,7	16,3± 24,5	ns	ns	ns
IL-6 (ng/l)	2,8 ± 1	4 ± 1,2	11,5 ± 25	<0,01	ns	ns
Leptin (ug/l)	40 ± 17	46 ± 17	39 ± 15	ns	ns	ns

Table. XII. Demographic, anthropometrics and metabolic parameters of obese patients in witch SAT samples were collected for i*n vitro* evaluation of adipogenic potential. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. T-CHL: total cholesterol. HDL: High Density Lipoproteins. LDL: Low Density Lipoproteins. TGL: triglycerides. hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

	ob N (n = 20)	ob pre-T2DM (n = 25)	ob T2DM (n = 23)	p ob N vs ob pre-T2DM	p ob N vs ob T2DM	p ob pre-T2DM vs ob T2DM
Sex (F/M)	17/3	21/4	13/10			
Age (years)	42 ±8	47 ± 10	52 ± 10	ns	<0,001	ns
HYP (n/tot goup)	7/20	11/25	21/23			
DLP (n/tot goup)	11/20	14/25	20/23			
OSAS (n/tot goup)	2/20	7/25	10/23			
BMI (Kg/m^2)	49 ± 11	48 ± 8	50 ± 9	ns	ns	ns
Waist(cm)	128 ± 15	133 ± 12	138 ± 18	ns	ns	ns
Blood Glucose (mmol/l)	5,0 ± 0,3	5,7 ± 0,6	10,1 ± 4,7	<0,0001	<0,0001	<0,001
Insulin (mU/l)	17 ± 11,5	30 ± 24	-	<0,05	-	-
HOMA-IR	3,9 ± 2,8	7,8 ± 6,9	-	<0,05	-	-
HbA1c (mmol/mol)	-	-	63,3 ± 22	-	-	-
T-CHL (mg/dl)	191 ± 28	202 ± 40	192± 48	ns	ns	ns
HDL (mg/dl)	48 ± 9	49 ± 12	40 ± 12	ns	<0,05	<0,05
LDL (mg/dl)	123 ± 24	127 ± 45	120 ± 40	ns	ns	ns
TGL (mg/dl)	117 ± 46	159 ± 96	171 ± 73	ns	<0,01	ns
hsPCR (mg/l)	5,3 ± 3,5	8 ± 4,5	8,1 ± 2,8	<0,05	<0,01	ns
TNF-α (ng/l)	7,7 ± 2,5	8,3 ± 3	9,7 ± 3	ns	<0,05	ns
IL-6 (ng/l)	2,9 ± 1,2	3,5 ± 1,1	4,6 ± 2,5	<0,01	<0,01	ns
Leptin (ug/l)	41 ± 17	43 ± 16	38 ± 16	ns	ns	ns

Table XIII. Demographic, anthropometrics and metabolic parameters of obese patients in witch VAT samples were collected for i*n vitro* evaluation of adipogenic potential. BMI: Body Mass Index. Hyp: blood hypertension. DLP: dyslipidemia. OSAS: obstructive sleep apnea syndrome. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. T-CHL: total cholesterol. HDL: High Density Lipoproteins. LDL: Low Density Lipoproteins. TGL: triglycerides. hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Results were reported as means ± DS; statistical analysis was performed by paired Student's t-test.

Percentage of mature adipocyte cells, after 9 days of culture inversely correlated with IL-6 both in SAT and in VAT (R = -0,3 in SAT and in VAT; $p \leq 0,05$) No other statistically significant correlation was found between EPs percentage and anthropometric and metabolic parameters considered.

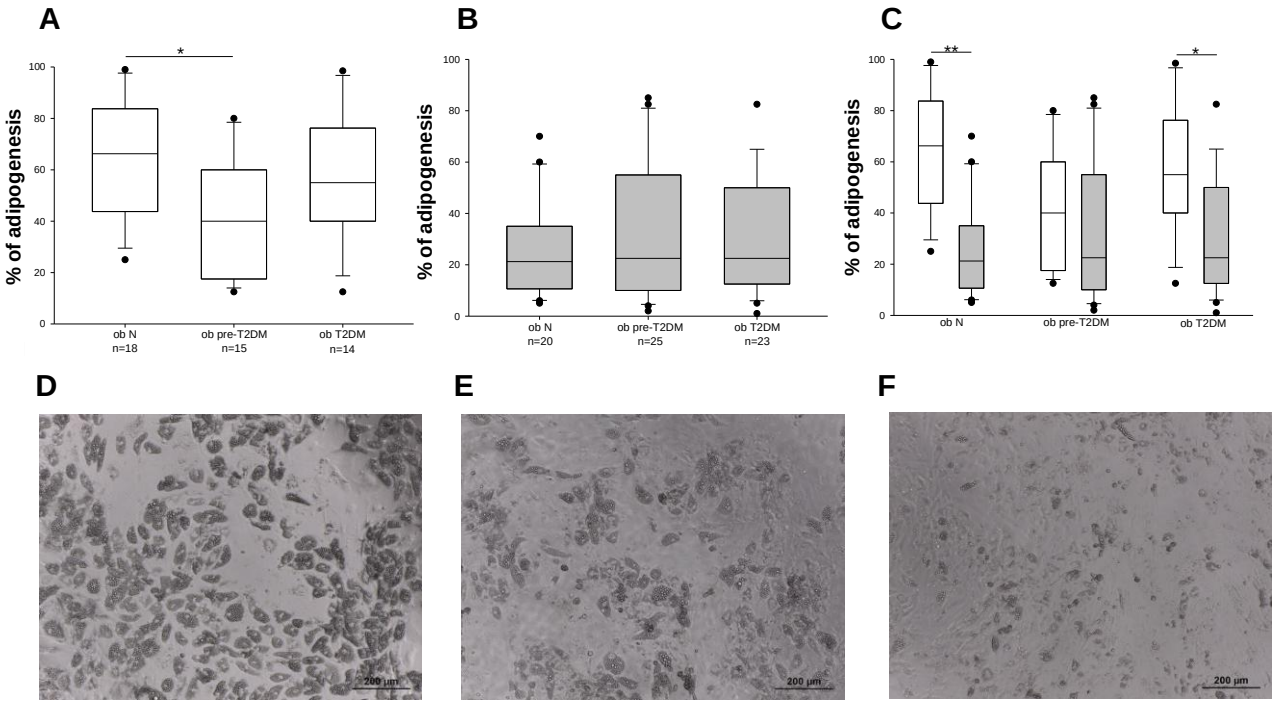


Figure 20. ***In vitro evaluation of adipogenic potential of stromal vascular fraction cells in obesity*.** Freshly isolated SVFs from SAT (A) and VAT (B) of the indicated groups were grown in adipogenic medium and after 9 days of culture percentage of differentiated cells were evaluated by optical microscope analysis (*p<0,05). C, percentage of differentiated cells in SAT vs VAT (*p<0, 05; **p<0,001). In D), E) and F) representative images of *in vitro* differentiated mature adipocytes at optical microscope at day 9 of adipogenic culture. In D, image of *in vitro* differentiated mature adipocytes in SAT of an ob N; in E, image of *in vitro* differentiated mature adipocytes in SAT of an ob T2DM and in F image of *in vitro* differentiated mature adipocytes in VAT of an ob T2DM Ob N: normoglycemic obese (SAT n= 18, VAT n= 20), ob pre-T2DM: prediabetic obese (SAT n= 15, VAT n= 25); ob T2DM: diabetic obese (SAT n= 14, VAT n= 23). Results are reported as box plot graph with median, minimum and maximum values; statistical analysis was performed by Mann-Whitney test, * p< 0.05.

5.1.5 Gene Expression Profile by RT-Real Time PCR.

We quantified the mRNA expression of several genes in SAT and VAT biopsies obtained from lean subjects and obese patients during surgical intervention. We observed in obese patients an increased expression of *PPARγ*, the transcription factor that mainly regulates adipogenesis and controls the expression of several adipose specific genes as *Adiponectin (Adipo Q)* and *FABP4*. Both in SAT and in VAT the ob T2DM group displayed a lower *PPARγ* expression in comparison with ob N and ob pre T2DM group suggesting a further reduction of the *in vivo* adipogenic potential and hyperplastic growth. Also the ob WL group showed a lower *PPARγ* expression as compared with that of ob N and ob pre-T2DM groups. We did not observed significant differences between *PPARγ* expression in the 2 depots analyzed neither in lean nor

in obese subjects (Figure 21). *VEGFA* expression resulted higher and more heterogeneous in normal weight subjects, especially in VAT, as reported in Figure 21. No significant differences were evident among the 3 obese groups that displayed a different degree of metabolic complications. The ob WL group showed a slightly increased in *VEGFA* expression even if statistically significant only when compared with ob pre-T2DM group.

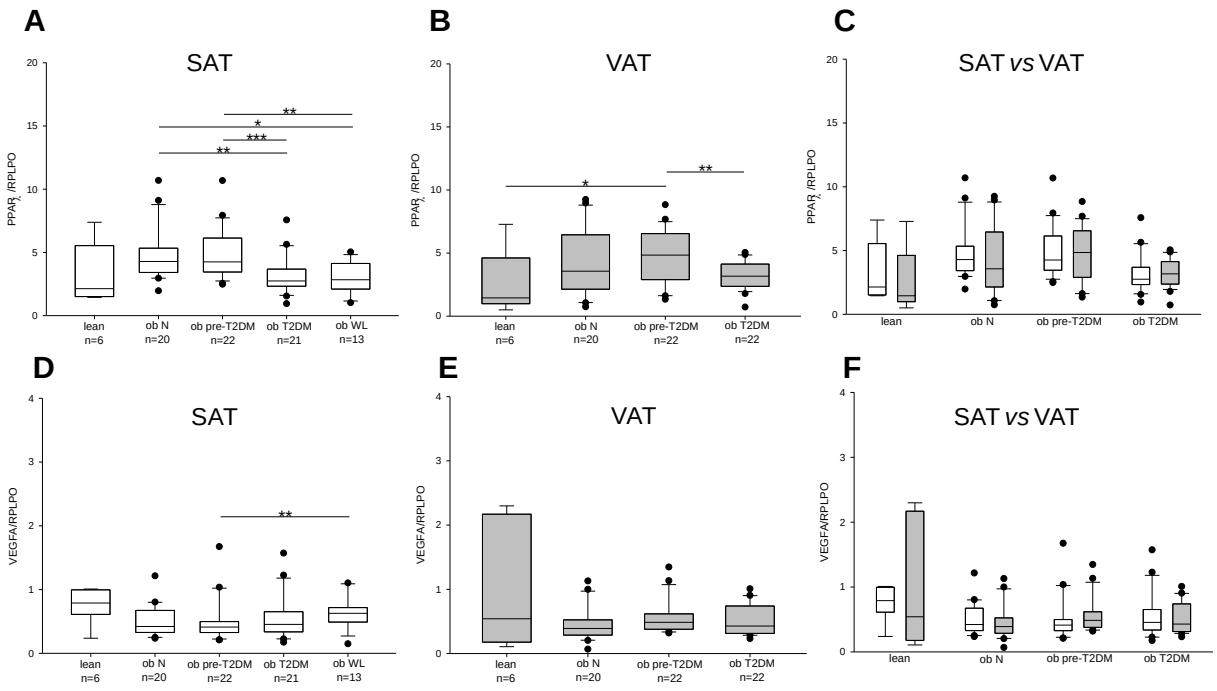


Figure 21. Gene expression profile in adipose tissue depots of lean subjects and obese patients. PPARγ (A, B, C) and VEGFA (D, E, F) expression were quantified in SAT (A, D) and VAT (B, E) biopsies of lean, obese normoglycemic (ob N), obese pre-diabetic (ob pre-T2DM), obese diabetic (ob T2DM) and obese after weight loss (ob WL) and normalized to RPLPO mRNA content. In panels C and F SAT (white plots) and VAT (grey plots) expressions were compared. The number of patients analyzed are reported in every plots (n=).Results were reported as box plot graph with median, minimum and maximum values. Statistical analysis was performed by Mann-Whitney test (*p<0,05;**p<0,01;***p<0,001).

Focused on the expression profile of the 3 obese groups in VAT, the AT depot highly vascularized, (Figure 22) we observed that ob pre T2DM and ob T2DM, even if had a similar BMI and Leptin blood level, displayed a significant higher quantity of *Leptin* mRNA than ob N group suggesting a different adipocyte function. Moreover we observed in Figure 22 a higher *VEGF-R2 (KDR)* expression in VAT of ob-pre-T2DM compared with ob N patients; this increase could represent a tentative to counteract the vasculature impairment that ob-T2DM patients were not able to further sustain. *HIF1A* displayed a similar expression profile in VAT

of obese patients correlating with its physiological role in stimulating the angiogenic processes (Figure 22).

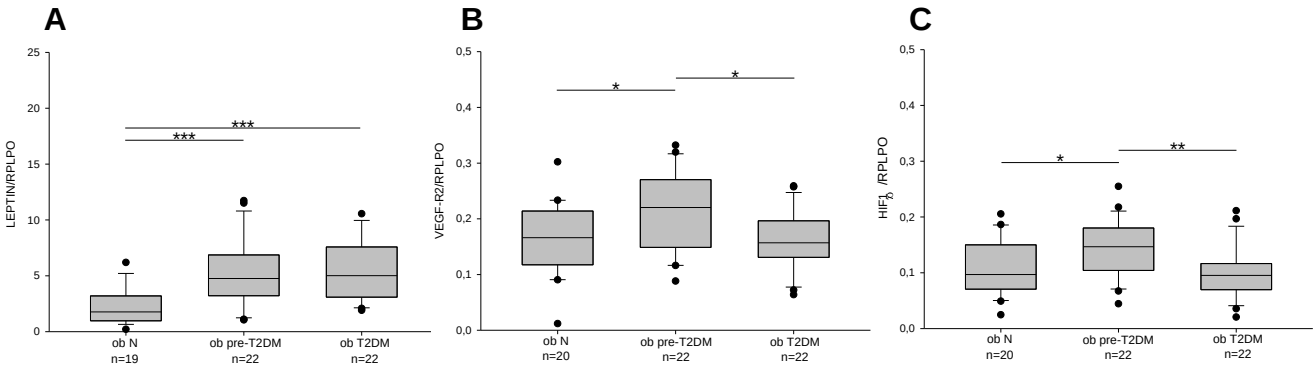


Figure 22. Gene expression profile in VAT of obese patients characterized by different metabolic complications. Leptin (A), VEGF-R2 (B) and HIF1α (C) expression were quantified in VAT biopsies of obese normoglycemic (ob N), obese pre-diabetic (ob pre-T2DM) and obese diabetic (ob T2DM) patients and normalized to RPLPO mRNA content. The number of patients analyzed are reported in every plots (n=). Results were reported as box plot graph with median, minimum and maximum values. Statistical analysis was performed by Mann-Whitney test (*p<0,05;**p<0,01;***p<0,001).

5.2 RESULTS (2)

5.2.1 Anthropometric and Metabolic Parameters one year after LSG

After surgery, 180 patients completed the OGTT. 11 patients didn't complete the test for gastric intolerance. In 6 patients the test was stopped earlier for the occurrence of a severe symptomatic hypoglycaemic event. Data analysis was performed on 180 patients who were able to complete the OGTT. Demographic, anthropometrics and metabolic parameters before and after LSG are reported in Table XIV**.**

	Before	**After**	**P value**
Age, year	43 ± 11	44 ± 11	/
Female, n., %	140 (71%)	/	/
Waist, cm	132 ± 16	103 ± 16	< 0.0001
Weight, Kg	133 ± 27	92 ± 22	< 0.0001
BMI, Kg/m^2	47.4 ± 7.3	32.7 ± 6.4	< 0.0001
%EBMIL	/	68.4 ± 22.8	/
Blood Glucose, mmol/l	5.4 ± 0.7	4.6 ± 0.4	< 0.0001
Insulin, mU/l	19.9 ± 13.6	7.6 ± 5.8	< 0.0001
HOMA-IR	4.9 ± 3.6	1.6 ± 1.4	< 0.0001
Total Cholesterol, mg/dl	189 ± 38	180 ± 35	< 0.001
HDL-Cholesterol, mg/dl	49 ± 13	58 ±14	< 0.0001
Triglycerides, mg/dl	114 ± 60	77 ± 32	<0.0001
hs-PCR, mg/l	8.9 ± 7.8	2.6 ± 3.2	<0.0001
IL-6, ng/l	3.19 ± 2.93	2.69 ± 2.5	<0.02
TNF-α, ng/l	8.51 ± 3.6	6.8 ± 3.48	<0.0001
Leptina ug/L	42 ± 15	14 ± 11	<0.0001

Table XIV Demographic, anthropometrics and metabolic parameters before and after LSG. LSG: Laparoscopic Sleeve Gastrectomy. BMI: Body Mass Index. EBMIL: Excess Body Mass Index Loss. HOMA-IR: Homeostasis Model Assessment. LDL: Low Density Lipopreteins. HDL: High Density Lipoproteins. Hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Paired Student's t-test was performed.

In all patients there was a significantly weight loss, with a %EBMIL amounting to 68.4 ± 22.8% (range 23-131%) one year after LSG. A highly significant improvement in HOMA-IR (4.9 ± 3.6 vs 1.6 ± 1.4; $p<0.0001$) and in lipid profile occurred. All inflammatory parameters (IL-6, TNF-α and hs-PCR) decreased significantly after weight loss. Glucose and insulin curves during OGTT performed before and after surgery are shown in Figure 23**.** Both

parameters and, in addition, c-peptide curve during OGTT (data not show), improved after surgery.

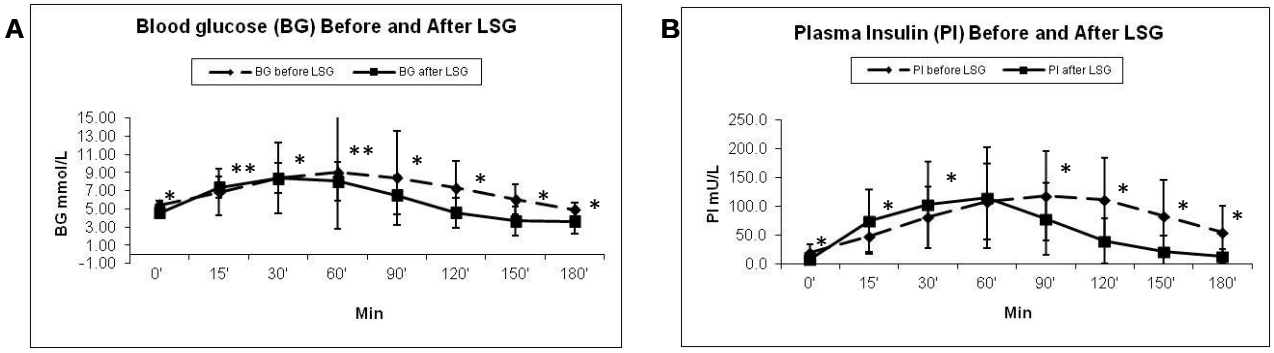


Figure 23 Glucose (A) and insulin (B) curves during OGTT performed before and after LSG. Dotted line indicates values obtained before LSG and solid line values obtained after LSG. Paired Student's t-test: *p<0.001; **p<0.05.

5.2.2 Prevalence of OGTT-related Hypoglycaemia one year after LSG

Before LSG, 2, 5% (5/197) of the patients experienced a hypoglycaemic event during the 3 hour OGTT. After surgery, the proportion of patients experiencing at least one hypoglycaemic event increased to 30, 6% (55/180) (p<0.001) (Figure 24 A), rising to 32, 8% considering the six patients in whom OGTT was suspended. After LSG, no patient had a hypoglycaemic and/or severe hypoglycaemic episode fasting and 30 minutes after glucose load. The highest frequency of hypoglycaemic events and severe hypoglycaemic events were observed 150' after OGTT, in 27/66 (40.9%) subjects and 9/66 (13.6%) subjects respectively (Figure 24 B).

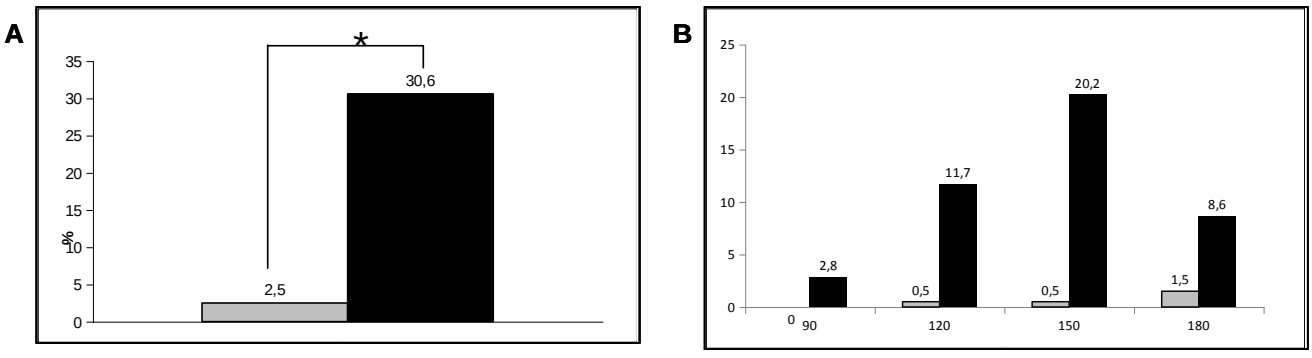


Figure 24. LSG and hypoglycaemia. Percent of patients affected by severe hypoglycaemia (A) and their distribution (B) during OGTT before (grey) and after (black) LSG. *p<0.001

5.2.3 Anthropometric and Metabolic Parameters, before and one year after LSG, in Patients With and Without Hypoglycaemic Events

We then divided our population in two groups: patients with (Hip) and without (N-Hip) a hypoglycaemic event after LSG (blood glucose levels ≤ 2.7 mmol/L). Baseline characteristics of these two groups were analysed in Table XV.

	Without	With	P value
Waist, cm	134±16	130±16	n.s.
Weight, Kg	136±29	128±22	0.05
BMI, Kg/m^2	48.4±7.9	46±5.7	0.02
Blood glucose, mmol/l	5.5±0.7	5.2±0.6	0.09
Insulin, mU/L	19.4±14.2	22.3±13.3	n.s
HOMA-IR	4.8±3.8	5.3±3.6	n.s.
Total Cholesterol, mg/dl	193 ± 39	184 ± 36	n.s.
LDL-Cholesterol, mg/dl	122±35	114±31	n.s.
HDL-Cholesterol, mg/dl	50±13	46±16	0.05
Triglycerides, mg/dl	109±59	132±65	0.02
hs-PCR, mg/l	9.1±8.1	8.9±7.5	n.s.
IL-6, ng/l	3.0±1.8	3.7±4.7	n.s.
TNF-α, ng/l	8.6±4	8.5±3	n.s.
Leptina ug/L	41,9±15.9	41.6±15.8	n.s.

Table. XV: Baseline characteristics of patients with and without a hypoglycaemic event after LSG (blood glucose levels ≤ 2.7 mmol/l). LSG: Laparoscopic Sleeve Gastrectomy. BMI: Body Mass Index. HOMA-IR: Homeostasis Model Assessment. LDL: Low Density Lipopreteins. HDL: High Density Lipoproteins. Hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Unpaired Student's t-test was performed.

There was no difference between the two groups in terms of waist, fasting blood glucose, plasma insulin, HOMA-IR, total-cholesterol, LDL-cholesterol, hs-PCR, IL-6, TNF-α and leptina. Hip patients were, at baseline, younger than N-Hip patients (40±11 yrs vs 46±10 yrs; $p<0,001$), with a lower weight, Kg, and BMI, Kg/m^2, (128±22 vs 136±29; 46±5.7 vs 48.4±7.9; $p<0,05$) and with a worse lipid profile; indeed, Hip patients had lower HDL cholesterol, mg/dl, and higher triglycerides, mg/dl, compared to N-Hip (46±16 vs 50±13 and

132±65 vs 109±59 respectively; p<0,05). Blood glucose and plasma insulin curves after OGTT before and after LSG in these two groups are shown in Figure 25 A-B.

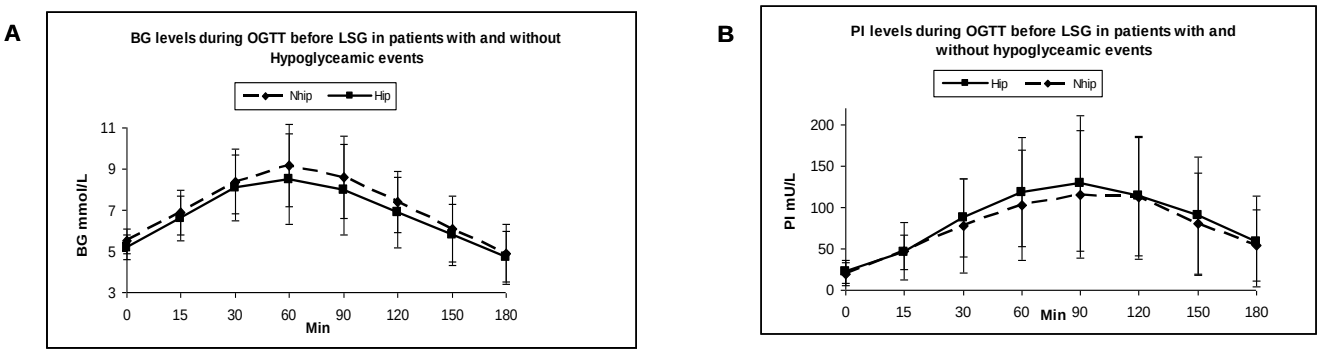


Figure. 25 Glucose (A) and insulin (B) curves during OGTT performed before LSG in patients with (Hip) and without (NHip) hypoglycaemic events. Dotted line indicates NHip patients and solid line values obtained in Hip patients. There are no significant differences. Unpaired Student's t-test was performed; p<0, 05.

Glucose AUC before surgery was significantly lower in patients with hypoglycaemic events after LSG compared to patients without hypoglycaemic events after LSG (glucose AUC Hip vs NHip: 1278±246 vs 1355±222 mmol/L/h, p=0.05); while insulin AUC before surgery did not differed between the two groups (insulin AUC Hip vs NHip: 17529 ± 8935 vs 15735 ± 9206 mU/L/h, p= 0.23).

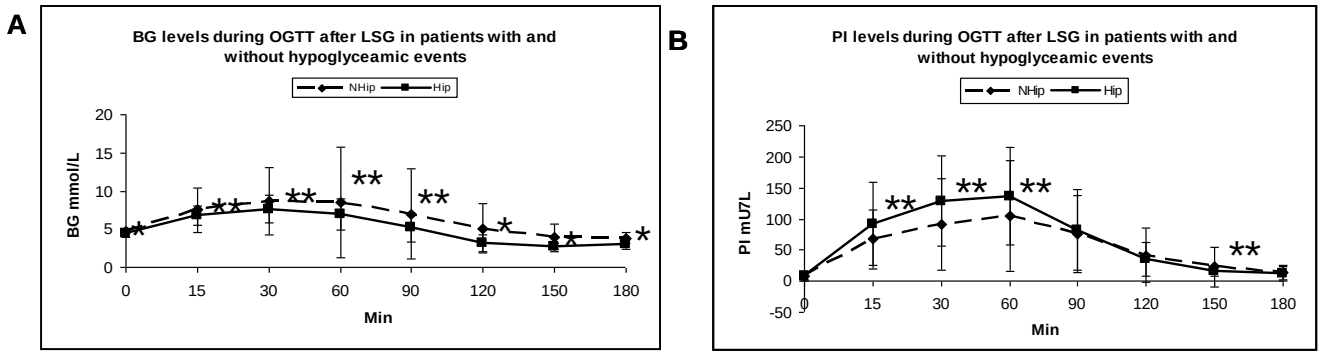


Figure 26 Glucose (A) and insulin (B) curves during OGTT performed after LSG in patients with (Hip) and without (NHip) hypoglycaemic events. Dotted line indicates NHip patients and solid line values obtained in Hip patients. Unpaired Student's t-test was performed; *p<0, 0001; **p<0,005. Unpaired Student's t-test was performed.

Considering the results of the OGTT performed one year after LSG, patients with a hypoglycaemic event after LSG had significantly lower levels of blood glucose from fasting state and during all OGTT (Figure 26, A). Insulin plasma levels were higher at 15, 30, 60 minutes after glucose load and lower at 150 minutes after glucose load in Hip patients (Figure 26, B). A similar pattern has been observed for c-peptide levels (data not shown). Glucose AUC after surgery was lower in patients with hypoglycaemic events (887±179 vs 1135±666 mmol/L/h, p<0.05), whereas insulin AUC was not different (11150±4974 vs 10218±6741 mU/L/h, p<=0, 38) compared to NHip patients.

	Without	**With**	**P value**
Waist, cm	107±16	95±13	0.0001
Weight, Kg	97±22	83±17	0.0001
BMI, Kg/m^2	34±6.3	29±4.8	0.0001
%EBMIL	62±21.	80±20	0.0001
Blood glucose, mmol/l	4.7±0.4	4.4±0.4	0.0001
Insulin, mU/L	7.9±5.6	7.4±6.9	0.02
HOMA-IR	1.7±1.2	1.5±1.7	n.s.
Total Cholesterol, mg/dl	184 ± 35	175 ± 36	n.s.
LDL-Cholesterol, mg/dl	110±32	103±33	n.s.
HDL-Cholesterol, mg/dl	59±14	57±12	n.s
Triglycerides, mg/dl	77±31	77±34	n.s
hs-PCR, mg/l	3.0±3.5	1.7±2.7	0.009
IL-6, ng/l	2.7±2.5	2.7±2.8	n.s.
TNF-α, ng/l	7.0±3.6	6.4±3.5	n.s.
Leptin ug/L	16.2±12.9	9.7±6.7	0.0001

Table XVI Anthropometrics and metabolic parameters after LSG in patients with and without hypoglycaemic events. LSG: Laparoscopic Sleeve Gastrectomy. BMI: Body Mass Index. EBMIL: Excess Body Mass Index Loss. HOMA-IR: Homeostasis Model Assessment. LDL: Low Density Lipoproteins. HDL: High Density Lipoproteins. Hs-PCR: High sensitive C-Reactive Protein. IL-6: Interleukin-6. TNF-α: Tumor Necrosis Factor-alpha. Unpaired Student's t-test was performed.

Patients experiencing hypoglycemic events after surgery had a significantly higher weight loss than patients without events (%EBMIL: 80±20 vs 62±21 %; p<0.0001) and a lower waist circumference (95±13 vs 107±16 cm; p<0.0001), weight (83±17 vs 97±22 Kg, p<0, 0001), BMI (29±4.8 vs 34±6.3 Kg/m^2) and leptin (9.7±6.7 vs 16.2±12.9 ug/l, p<0, 0001). Moreover, Hip had lower plasma levels of hs-PCR compared to NHip (1.7±2.7 vs 3.0±3.5 mg/l) as

showed in Table XVI. The delta weight and delta BMI was higher in Hip than in NHip group one year after the operation.

5.2.4 Predictors of Hypoglycaemic Events before Surgery

In order to find the independent predictors of the occurrence of a hypoglycaemic event after surgery, we considered all parameters significantly different between Hip and NHip subjects before LSG, included fasting blood glucose and glucose levels 120' after glucose load because they were lower in Hip than in NHip subjects even if no significantly (p=0,09 and p=0,06). The independent predictors were investigated with the use of a multiple logistic regression analysis model (see Statistical Analysis).

Low age (p<0, 05), low fasting blood glucose levels (p<0, 05), and high triglycerides levels (p<0, 01) before LSG, were found to be independent predictors of the occurrence of a hypoglycaemic events after surgery (r^2=0,131) (Table XVII)

Indepent variables	Correlation Coefficents	P value
Age, years	-0.057	0.002
BMI, Kg/m2	0.063	n.s.
Fasting BG, mmol/l	-0.711	0.018
BG 120’ after glucose load, mmol/L	0.035	n.s.
HDL-Cholesterol, mg/dl	-0.040	n.s.
Triglycerides, mg/dl	0.008	0.009

Table XVII: Multivariate prediction of hypoglycaemic events after LSG (blood glucose levels ≤ 2.7 mmol/l). r2 = 0.13. The occurrence of hypoglycaemia was used as the dependent variable. Sex (male =0; female =1), age, BMI before surgery, and the baseline variables found to have a significance difference between patients with a without hypoglycaemic events after surgery were entered as independent variables in the multiple regression analysis.

6 DISCUSSION

6.1 DISCUSSION (1)

T2DM and obesity are global health care problems that are closely linked together. The concept of diabesity (obesity-T2DM) emerges by the estimation that about 90% of T2DM is attributable to weight excess (Hossain P, 2007), and this is the reason because it has been applied both in research and clinical settings. The precise mechanisms linking the two conditions remain unclear. In recent years an increasing number of works have studied common metabolic defects in T2DM and obesity with a particular focus on microvascular dysfunction. Indeed, microvasculature plays a central role in glucose homeostasis: an impaired capillary recruitment and capillary rarefaction may reduce glucose uptake and contribute to insulin resistance development (Levy BI, 2008).

Expanding AT requires new vasculature to receive an adequate oxygen supply to sustain tissue growth both by hypertrophy and by hyperplasia. Several studies suggest that obesity leads to an impaired angiogenesis and AT hypoxia, inducing an inflammatory and a profibrotic response that plays a pivotal role in the pathogenesis of metabolic complications related to weight gain, first of all insulin resistance and diabetes (Corvera S, 2014; Cao Y, 2013; Lee BC, 2014). Structural and functional abnormalities, such as lower capillary density (Pasarica M, 2009; Gealekman O, 2011), increased adipocyte-capillary distance due to adipocyte hypertrophy (Halberg N, 2009) and increased cell O2-consumption (Lee YS, 2014), have been postulated as mechanisms leading to AT hypoxia. Furthermore low oxygen tension activates HIF1α pathway that, in obesity, is inadequate to sustain the neoagiogenetic response but triggers AT fibrosis contributing to a worse metabolic profile (Sung HK, 2013).

Moreover it is well showed in mice that obesity greatly affects adipose derived stem cells (ASCs) behaviour, impairing self renewal and plasticity (Pérez LM, 2013). How diabetes plays its role in this settings is widely unexplored. Recent evidences showed an alteration of angiogenetic potential and gene expression profile of ASCs in diabetic animals (Ferrer Lorente R, 2014; Rennert RC, 2014). All these recent findings highlight the close link between AT, microcirculation, angiogenesis and diabetes but utilized animal models or only few human subjects without a deep clinical evaluation of metabolic parameters and focusing mainly on subcutaneous AT (SAT). From a pathophysiological point of view it is well established that dysfunctional visceral AT (VAT) is one of the major determinant of metabolic complications of

obesity (Gesta S, 2007) while subcutaneous depots has been considered "metabolically healthy". Nevertheless it could be hypothesized that in the evolution of obesity into diabesity ,through metabolic impairment, SAT could become dysfunctional as VAT.

On the basis of these data, we planned to characterize both SAT and VAT measuring adipocytes size, capillary density, ASC and endotelial precursor number, and stromal vascular franction (SVF) adipogenic potential, in lean subjects and in obese patients divided in 3 groups: Normoglycemic (ob N), prediabetic (pre-T2DM) and Diabetic (T2DM) on the basis of clinical and laboratory evaluations. .

According with the literature (Pasarica M, 2009; Gealekman O, 2011), we found that in SAT obese patients had significant decreased in capillary density compared to lean subjects and, also in VAT, a depot which has not yet previously investigated in humans, at least to our best knowledge. An interesting result to be emphasized is that, while the number of vessels per mm^2 decreased of 40% between lean and obese SAT, the parallel reduction in VAT resulted higher, amounting to 54,5%. This finding supports both the hypotheses that obese AT is less vascularized than healthy AT and that SAT, from the point of view of vascularization, is less damaged than VAT. Differently from data showed by S. Corvera' group we found that VAT has a higher number of capillary per mm^2 both in lean subjects and in obese patients. In contrast to our expectations, we did not found any significant differences in capillary density among the 3 obese patient groups: neither in ob pre-T2DM compared to ob N nor in ob T2DM compared to both ob N and ob pre-T2DM (Figure 12).

Accordingly with these results, the *VEGFA* expression largely parallel the capillary density measured by histological vessel staining, confirming a reduction of vascularization in obesity both in SAT than in VAT and failing to shown a worsening effect of the metabolic complications and of overt T2DM on vascularization. Instead, we found significant differences in adipocyte size. In fact, adipocyte area (μm^2) was smaller in lean subjects than in obese patients both in SAT than in VAT and, more importantly, adipocyte size had an increasing trend in the 3 groups both in SAT and in VAT showing that adipocytes coming from ob T2DM patients are the largest ones (Figure 11). This result is particularly interesting because allow us to correlate the adipocyte size with the onset and the development of metabolic complications, suggesting that AT is further able to expand by hypertrophy probably generating fat cells more and more insulin resistant, independently of patient's BMI. In fact, as reported in (Table VII), ob N and ob T2DM patients selected for histological analysis displayed similar BMI and ob

pre-T2DM had a lower BMI than the other two obese groups. The drawing of Figure 27 well recapitulates our findings and represents our pathogenetic hypothesis for diabesity evolution at AT level. Moving from an healthy AT, of lean subjects, to an unhealthy AT, of obese patients and finally to an metabolic complicated-unhealthy AT, of obese T2DM patients , we observed a significant decrease in capillary density only in the first step and a progressive increase in fat cell size which characterize all steps analyzed. Regarding the AT vasculature, we have to take into account that our observation were only quantitative and we cannot exclude that capillary of ob N and of ob T2DM obese patients could differ in perfusion function or angiogenic factors responsiveness.

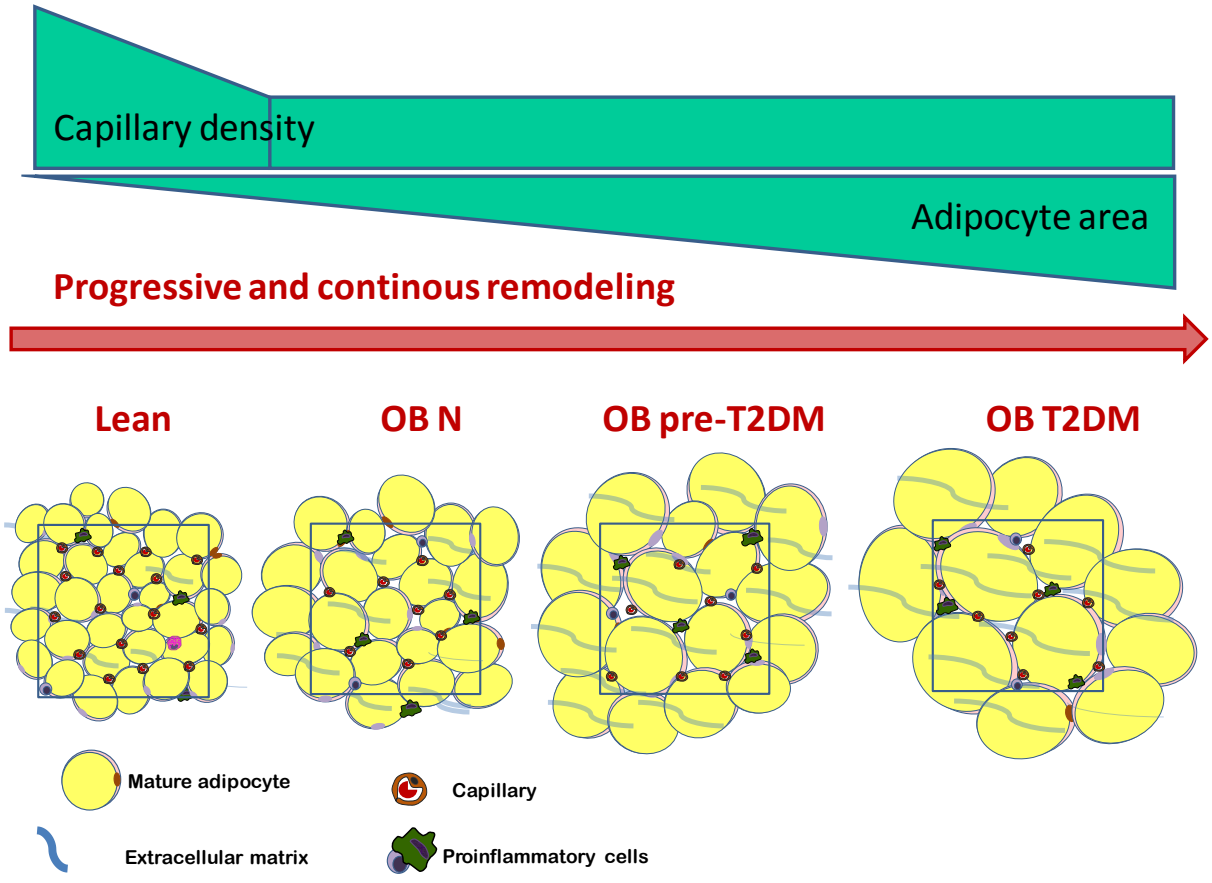


Figure 27. Schematic drawing of adipocytes and vasculature remodelling during diabesity.

On the other hands, the findings related to adipocytes are partially in agreement with previous report of Lundgren et al. (Lundgren M., 2007) which examined the relationship between fat cell size and insulin sensitivity in diabetic and non-diabetic subjects with a wide range of age

and BMI. This study showed that fat cell enlargement is associated with insulin resistance in non-diabetic individuals but not in T2DM subjects. Moreover, we observed that adipocyte area positively correlated with IL-6 blood level, underling the relationship between adipocyte hypertrophy and inflammation. It is worth noting that our findings are concordant both in SAT and in VAT, reinforcing the idea that not only VAT, but also SAT could be dysfunctional. F. H. J. Van Tienen and collaborators (Van Tienen FHJ, 2011) observed that the presence of large adipocytes is an indicator of decreased adipogenic potential in SAT and can be the trigger for increased macrophage infiltration and inflammatory process activation.

In fact these authors found that among T2DM subjects, preadipocytes isolated from abdominal SAT displayed decreased expression of genes involved in adipogenic differentiation. Consistent with these data, our results further showed that adipogenic differentiation impairment is already present in a pre-diabetic condition; in fact SVFs obtained from SAT of ob preT2DM and, unless in a minor extent, from ob T2DM displayed a lower adipogenic potential in comparison to ob N group (Figure 20).

More importantly, the ex-vivo quantification of ASCs(CD34+CD45- CD31- cells), Figure 17 showed that a pre-diabetic condition is sufficient to cause a significant decrease in the ASC, that persists also in T2DM obese patients, both in SAT and in VAT. It is interestingly that glucose impairment early affects ASCs; this could explain, at least in part, why patients with metabolic alterations preferentially expand their AT by hypertrophy than by hyperplasia. ASCs isolated from stromal vascular fraction (SVF) of our samples, were lower in lean patients compared to obese patients.

Accordingly with our results, the group of Isakson P. (Isakson, 2009) showed that the number of CD133-positive cells, containing pluripotent cells able to differentiate into adipose cell, isolated from SAT SVF positively correlate with BMI, suggesting that obese individuals could present a differentiation impairment rather than a precursors cells reduction.

Comparing the two AT depot, we found that VAT is more enriched of ASCs than SAT in all obese groups and in lean subjects. Differently, in mice underwent to high fat diet, Joe at al. (Joe, 2009), showed that adipogenic precursors were found eightfold more abundant in SAT compared to VAT, consistent with the observation of a prevalent SAT growth by hyperplasia and VAT growth by hypertrophy. However recent studies focused on VAT, both in diet-induced obese mice (Jeffery, Church et al. 2015) (Wang, Tao et al. 2013) and, more importantly, in humans (Arner, Andersson et al. 2013), showed a predominant expansion through hyperplasia

rather than hypertrophy, underlying the central role of ASCs in the pathogenesis of obesity in both depots.

As previously reported, we found that the i*n vitro* adipogenic potential of SVF obtained from VAT is lower than that obtained from SAT, but this finding was not correlated to the VAT ASCs number. It is likely that the VAT microenvironment deeply affects the differentiation capacity of ASCs to mature adipocytes. In addition, we found that in obese, but not in lean subjects, VAT ASCs display a significantly higher expression of CD105 and CD271 in comparison with SAT. The differences of surface marker expression profile of ASCs, could account for a differential, depot-specific function, of precursors cells.

Van Harmelen and colleagues (Van Harmelen V., 2004) found that age correlated negatively with proliferation only in SAT preadipocytes and not in the omental depot and he supposed that aging has distinct effects on preadipocytes from different fat depots and it could explain the loss of SAT and relative preservation of omental fat with aging.

In our study, even if ob T2DM were older than ob pre T2DM and ob N we did not find any correlation between age neither with percentage of ASCs, nor with percentage of mature adipocyte cells, after 9 days of culture. Considering the high numbers of samples analysed, we can affirm that not age but glucose profile affects both pre-adipocyte and their ability to differentiate, certainly in SAT and probably in VAT.

Moreover, in our study we observed that the number of endothelial precursors cells (EPs), defined as CD34+ CD31+ CD45- cells, was higher in SAT of obese patients compared with lean subject, whereas in VAT there were no significant differences (Figure 19). Both in SAT than in VAT, EPs did not differ in ob pre-T2DM and ob T2DM compared to ob N and this underlines that angiogenic potential is lesser affected by glucose impairment than adipogenic counterpart, as it was also observed with immunohistochemical analysis.

In SAT and VAT, EPs showed an opposite trend compared to ASCs; indeed, in obese groups, EPs were lower in VAT than in SAT. These results could suggest that VAT expansion is achieved preferentially by hypertrophy due to the reduction of the angiogenic potential which did not parallel the adipogenic potential represented by the in vivo higher number ASCs in comparison with SAT.

Another interesting result comes from our studies concerning ASCs of obese patients after weight loss (ob WL). Consistent with previous data, we found that ASCs are significantly higher in ob WL group compared both to obese groups and lean subjects, whereas, EPs are

significantly lower in ob WL than the 3 obese groups characterized by different metabolic profile. Moreover ob WL group showed a lower *PPARγ* expression as compared with that of ob N and ob pre-T2DM groups. We can supposed that in these patients the relevant WL achieved could stimulates the increase of ASCs as a reaction to important fat mass reduction, but the AT expansion and hyperplasia are counteracted by the lowering of *PPARγ* expression and by the concomitant limitation of EPs number. Another hypothesis to explain the high percentage of ASC quantified in AT of ob WL is that hypertrophic adipocytes, after lipid mobilization due to massive WL, could dedifferentiate again into preadipocytes as postulated by Baptista L. et al. (Baptista L., 2015), probably through the involvement of pericytes (Silva K., 2015).

In fact, a fourfold increase in SAT supra-adventitial cells was described in ex-obese patients compared to obese together with an increase in pericytes's number in both groups compared to lean subjects (Silva K., 2015). Many recent studies support the idea that ASCs tend to maintain their features even after drastic WL, probably due to epigenetic modulations occurred during the obese state, partially explaining the mechanisms involved in weight regain over time. Even if microenvironment changes in inflammatory status have been described in SAT after WL due to surgical intervention, with a decrease in some cytokines levels, such as IL-6 and C-reactive protein, post-bariatric ASCs seem to maintain high levels of other factors such as MCP-1, partly sustaining a macrophages tissue infiltration (Silva K., 2015).

However, the metabolic improvement observed after WL partly depends on the restoration of AT from features present before the obesity development. A comparative study of SAT from obese, obese underwent to WL due to diet intervention and normal weight subjects suggested that a full adipose cell re-programming has been induced after weight loss, with a reduced DNA-damage and consequent longer cell survival, an extended replicative lifespan and a reduced adipogenic commitment.

The variables that probably strongly determine adipose cell behavior after WL, causing a minimal, partial or complete pre-obesity cell pattern restoration, could be represented by the duration of the disease and the presence/absence of obesity-associated comorbidities, which contribute to a stabilization of epigenetic modifications and thereby to a cell memory reinforcement (Mitterberger MC,. 2014).

In conclusion, our study, confirmed that obese AT is less vascularized than lean AT and showed for the first time that T2DM does not represent an aggravating factor to the vascular reduction already present in obesity. On the contrary T2DM and also prediabetic condition are able to

further modify the AT architecture, remodeling the mature adipocyte size and the adipogenic potential mediated by ASCs importantly reducing the AT hyperplastic growth capacity. Our results allow us to assume that the *primum movens* in the diabesity evolution at AT level has to be searched more in the adipocyte/preadipocytes remodeling than in the vasculature/angiogenesis impairment and that both depots, SAT and VAT, equally play a pivotal role in the metabolic disease progression.

6.2 DISCUSSION (2)

Hypoglycemic events are a well-established complication of upper gastro-intestinal surgery. In particular is well known that the frequency of asymptomatic hypoglycemia after RYGB may be over 30% (Service GJ., 2005). Similar outcomes are reported for LSG in only two studies (Papamargaritis D., 2012; Natoudi M., 2014) in a small number of patients and little none is known about clinical predictors. In our study we aimed to identify how many subjects develop hypoglycemia after LSG and if we can find any predictors of these events before surgery in non-diabetic obese patients.

We observed that 5 of 197 (2, 5%) patients, have experienced at least one severe hypoglycemic event (BG ≤ 2.7 mmol/L) during 3 hour OGTT before bariatric surgery. This data is consistent with data present in literature for obese patients where hyperinsulinemic hypoglycemia is described (Pigeyre M., 2015).

After LSG, 61 of 186 (32, 8%) patients have experienced at least one hypoglycemic episode during OGTT. These data are just partly comparable with the results reported by Papamargaritis and Natoudi (Papamargarits D., 2012; Natoudi M., 2014) because both authors had subjected patients to a 2 hour OGTT and have found 4 of 12 (33%) patients with BG ≤ 3, 3 mmol/L at 90 or 120 minutes after glucose load and 6 of 30 (37, 5%) patients with BG ≤ 3, 3 mmol/L at 90 or 120 minutes, respectively. In our study the highest frequency of severe hypoglycemic events were observed 150’ after OGTT, in 20,2% of subjects, while, at 90' and 120' minutes after glucose load, 2,8% and 11,7% of our patients have had a hypoglycemic events.

In our recent study (Nannipieri M., 2016), we demonstrated that all patients with neuroglycopenic symptoms had, at least one value of blood glucose level ≤ 2, 7 mmol/L during OGTT. Certainly, OGTT overestimates hypoglycemic events but, considering a lower cut-off of hypoglycemia compared to other studies (2,7 mmol/L vs 3,3 mmol/L), and, considering the

large number of patients included in this study, we can affirmed that hypoglycemia is a common complication one year after LSG. Moreover, even if we didn't any specific test in order to quantify the number of patients reporting hypoglycemic symptoms under free-living conditions, after blood exams, all patients did a medical interview, and, about one third of our patients have reported symptoms consistent with hypoglycemia, in particular, headache, dizziness, irritability and sweating. Most of symptomatic patients were those who, during OGTT, had blood glucose levels ≤ 2, 7 mmol/L; indeed, only 4 patients who reported symptoms consistent with hypoglycemia had, during OGTT, glucose levels > 2, 7 mmol/L. Therefore, we can conclude that mild hypoglycemia after OGTT does not reflect the real clinical status and we can suggest that OGTT could be a good diagnostic test if carried out over 3 hours and if the diagnostic threshold for hypoglycemia is lowered at 2.7 mmol/L.

Moreover, several studies described an early dumping syndrome after RYGB (Hamer H.F., 2012); even if we didn't apply any questionnaire for dumping, we find hypoglycemia later than one hour and it could be explain because in LSG the pyloric valve and the duodenal feedback inhibition of gastric empting are preserved.

These two anatomical differences in LSG could slow down gastric empting and so avoid the typical symptoms of early dumping syndrome. Recently, Lee Clare J (Lee CJ., 2015) on the basis of validate questionnaires, demonstrated that RYGB confers some increased risk between the presence of hypoglycemia symptoms before and after surgery while these risk was not find in subjects underwent to LSG. So we can think that LSG could be seen as a good potential alternative. Moreover, these findings are according with Papamargaritis work that demonstrated an increase of late dumping symptoms at 6 and 12 months after LSG.

Another aim of our study was to analyze the differences in clinical and metabolic characteristics between non diabetic morbidly obese patients who developed or not post-OGTT hypoglycaemia one year after LSG. According with the recent work of Papamagaritis (Papamergaritis D., 2016) performed on 18 non diabetic obese patients six month after LSG, we found that patients experienced hypoglycemia had a greater weight loss and a greater reduction in waist compared with NHip patients (Table XVI).

After bariatric surgery, an increase risk of traumatic deaths and an increase in depression have been described in patients with hypoglycemia. In rare cases, pancreasectomy for untreatable severe hypoglycemia has been required years after RYGB. For these reasons, it is very important to identify patients at high risk of hypoglycemia in order to educate them to

recognize symptoms and to use adequate nutritional habits. Our findings highlight that patients with greater weight loss and lower BMI after surgery have a higher risk to develop hypoglycemic events after surgery, but the identification of baseline pre-operative predictors remains elusive. In fact, even if in our study we found that baseline younger age, lower fasting blood glucose and higher triglycerides have been shown to be independent predictors of hypoglycemia after surgery, their predictive power is really small.

The identification of more reliable predictors would require a more extensive investigation of the pathophysiological events leading to the development of hypoglycemia after LSG. As we described in our recent, in a smaller group of patients (Nannipieri M., Belligoli A., 2016), in all subjects, LSG anticipated the time of post-OGTT glucose peak and lowered the plasma glucose nadir through more rapid dumping of gastric contents into the small bowel. In the Hip subjects, however, the features predisposing to hypoglycemia were accentuated as compared with NHip subjects. The relative potency of these factors probably varies among subjects and is difficult to gauge. Firstly, post prandial hypoglycemia (PPHG) may be detected beyond the time frame of the current study.

Secondly, in some individuals a lesser weight loss might protect against PPHG despite a high load of predisposing factors; in yet other subjects, gastrointestinal hormone release or action may be defective or pre-existing insulin sensitivity be impaired. Thirdly, gastric emptying may change over time because of long-term adaptations of motility or ensuing autonomic neuropathy (*e.g.,* long-term and/or uncontrolled diabetes). Finally, the pattern of predisposing factors may differ depending on whether PPHG manifests itself only once or repeatedly and whether it is mild-moderate or severe. Additional factors that were not measured in our study might have had a part.

For example, (1) lack of reduction of ß–cell mass, which was constitutively increased during the obese state prior to surgery, (2) gut hormonal activation of new ß-cell formation due to surgically induced changes in the secretion of insulinotropic incretins, or other regulatory peptides, (3) abnormal counterregulatory hormonal responses, (4) changes in gut microbiota, and (5) changes in bile acid composition.

In conclusion, these findings confirm a high incidence of severe hypoglycemia after a provocative test (OGTT) 1-year after LSG. Patients with hypoglycemic events have a lower weight and BMI and a greater %EBML after LSG. Hypoglycemia is more frequent in patients having lower age, lower fasting blood glucose levels, and higher triglycerides levels before

LSG but these predictors do not have such a marked correlation to contraindicate LSG. Whether hypoglycemic events are so frequent in daily life of patients underwent LSG as shown by OGTT is something that needs a deep investigate and we need to deep investigate also the pathophysiological events leading to the development of hypoglycemia after LSG.

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