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Morphology of visceral and subcutaneous adipose tissue of obese subjects

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SUMMARY

In case of positive energy balance, a progressive pathological remodeling of the adipose tissues occurs: adipocytes increase their volume (hypertrophy) leading to a consequent increase of fat mass. Hypertrophy of adipose cells is associated with cellular abnormalities and a massive infiltration of macrophages which induce a state of chronic low-grade inflammation that leads over time to insulin-resistance and eventually type 2 diabetes. Another feature of obesity is remodeling of the extracellular matrix (ECM) with an excessive accumulation of fibrosis that increases the total stiffness of the obese adipose tissue and reduces expandability of the tissue. Some aspects regarding the development and the progression of fibrosis in adipose tissues in humans remain to be elucidated. In this regard, we performed a systematic study of visceral and subcutaneous fat biopsies from 33 obese patients undergoing bariatric surgery, compared with fat biopsies from 6 lean control patients. We detect that a large percentage of omental and subcutaneous adipocytes are Perilipin1 (PLIN1)-negative, and a positive correlation between the amount of PLIN1-negative adipose cells is found in both adipose depots considered. The amount of non-viable adipose cells is significantly correlated with total fibrosis for all obese patients, in fact PLIN1-negative adipocytes, single or grouped, are surrounded by fibrotic bundles. Pyroptosis, the main mechanism of cell death of murin hypertrophic adipocytes, is not revealed within fibrotic regions. Furthermore, TEM (Transmission Electron Microscopy) analyses demonstrate that dense collagen fibrils are closely associated to the external surface of basal membrane of adipocytes, more in obese than lean subjects, consistent with hyperproduction of collagen fibrils by adipocytes themselves, and signs of cellular stress in degenerating adipocytes were also found.

Finally, data support the idea that a large proportion of hypertrophic adipose cells die, due to hyperproduction of collagen fibrils and they consequently recruit inflammatory cells to clean up the adipose tissue. Macrophages are prevalent mainly in the parenchyma and only to a lesser extent in Crown-like Structures (CLS), unlike the mouse models. They probably induce insulin-resistance, and this could explain the pathogenetic hypothesis of the link between obesity and diabetes. Therefore, in humans we hypothesize that, in addition to death of pyroptosis, there could be another type of cell death for obese adipocytes, related to fibrosis. The clinical significance of this new aspect of the histopathology of adipose tissue in obese people remains to be indagated.

INTRODUCTION

1. Obesity

1.1 Obesity and clinical features

World Health Organization defines obesity as a disease characterized by an excess deposition of white adipose tissue in the body, due to an imbalance between calorie intake and energy expenditure, involving numerous risks to health and leading to a reduction in life expectancy (WHO, 2020). Thus, when the calories assumed are greater than the calorie expenditure, our body progressively stores lipids, leading to the condition of overweight and eventually obesity. In addition to excessive food intake, physical inactivity is also a key factor that over time leads to obesity.

The obesity condition is classically defined by The National Institute of Health based on BMI (Body Mass Index), a biometric parameter which compares weight and height of humans, calculated as weight in kilograms divided by height in meter squared. It is now considered internationally as the only measurement system to establish whether a person is lean, overweight or obese, based on "threshold values". Therefore, people with a BMI between 25 and 29,9 kg/m² are classified as overweight individuals while a value of BMI greater than 30 kg/m² denotes an obesity condition (WHO, 2000). BMI, however, is not a true index of fat mass because it is unable to distinguish muscle weight from weight associated with the abnormal accumulation of body fat. Despite its limitations, BMI is currently the most commonly used measure to diagnose obesity in adults. Waist circumference or waist-to-hip ratio, instead, could be considered an effective measure of body fat distribution (Nimptsch et al. 2019).

Obesity can be identified into two forms based on the distribution of white adipose tissue: "apple-shaped obesity", also called visceral obesity, is characteristic of men and it consists of an accumulation of fat in the abdominal cavity. "Pear-shaped obesity", typically described as female obesity, is a form of obesity in which adipose tissue is predominantly distributed in the subcutaneous region of the thighs and hips (Tchernof et al. 2013).

Visceral obesity is the most severe form as it is related to the onset of comorbidities, including insulin resistance, diabetes and cardiovascular diseases. On the other hand, gynoid obesity is the form least associated with complications. Indeed, different data suggest a greater pathogenicity of adipose tissue visceral than subcutaneous (Tchernof et al. 2013, Poirier et al. 2003). It has been noted in humans with a high value of WHR ("Waist hip to ratio"), index of accumulation of adipose tissue abdominal, which is obtained by making the relationship between waist circumference and hip circumference, is more likely to correlate with comorbidities (such as diabetes) despite a low BMI. Conversely a low

WHR correlates to a low probability of comorbidity, despite a high BMI (Ohlson et al. 1985, Nimptsch et al. 2019).

1.2 Obesity and epidemiological evidences

Obesity is currently defined as the most important problem of world public health because over the past ~50 years its incidence has increased to pandemic levels and it represents a risk factor for the onset of other chronic pathologies responsible for 86% of the deaths in Europe (60% worldwide) (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight).

The most recent scientific evidences indicate that overweight and obesity is increasing dramatically around the world with an exponential spread and is mainly affecting teenagers and childrens. Indeed, a recent study states that obesity affects 33% of people in the United States and is estimated to reach over 50% of the population by 2030, due to the rising rate of obesity among adolescents (Andolfi et al. 2018).

According to other recent data provided by the World Health Organization (WHO), more than 1.9 billion adults over the age of 18 were overweight in 2016 and of these, over 650 million adults were obese (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight).

In Italy, the epidemiological inquiry "Rapporto Osservasalute 2016" (referred to ISTAT data collected in 2015) supports that 35.3% of adult population is overweight, while 9.8% is obese and the territorial difference is remarkable: the southern regions show a higher percentage of obese adults, compared to those in the north (https://www.osservatoriosullasalute.it/wp-content/uploads/2017/05/ro-2016.pdf).

1.3 Obesity and associated comorbidities

Obesity is a worldwide public health problem due to its widespread and growing incidence but also and above all because it is considered an important risk factor for numerous metabolic disorders, first of all Type 2 Diabetes but also insulin resistance, metabolic syndrome, dyslipidemia, atherosclerosis, cardiovascular diseases, hypertension, non-alcoholic fatty liver diseases. In addition, obesity can predispose to the onset of several other diseases that are not classified as metabolic, such as some forms of cancer, osteoarthritis, obstructive sleep apnea and neurodegenerative diseases (Bray 2004, Apovian 2016).

Therefore, for all these complications, obesity is associated with an increased risk of reduction lifespan (Haslam et al. 2005). In this regard, a loss of body weight, through diet and exercise, reduces all causes of mortality in obese subjects (Polyzos et al. 2019).

- The term Metabolic Syndrome indicates a clinical condition, and not a disease per se, in which many of the comorbidities of obesity coexist. Therefore, we speak of metabolic syndrome when in an individual there are at least three of the following factors including abdominal obesity, hypertension, insulin resistance, impaired glucose metabolism and dyslipidemia (Haslam et al. 2005, Samson et al. 2014).

- Of all the complications, Type 2 Diabetes Mellitus is (T2DM) the most strongly associated with obesity, indeed the term "Diabesity" has been coined. 90% of diabetic individuals are obese or overweight but not all obese people have diabetes (Chadt et al. 2000). It is typical of adulthood in fact it affects individuals aged between 40 and 65 years.

  Type 2 diabetes is also referred to as insulin-independent diabetes, because it is not caused by an insufficiency in insulin production, such as in Type 1 Diabetes Mellitus, but by a functional deficit and numerical insufficiency of insulin receptors. Thus, insulin is physiologically produced by the pancreas but a prolonged overeating lead to a diminished responsiveness of the skeletal muscle and adipose tissue receptors to insulin that prevent their action of glucose uptake into cells (defined condition of insulin resistance). During the first phase of insulin resistance the pancreatic beta cells compensate by producing more insulin (stage of hyperinsulinemia), thus maintaining blood sugar at normal levels, but subsequently the pancreas is no longer able to compensate and there is the onset of diabetes (fasting blood sugar ≥ 126 mg / dl) (Groop 2000, Fletcher et al. 2002).

- Dyslipidemia is associated with visceral obesity and it is one of the main disorders of the metabolic syndrome (Despres et al. 2001). It involves a change in the amount of lipids circulating in the blood: in particular there is an increase in triglycerides, a decrease in HDL (high density lipoprotein) cholesterol, relatively normal or slightly increased levels of LDL (low-density lipoprotein) cholesterol, and high levels of LDL particles which are smaller and denser than normal (Klop et al. 2013, Tchernof et al. 2013). These are all risk factors for cardiovascular disease, along with high blood pressure and increased blood sugar levels (Klop et al. 2013)**.**

- A high mortality rate in overweight people is caused by cardiovascular diseases (CVD) such as coronary syndrome, stroke and peripheral vascular disease. Indeed, there is a significant correlation between abdominal obesity, excess visceral adiposity and cardiovascular disease. It has been noted that there is a strong effect of weight loss on the reduction of total cholesterol, LDL cholesterol and triglyceride levels (Dattilo et al. 1992).

- Some studies reveal that obese patients have higher rates of hypertension compared with normal-weight individuals (Sarzani et al. 2014, Chiang et al. 1969), but not every obese

patient is hypertensive (Poirier et al. 2003). Hypertension is one of the most important risk factors for a number of cardiovascular complications. In addition, metabolic syndrome and insulin resistance potentiate the damaging impact of hypertension on target organs and CVD risk (Sarzani et al. 2012, Stamler et al. 1978).

- NAFLD (nonalcoholic fatty liver disease) is a complication of obesity characterized by a series of morphological and functional changes in the liver such as elevated levels of liver enzymes, hepatomegaly and abnormal histology with a typical steatosis (vesicles of fat, consisting mainly of triglycerides accumulate within the hepatocytes) (Pierantonelli et al. 2019, Bray 2004). NAFLD include both simple steatosis (SS) and non-alcoholic steatohepatitis (NASH), much more deleterious because it can evolve into cirrhosis and eventually into cellular hepatocellular carcinoma (Svegliati-Baroni et al. 2019, Polyzos et al. 2019). More specifically, in patients with NASH, immune cells infiltrate the liver parenchyma leading to chronic low-grade inflammation. When inflammation is prolonged, the fibrosis process begins which gradually leads to liver cirrhosis (Polyzos et al. 2019).

- Obstructive sleep apnea (OSA) is a medical condition characterized by frequent interruptions in breathing during sleep due to total or partial obstruction of the upper airways. It is common in obese subjects with a prevalence of 50-60%, although it is higher in obese diabetic patients (Drager et al. 2013). This alteration of lung activity is due to the reduction in residual lung volume which is associated with increased visceral fat and increased abdominal pressure on the diaphragm (Drager et al. 2013).

- Obesity increases the risk of developing various malignancies (Avgerinos et al. 2019, Vucenik et al. 2012) and due to the increasing number of obese individuals, the worldwide incidence of cancer will continue to rise (Avgerinos et al. 2019). The tumors most frequently associated with excess body weight are breast, ovarian, thyroid, colorectal, pancreatic cancers and hepatocellular carcinoma (Avgerinos et al. 2019).

2. Anatomy of adipose organ

Adipose tissues, classically defined as connective tissue without a specific anatomy, has actually a well-defined anatomy, with a specific vascularization and innervation and a high physiological plasticity (Cinti 1999) (Cinti 2012).

Since an organ is generally defined as an anatomically dissectible structure consisting of at least two tissues that cooperate for a function, it is possible to speak of "Adipose Organ" (Cinti 2005, 2018) (Cinti 1999). Indeed, it made up of two types of tissue that are contained in a large structure with all the features of a real organ, which can be anatomically dissected as a unitary structure in mice.

Therefore, White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT) are the two tissues that constitute the Adipose Organ. They are mainly contained in two different body regions in both mice and humans which are the subcutaneous compartment and the visceral compartment. In particular, the subcutaneous depots are located under the skin whereas the visceral depots are present in the trunk: thoracic and abdominal regions. For this reason, in mammals, adipose tissues can be considered a multi-depot adipose organ (Cinti 2001, 2005, Giordano et al. 2014).

Most areas of adipose depots are white and belong to white adipose tissue while others are brown and correspond to brown adipose tissue. However, looking carefully with light microscopy, it turns out that the depots that apparently consist only of white, or only of brown, adipose tissue are actually mixed deposits, even if some adipose depots are really formed by pure WAT and others by pure BAT (Vitali et al. 2012, Cinti 2018).

But why are adipose tissues contained within the same organ? What is their functional cooperation? To answer this question, it is necessary to see how they are structured and how the adipose organ changes in different functional conditions.

Briefly, adipose tissues are made up by cells rich in lipids, for this reason defined as "adipo-cytes". There are two types of adipocytes which differ in morphological, functional, biochemical and tissue characteristics, concerning vascularization and innervation, and they are distinguished into white, or unilocular, adipocytes and brown, or multilocular adipocytes.

2.1 White adipose tissue

White adipose tissue, or WAT, is so called as it appears white in small mammals, but yellow in humans in anatomical dissection (Cinti 2018). It is composed mainly from white adipocytes but other cell types are also present including vascular cells, nerve cells, fibroblasts and moreover, in the interstitium, there are always immune cells such as macrophages, lymphocytes and mast cells (Cinti 2012).

White adipocytes are cells of about 50-70 μm in diameter, spherical in shape as they are formed by a single lipid droplet, rich in triglycerides, that occupies 90% of the cell volume, for this reason they are also defined unilocular adipocytes (Cinti 2018). This morphology is easily recognizable by scanning electron microscopy (*Figure 1*). The lipid droplet appears clearly visible by electron microscopy as it is delimited by a dense line, due to the presence of proteins essential for the metabolism of fatty acids, first of all Perilipin 1 which regulates lipolysis (Blanchette-Mackie et al. 1995, Brasaemle 2007).

Furthermore, they have a thin cytoplasm with a flattened nucleus at the periphery and the mitochondria have an elongated morphology with a few randomly arranged cristae. Outside

the cell membrane, the adipocytes are delimited by a typical external lamina defined basal membrane, consisting mainly of type IV collagen (Pierleoni et al. 1998).

This specific morphology allows white adipocytes to perform their main function which is to store highly energetic molecules, such as fatty acids, to supply energy to the body for survival in the intervals between meals.

It also has an endocrine function as it produces several hormones, among which one of the most important was discovered in 1994 and is Leptin. It has an important role in controlling energy accumulation in the body because it reflects the amount of white adipose tissue and it induces the search for food when its levels are low (Zhang et al. 1994, Cinti et al. 1997). The control mechanism of leptin secretion appears mainly linked to the amount of white adipose tissue present in the body, therefore in the obese subjects leptinemia is generally high and it is hypothesized that in these subjects a leptin resistance is established (Maffei et al. 1995).

2.2 Brown adipose tissue

Brown adipose tissue, or BAT, appears of this color due to the high mitochondrial density, high innervation and vascular network. It is mainly formed by brown adipocytes which are polygonal cells, smaller than white adipocytes in which the cytoplasmic lipids are organized in many small droplets, for this reason they are also called multilocular adipocytes (Cinti 2018) (*Figure 1*). Brown adipocytes, like white adipocytes, are also characterized by the presence of an external lamina on the outer side of the cell membrane. However, the two cell types differ not only in the morphology of the lipid accumulation but also in the morphology and biochemical composition of the mitochondria. In BAT the mitochondria are numerous, large, round in shape with many laminar cristae and they are rich in UCP1 (Uncoupling Protein 1), a thermogenic protein exclusively expressed in these cells and considered the main marker of metabolically active brown adipocytes (Cinti 2017). This specific morphology is essential to perform their function which is to dissipate lipids in the form of heat: this is possible thanks to the activity of UCP1 which uncouples oxidative phosphorylation from ATP synthesis and therefore results in thermogenesis (Cannon et al. 2004, Frontini et al. 2007, Cinti 2017). So, BAT uses the same substrate of WAT that is triglycerides which, however, are burned in the mitochondria and the more they burn the more they produce heat. To conclude, we can say that white adipocytes and brown adipocytes have two opposite functions: the

former accumulate lipids for survival, while the latter dissipate them by producing heat to distribute it to vital organs, but both nourishment and heat are essential for human survival.
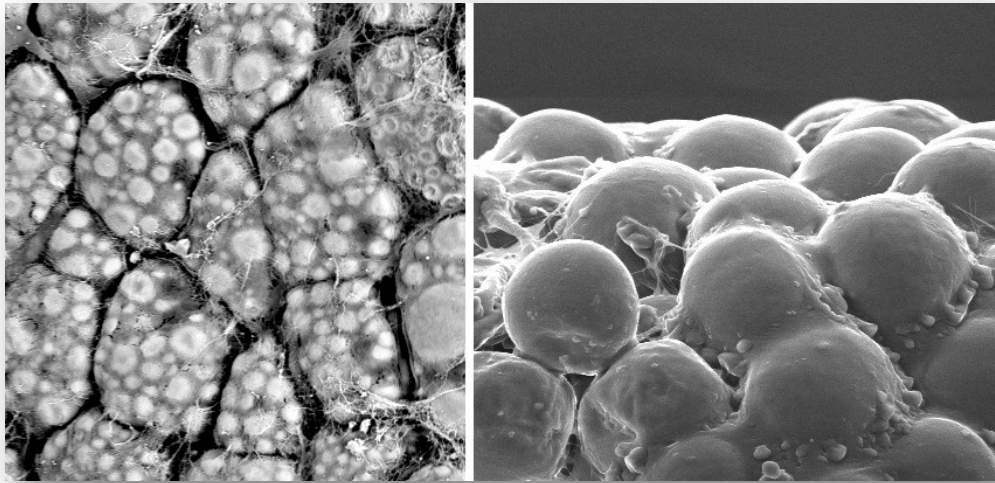

Figure 1: *Brown adipocytes (left) and white adipocytes (right) by scanning electron microscopy. Cinti S. Obesity, T2 Diabetes and The Adipose Organ, Springer 2018.*

3. Plasticity of adipose organ

The main functions of this organ are thermogenesis, performed by BAT, and the energy reserve function performed by the WAT, as it allows the storage of high-energy molecules necessary for the survival in the intervals between one meal and another (Cinti 2018). Therefore, the two adipose tissues perform different functions and to answer the initial question about why they are present in the same organ and what their functional cooperation is, numerous studies were carried out including those of the group of Professor Cinti. The hypothesis most considered to explain the simultaneous presence of the two adipose tissues is that they have the property of interconverting one into the other, for this reason the adipose organ is known to have a high plasticity (Cinti 2009a, 2011). In fact, under appropriate stimulation, such as prolonged exposure to cold or a chronic positive energy balance, the brown or white component, not directly involved in the adaptation response, converts to the phenotype that the body needs most at that moment. Therefore, to satisfy these needs, mechanisms of plastic remodeling are activated so during exposure to chronic cold the amount of BAT in the organ could increase, phenomenon called "browning", while in case of exposure to an obesogenic diet increases "whitening" (Smorlesi et al. 2012, Giordano et al. 2017). This just described is a tissue remodeling mechanism, known as "transdifferentiation", that is a direct transformation of the brown adipocytes in white adipocytes and vice versa, depending on the body's needs (Cinti 2009b, Giordano et al. 2014). Transdifferentiation is a reversible phenomenon of cooperation between the two adipose

tissues in which differentiated mature adipose cells reprogram their genome in a reversible way without passing through a stage of dedifferentiation, and therefore they change phenotype and function in response to physiological stimuli (Barbatelli et al. 2010, Cinti 2011, 2009a).

3.1 Browning phenomenon

The main function of brown adipose tissue is the production of heat or thermogenesis. This function is made possible thanks to the presence of the Ucp1 protein placed in the inner membrane of mitochondrial cristae. Therefore, the physiological stimulus to brown adipocyte activity is exposure to cold, that is, exposure to a temperature below thermoneutrality (Frontini et al. 2007).

In particular, after exposure to cold, the release of noradrenaline by the sympathetic fibers, present in the adipose parenchyma, activates the β3 adrenergic receptors of brown adipocytes which in turn stimulate lipolysis and activate Ucp1 (Giordano et al. 1996, Nicholls et al. 1984). Exposure to cold in addition to activating Ucp1, also determines the neosynthesis of Ucp1 and the neosynthesis of mitochondria. Thus, activated brown adipose tissue changes its morphology: lipid droplets become smaller, mitochondria assume a spherical shape, and finally both the number and density of mitochondrial cristae increase (***Figure 2***) (Ricquier et al. 1978, Cinti 2018).

However, when exposure to cold becomes chronic (days or even months), the amount of brown tissue present is no longer sufficient for thermogenetic needs, and for this reason the transdifferentiation of white adipose tissue into brown adipose tissue occurs (the so-called "browning") (***Figure 3***) (Giordano et al. 2014, Cinti 2002, 2009b).
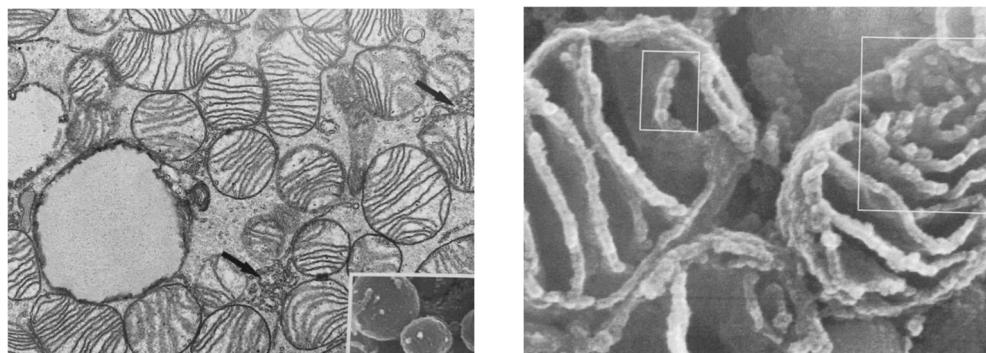

Figure 2: *Morphology of mitochondria in activated brown adipocytes by Trasmission Electron Microscopy (TEM) (left) and by High-Resolution Scanning Electron Microscopy (HRSEM) (right). Cinti S. Obesity, T2 Diabetes and The Adipose Organ, Springer 2018.*

In addition, it has been shown that not only exposure to cold, but also physical activity induces browning (De Matteis et al. 2013). In fact, the muscle produces a hormone called irisin, which is able to transform white adipocyte in brown (Bostrom et al. 2012) and also causes a thickening of the bones

(Colaianni et al. 2015), in fact it has also been shown that prevents osteoporosis (Colaianni et al. 2017).

3.2 Whitening phenomenon

The white adipose tissue has the function of storing energy thus allowing survival between meals, in fact, when the animal undergoes a normal diet, it accumulates lipids through the unilocular adipose cells. However, in case of chronic positive energy balance when the body is subjected to an obesogenic diet, the adipose organ accumulates as much as possible these energies essential for survival, because it cannot refuse the fuel storage in anticipation of a possible period of fasting. One of the mechanisms that allows storing these energetic molecules is the transdifferentiation of brown adipocytes into white adipocytes (the so-called "whitening") (*Figure 3*), in addition to hypertrophy and hyperplasia (Kotzbeck et al. 2018).
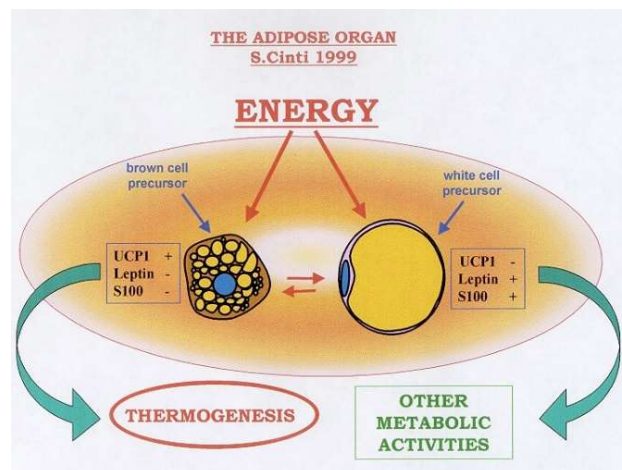

Figure 3: Theory of transdifferentiation of white adipocytes into brown adipocytes and vice versa. Cinti S. The Adipose Organ. Kurtis, Milan, 1999.

4. Adipose organ in humans

Unlike the mouse model, the dissection of adipose tissue in humans is very complicated. However, since some years, there has been a 3D human anatomy system, in which it is possible to study human anatomy on digitized cadavers, therefore it is possible to perform a three-dimensional reconstruction of all human organs including the adipose organ. Preliminary data by our group show how the human adipose organ is comparable to that of the mouse, as the subcutaneous depots occupy the extremities of the body whereas the visceral depots are in the central part and, even, also in humans, there are depots of brown adipose tissue in the part corresponding to the region more cranial of the mouse (*Figure 4*).
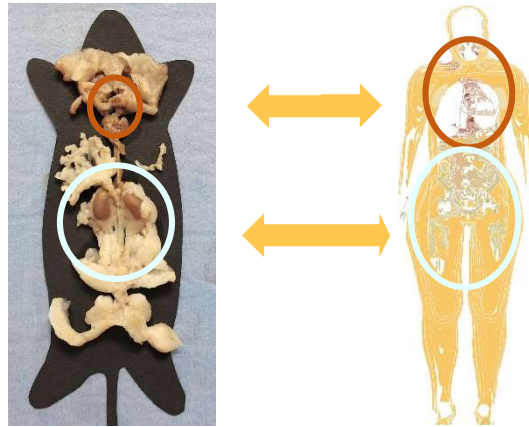
Figure 4: Correspondence of adipose depots in humans and mice. Cinti S. Obesity, T2 Diabetes and The Adipose Organ, Springer 2018.

In particular, brown adipose tissue is not abundant in adult humans as it is in small mammals, however it is possible to find it in the supraclavicular, interscapular, mediastinal and perirenal regions (van Marken Lichtenbelt et al. 2009). Moreover, it tends to be more present in young and lean individuals (Zingaretti et al. 2009). On the contrary, there are considerable quantities of BAT in newborns, and this condition is restored in adults in situations such as exposure to cold (Saito et al. 2009, Efremova et al. 2020) or in case of pheochromocytoma (Frontini et al. 2013).

4.1 Plasticity of adipose organ in humans

The phenomenon of transdifferentiation observed in humans concern the direct transformation of the white adipose tissue in brown adipose tissue both in pathological conditions, such as the pheochromocytoma, and in physiological conditions, such as exposure to cold.

People with pheochromocytoma, adrenaline and noradrenaline secreting benign tumor, present on PET (Emission Tomography of Positron: a technique able to detect tissues, such as BAT, able to up-take marked glucose) a large amount of activated BAT. Interestingly, following the resection of the tumor, no signs of uptake are observed, demonstrating that BAT has functionally switched off because there are no longer hormones activating brown fat cells (Kuji et al. 2008). Furthermore, the work of Frontini et al. 2013 shows that in pheochromocytoma patients it is possible to detect brown adipose and paucilocular cells, UCP1-positive, within the omentum, typically a pure white adipose depot. Of note, all data indicative for neo-formation of brown adipocytes (proliferative or developmental markers) were negative and the presence of unequivocal transitional stages, including the UCP1 positive unilocular cells, confirmed the direct transition between white and brown phenotype in this clinical condition.

However, pheochromocytoma is a pathological condition, and in order to verify what happens to the human adipose organ in case of a physiological stimulus, Efremova et al. studied Siberian subjects.

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In this paper they show that Siberians, chronically exposed to low temperatures, have a much higher quantity of brown adipose tissue than that present in an adult usually exposed to milder temperatures. Visceral adipose tissue biopsies from these subjects show the presence of real brown adipocytes and paucilocular adipocytes, UCP1-positive, demonstrating that there is an interconversion of white adipocytes in brown adipocytes in physiological conditions. Therefore, not only the pathological conditions induce a direct conversion of white adipocytes into brown adipocytes, but there is a very clear response of human adipose tissue also to environmental physiological stimuli such as exposure to low temperatures (Efremova et al. 2020).

5. The obese adipose organ

The adipose organ when is constantly subject to an energy balance positive, to meet the needs of the organism to accumulate all the possible energies in anticipation of a possible fast, goes to meet a pathological remodeling with a series of morphological and functional alterations, such that it becomes a dysfunctional organ. The progression towards obesity is characterized by both an increase in the number and an increase in the size of white adipocytes, phenomena respectively defined as hyperplasia and hypertrophy (Johnson et al. 1972, Klyde et al. 1979, Cinti et al. 2005, Cinti 2018). Over time this tissue remodeling is responsible for an increase in the weight and size of the organ, as well as cellular and tissue abnormalities.

5.1 Chronic low-grade inflammation

A fundamental aspect that characterizes the obese adipose tissue is the remarkable macrophage infiltration, responsible for the chronic inflammatory state of the obese individual. In 2003, two independent groups (Weisberg et al. 2003, Xu et al. 2003) demonstrated that obese adipose tissues, both in animals and in humans, are infiltrated by inflammatory cells, especially macrophages, leading a chronic low grade inflammation and that this infiltration corresponds to the onset of insulin resistance. In addition, they discovered a positive correlation between the size of adipocytes and the number of macrophages infiltrating white adipose tissue. But why do macrophages infiltrate the adipose tissue? In 2005 Cinti et al. answered to this question with a study in which they demonstrated by immunohistochemistry that more than 90% of macrophages localized almost exclusively around what could appear to be adipose cells forming typical structures, the so-called Crown-like Structures (CLS) and that these CLS are much more numerous in the animals and in the obese individuals than the lean (*Figure 5*) (Cinti et al. 2005).
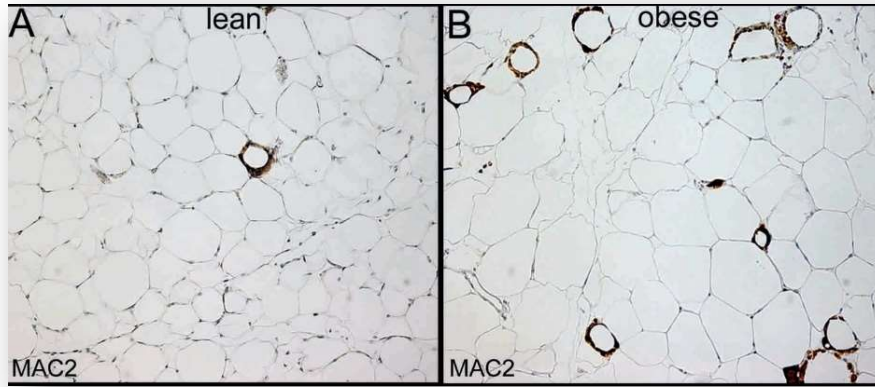
Figure 5: Immunohistochemistry showing MAC2 immunoreactive macrophages, arranged to form Crown-Like Structures in lean and obese mice. Cinti et al., 2005 J Lipid Res.

Subsequently, electron microscopy and immunohistochemical analysis, revealed that a CLS is the site of a dead adipocyte, and that macrophages are essential to reabsorb cellular debris. It is also shown that CLS are more present in the visceral adipose depot than the subcutaneous one, suggesting therefore that the visceral adipose cells have a "critical death size" smaller than the subcutaneous adipocytes (Murano et al. 2008).

A further demonstration that CLS are related to adipocyte death, was a transgenic model in which, by injecting a specific drug into mice, caspase 8 is expressed (a protein that induces cell death), specifically in adipose tissues. By studying the time course of events happening in fat after death induction, it was shown that there was a progressive loss of PLIN1 (therefore now considered a marker of viable adipocytes) and a subsequent infiltration of fat by macrophages. The number of CLS increased progressively and, in the end stages (after about seven days), all dead adipocytes gave rise to a CLS. These data strongly confirmed the idea that obese adipocytes die and give rise to remnants that must be reabsorbed under the form of CLS (Pajvani et al. 2005, Murano et al. 2013).
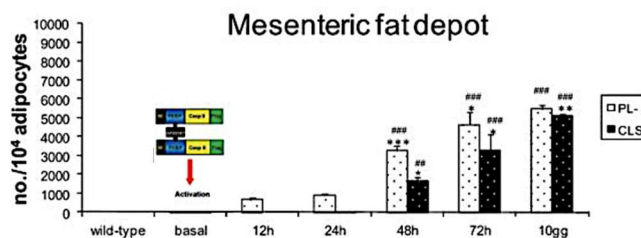

Figure 6: Time course of histomorphological changes in adipose tissue of a transgenic model ("fat attac") in which the administration of a dimerizator induces death specifically in adipose cells. Murano et al., 2013 Nutr Metab Cardiovasc Dis.

In order to understand the cause of death of obese adipocytes, a model of genetically modified mice, lacking the enzyme Hormon Sensitive Lipase (HSL), important for lipolysis, was studied. In this animal model, the fat cells accumulate lipids but do not release them: they become hypertrophic and then die, although the animals are not obese but are thin. This study was the demonstration that cell death is not due from obesity per se but depends only by the hypertrophic state of adipocytes. (Cinti et al. 2005). But what is the molecular mechanism of death of obese adipose cells?

Giordano et al. have demonstrated, through morphological and molecular studies, the mechanism through which hypertrophic adipocytes die. They have highlighted and quantified a whole series of organular alterations in hypertrophic adipose cells, such as mithochondria dismorphisms, dilated RER, glycogen accumulation, cholesterol crystals. All these alterations were higher in the visceral than in the subcutaneous depot. Among these alterations, the cholesterol crystals have a particular importance because they have been shown as responsible for the activation of a protein complex called NLRP3 inflammosome. It, in turn, activates the caspase-1, responsible for the activation of interleukins IL1β and IL18 that lead the cell to death. This particular mechanism of death is called "pyroptosis" (Giordano et al. 2013).

Several data suggest a greater pathogenicity of visceral than subcutaneous adipose tissue. It has been noted in humans that a high value of WHR ("Waist hip to ratio"), is more likely to correlate with comorbidities, such as diabetes, despite a low BMI. In contrast, a low WHR correlates to a low probability of comorbidity, despite a high BMI (Ohlson et al. 1985). Furthermore, considering the well-known positive correlation between adipocytes size and number of infiltrating macrophages (Weisberg et al. 2003), it was expected an higher infiltration of subcutaneous fat than visceral fat because subcutaneous adipocytes are larger than visceral adipocytes. However data showed an higher number of macrophages (CLS) in visceral than subcutaneous fat (Murano et al. 2008). These data suggested the "critical death size theory" suggesting a lower critical death size for visceral fat (Cinti 2009a). The hypothesis to explain the difference was that visceral fat in adults derive from conversion of brown adipocytes in white adipocytes and the subsequent experimental data using ATGL KO mice fully confirmed the theory (Kotzbeck et al. 2018). These data seem to offer an explanation to the well known clinical data that "apple shaped" visceral obesity, typical of men, is more pathogenic and often complicated by collateral diseases (first of all type2 diabetes), than subcutaneous obesity "pear-shaped obesity" characteristic of women.

5.2 Remodeling of the extracellular matrix

Physiologically adipose cells are embedded into a network of proteins of extracellular matrix (ECM), necessary to maintain the structural integrity of these cells and therefore to provide mechanical

support. In addition, extracellular matrix has a key role in adipogenesis (Sun, Tordjman, et al. 2013, Khan et al. 2009).

During the development of obesity, profound changes occur in the composition of the ECM which undergoes a real structural remodeling. These modifications concern an abnormal deposition of collagen fibrils or fibrosis. Therefore, a variation of the protein components of the extracellular matrix occurs that increase their expression leading to an excessive accumulation in the entire adipose tissue (DeBari et al. 2020, Marcelin et al. 2019). Thus, in case of positive energy balance, this increased protein of ECM deposition, such as various types of collagen, induces a state of local fibrosis and it results in an increased total rigidity of adipose tissue and a reduced expandability. Increased ECM stiffness of adipose tissue counteracts the continuous pressure of adipocytes to expand (Divoux et al. 2011, Khan et al. 2009). These two opposing actions could cause, over time, an increasing mechanical stress until triggering mechanisms that determine the death of adipocytes and the consequent inflammation.

Type 1, 3 and 6 Collagen appear particularly abundant in obese adipose tissue (Divoux et al. 2010, Divoux et al. 2011, Sun, Tordjman, et al. 2013, Khan et al. 2009). In particular, Collagen 6 is abundantly produced and secreted by adipocytes (Scherer et al. 1998, Iyengar et al. 2005, Mariman et al. 2010). It is the type of collagen most expressed in different adipose depots in mice (Khan et al. 2009) and it has been found to increase in human obesity and so its expression correlates with BMI (Pasarica et al. 2009). Collagen 6, unlike Collagen 1 and 3 that are fibrillar, is a type of non-fibrillar collagen that plays a structural role in stabilizing fibrillar collagens (Sardone et al. 2016). It consists of three polypeptide chains (α1, α2, α3), encoded by the genes Col6a1, Col6a2, Col6a3, which assemble to form a heterotrimer. The heterotrimers, in turn, associate first in dimers and then in tetramers which are secreted in the ECM (Sardone et al. 2016, Karousou et al. 2014) giving rise to microfibrils with 100 nm periodicity (Sardone et al. 2016, Bruns et al. 1986).

However, the literature is controversial regarding the production of Collagen 6 in obesity. The above-mentioned works support an increase in the expression of Collagen 6, but other works support the contrary. Dankel et al., demonstrate that the gene expression of Col6a3 correlates with insulin resistance regardless of BMI; that it is greater in obese compared with lean subjects but that it negatively correlates with the size of the adipocytes, thus small adipocytes produce more Collagen 6 compared to large adipocytes (Dankel et al. 2014). McCulloch et al., argue that Collagen 6 is reduced in obese subjects, both in visceral and subcutaneous adipose tissue, compared to lean individuals, and that its expression increases following weight loss, induced by diet or bariatric surgery (McCulloch et al. 2015), also in line with other works (Dankel et al. 2010, Henegar et al. 2008).

Therefore, the link between obesity and fibrosis in humans is not yet completely clear and further studies on the origin and mechanisms that induce fibrosis are needed. However, fibrosis appears to be a response to adipocyte hypertrophy, since after the increase in the size of the adipose cells, an upregulation of the ECM components is triggered (Khan et al. 2009).

Probably the factor that leads from hypertrophy to fibrosis is hypoxia, since in obesity when the adipocytes expand, they reach the diffusional limit of oxygen and oxygenation is reduced. Under these conditions the adipose tissue becomes hypoxic because the vascular system is unable to keep up with the increase in size, thus an increase in expression of Hypoxia-Inducible Factor 1α (HIF1α) occurs (Sun, Halberg, et al. 2013, Crewe et al. 2017). HIF1 is the key reactive protein of hypoxia. It consists of two subunits, α and β. HIF1β is constitutively expressed; HIF1α is oxygen-sensitive, so in normoxigenation conditions of fat it is degraded by prolyl hydroxylation while under hypoxic conditions its expression is induced due to a reduced level of prolyl hydroxylation (Halberg et al. 2009, Sun et al. 2011). Moreover, it has been found that an overexpression of HIF1 α leads to the condition of fibrosis with an upregulation of collagen. Thus, hypoxia could be implicated in the development of fibrosis (Halberg et al. 2009, Divoux et al. 2011).

Therefore, fibrosis and hypoxia are two of the main correlated hallmarks of dysfunctional obese adipose tissue that prevent a healthy expansion during positive energy balance. However, many aspects of their interrelationships are still unknown and a deeper knowledge of histopathology of obese adipose tissue could help to detect new therapeutic targets to combat obesity and its related diseases.

AIM OF THE PROJECT

The obese adipose organ in humans and mice, constantly subjected to a state of caloric excess, becomes dysfunctional and undergoes a pathological remodeling mainly due to hypertrophy and hyperplasia of adipocytes, which over time determines cellular and tissue abnormalities. These are responsible for a chronic inflammatory state that induces insulin resistance, hyperinsulinemia, type 2 diabetes mellitus and various related complications (Hotamisligil 2017, Giordano et al. 2016). Therefore, one of the most studied fundamental problems related to obesity is precisely the pathogenesis of chronic low-grade inflammation of adipose tissues.

The purpose of this thesis is to add new morphological studies of human obese adipose tissues, with particular attention to phenomena of inflammation and fibrosis. In particular, the main aim is to investigate whether inflammation in the adipose tissue of obese subjects, in addition to being due to the proven death for pyroptosis (Cinti et al. 2005, Giordano et al. 2013), could also be caused by other phenomena related to fibrosis.

The extreme variability of genetic background in humans imposes the study of a high number of subjects also in consideration that published work in this field mainly report a small number of studied subjects.

MATERIALS AND METHODS

1. Patients and experimental conditions

Adipose tissue biopsies were obtained from 33 obese patients undergoing bariatric surgery, at Ospedali Riuniti of Ancona, Italy. During surgery a sample of Omental WAT (oWat) and Subcutaneous WAT (scWAT) from each patient were collected, for a total of 66 fat biopsies. In all cases informed written consent was obtained. Ethical Committee approved the study (Prot 208166 30/09/2008).

The 33 obese patients are mainly women: 22 women and 11 men, with an average age of 44 ± 8.5 years and BMI 43.6 ± 6 kg/m^2. Obese individuals were compared with 6 lean subjects undergoing cholecystectomy (age 61 ± 6.6 years; BMI 23.9 ± 2.7 kg/m^2; waist circumference < 88 cm for women and < 102 cm for men), from which a sample of visceral and subcutaneous fat was taken. Clinical data are reported in *Table 1.*

Table 1: Clinical data of obese patients

Patient	Sex (M/F)	Age (Years)	BMI (kg/m^2)	Diabetes
C. C.	F	38	50	YES
Z. C.	M	27	41	NO
P. O.	M	43	43	NO
M. M.	F	42	50	NO
B. E.	M	42	43	NO
R. G.	M	45	40,9	YES
B. E.	F	57	42,6	NO
L. B.	F	44	46	YES
P. S.	M	45	41	YES
U. G.	M	55	59,1	YES
P. F.	M	33	60	NO
M. R.	F	46	40	NO
M. E.	F	44	40,8	NO
V. M.	F	26	36,4	NO
T. A.	F	42	40	YES
P. G.	M	33	43,3	NO
H. E.	F	41	40	NO
B. C.	F	53	43,25	NO
M. S.	F	42	38	NO
P. A.	F	55	43,3	NO
G. A.	F	32	43,6	NO

P. M.	F	47	29,76	NO
B. L.	F	32	48	NO
B. A.	M	44	45,6	NO
P. M.	F	56	41	YES
B. G.	M	56	44.5	NO
Q. M.	F	46	48,28	NO
G. G.	M	48	38,9	NO
C. O.	F	56	43,9	NO
S. L.	F	52	44,6	NO
P. A.	F	54	43,6	YES
D. F.	F	46	42	NO
M. P.	F	46	45	YES

2. Light microscopy

For the morphological study in light microscopy all the samples of visceral and subcutaneous fat were processed with the same treatment. Briefly, all the adipose depots were first fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 overnight at 4 ° C and then dehydrated through passages in alcohol with increasing concentration, clarified in xylene and finally embedded in paraffin blocks (Cinti et al. 2001). From each adipose biopsy serial paraffin sections (3 μm thick) have been cut on a sliding microtome, posed on glass slides, and dried.

2.1 Immunohistochemistry

Paraffin sections, 3 μm thick, were rehydrated and reacted with 0.3% H_2O_2 (in distilled water for 5 min) to block endogenous peroxidase, rinsed in PBS and treated with 2% normal serum blocking solution (diluted in PBS for 20 min) to block non-specific binding of the secondary antibody. Then sections have been incubated with primary antibodies (in PBS overnight at 4°C) (***Table II***). After rinsing in PBS, biotinylated secondary antibody was added (1:200 in PBS for 30 min; Vector Laboratories, Burlingame, CA). Sections were rinsed again in PBS and incubated with Avidin-Biotin peroxidase complex (1:100 in PBS for 60 min; Vectastain ABC kit Vector Laboratories). Sections were rinsed in PBS several times and finally the substrate Sigma Fast 3,3-diaminobenzidine (Sigma-Aldrich, Vienna, Austria) was added for 3 min. After immunohistochemical staining, sections were counterstained with hematoxylin, dehydrated in ethanol, cleared with xylene and covered with Eukitt® mounting medium (Sigma-Aldrich). For negative control the primary antibody was omitted, and staining was never observed in these samples. Unmasking procedures, with citric acid at 96 °C for 20 minutes, were used for immunohistochemical detection of CD68.

Table II: The different primary antibodies used in this study are shown.

Marker	Host/isotype	IHC	WB	Manufacturer
Perilipin 1	Polyclonal rabbit	1:800		Abcam 3526
CD68	Monoclonal mouse	1:200		Dako M0814
Caspase-1	Monoclonal mouse	1:200		Novus Biologicals 14F468
Hif1α	Monoclonal mouse		1:2000	Thermofischer MA1-516

2.2 Sirius Red staining

Sirius Red staining is one of the best-known histochemical techniques able to highlight the collagen fiber network, which appear red on paraffin sections (Malkusch et al. 1995).

The samples were dewaxed in xylene and rehydrated with sequential immersions in alcohols with decreasing concentration. Later they were incubated with Sirius Red dye for one hour at room temperature. After abundant washing in water, to remove excess dye, the sections were contrasted with Hematoxylin for 2 minutes, dehydrated and finally mounted with Eukitt.

2.3 Morphometric analysis

Tissue sections, used for the different staining techniques just described above, were observed with a light microscope (Nikon Eclipse E800) and then digital images were captured with Nikon camera (DXM 1200). All images were analyzed with ImageJ morphometric program (RRID:SCR_003070). First of all, to check the size of the adipocytes, the average area of 200 adipocytes, immunoreactive for Plin1, per patient was measured at 10x magnification. Then, in order to verify whether fibrosis can be related to death of adipocytes, it was considered useful to quantify the perilipin-negative areas and then relate them to the fibrotic areas.

- For the quantification of immunonegative areas for Perilipin 1, the visceral and subcutaneous adipose tissue of each patient was captured at 4x magnification in a different number of pictures based on the section size (in average 10 pictures / biopsy). With ImageJ the percentage of negativity to Perilipin 1, for both adipose depots of each patient, was measured. In particular, in each image the total tissue area was first calculated and, subsequently, the negative tissue area. Finally, the mean of negativity to Perilipin 1 of adipocytes of all patients, in both omentum and subcutaneous fat, was calculated.

- For the quantification of fibrosis, visceral and subcutaneous adipose tissues stained with Sirius Red were captured at 4x magnification with an average of 10 pictures / biopsy. The percentage of total fibrosis of all fat samples for all patients was measured using ImageJ software. In particular to quantify the collagen content, stained red, the image is converted first to a grayscale and then "isolated" the color red using the Threshold. At this point the percentage of total fibrosis was calculated. It was expressed as the ratio of the red stained area (fibrotic) to the total tissue area.

- Furthermore, to study the inflammatory state that characterizes the obese adipose tissues considered, a quantification of CD68+ macrophages was made. About 20 random fields at 20x magnification were captured for each tissue section and then CD68 positive macrophages, distributed in five tissue districts (see below), were counted with ImageJ and normalized to 10^4 adipocytes. More precisely, the five districts where macrophages have been counted: 1) around the dead adipocytes (arranged in CLS), 2) within fibrotic areas, 3) into the parenchyma, 4) inside the vessels and 5) around the vessels. For each patient the percentage of CD68+ macrophages, present in each of the five tissue regions analyzed, was calculated, and finally the total average for both the visceral and the subcutaneous fat was also calculated. Moreover, CLS density (CLS per 10^4 adipocytes) was also determined (Cinti et al. 2005).

3. Transmission electron microscopy

For the ultrastructural study omental and subcutaneous fat samples were also analyzed by Transmission Electron Microscopy (TEM). First of all, small tissue fragments were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L PB, pH 7.4 for 4 h at room temperature. Then they were postfixed in 1% osmium tetroxide (for 1 h at 4°C), dehydrated through steps with acetone at increasing gradation of and embedded in a mixture of Epon-Araldite. For each sample were cut semithin sections, 1,5 μm thick, and stained with toluidine blue. Thin sections (60 nm) were obtained with an MT-X ultratome (RMC; Tucson, AZ), stained with lead citrate and examined with a Philips CM 10 transmission electron microscope (Philips, Eindhoven,Netherlands). For each patient at least three different thin sections of different areas selected on semithin sections stained with toluidine blue were observed.

4. Quantitative real time-polymerase chain reaction

Frozen tissues were disrupted with Trizol reagent throught high-speed shaking (50 Hz for 5 minutes) in plastic tubes with stainless steel beads using TissueLyser LT (Qiagen, Milano). Total RNA was

extracted using RNeasy Micro kit (Qiagen, Milano, Italy) according to the respective manufacturer's instructions. To determinate the messenger RNA (mRNA) levels, 1 μg RNA was reverse-transcribed with a High-Capacity cDNA RT Kit with RNase inhibitor (Applied BioSystems, FosterCity, CA) in a total volume of 20 μl. qRT-PCR was performed in triplicate using TaqMan Gene Expression Assays (Applied BioSystems). All TaqMan probes were shown in **Table III**. Reactions were carried out in a Step One Plus Real Time PCR System (Applied Biosystems) using 50 ng RNA in a final reaction volume of 10 μl. The thermal cycle protocol was the following: initial incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 20 s. To rule out genomic contamination, a control reaction was included for each sample in which reverse transcriptase was omitted in the amplification mix. Relative mRNA expression was determined by the ΔCt method ($2^{-\Delta Ct}$) using TATA box-binding protein (TBP) levels as the endogenous control. Data are presented as histograms reporting the mean ± SEM.

Table III: List of the TaqMan probes used in qRT-PCR.

Gene	Assay ID
HIF1α	Hs00153153_m1
COL6a3	Hs00915125_m1
MCP1 (CCL2)	Hs00234140_m1
TBP	Hs00427620_m1

5. Western Blotting

Proteins were obtained using RIPA buffer, containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 1 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonylfluoride, and 50 mg/ml aprotinin. Tissue fragments were homogenized and cleared by centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Richmond, CA, USA) and so an equal amount of proteins were loaded onto homemade polyacrylamide gels. Proteins were first seperated using Sodium Sodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane using a trans-blot turbo transfer system (Bio-Rad). To check loading and transfer efficiency, bands were visualized with Ponceau S solution (Santa Cruz Biotechnology, Santa Cruz,

CA, USA). Membranes were incubated in 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h to block not specific binding of antibodies and subsequently incubated overnight at 4°C with the primary antibody HIF1α (1:2000 in TBS) (***Table II***). Membranes were washed with TBS-T three times and incubated with the anti-mouse secondary antibody (in TBS-T for 2 h). Finally, membranes were developed using clarity ECL substrate and the bands were visualized with the Chemidoc Imaging system (all from Bio-Rad). The immunoreactive bands were quantitated with Bio-Rad's Image Lab software. Where appropriate, membranes were stripped, washed, and re-probed for total protein content.

6. Statistics

Data were analysed using GraphPad PRISM (8.4) and were reported as mean ± standard error of the mean (SEM). Student's t-tests, in case of two groups, and 1-way ANOVA, in case of more then 2 groups, were used. Moreover, the Pearson correlation coefficient (r) and its significance (p) were calculated between variables. Group differences were considered significant for $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS

1. The human obese adipose tissue contains a large amount of non-vital adipocytes

Previous work showed that obese adipose tissue is hypertrophic and contain dead adipocytes forming CLS (see introduction chapter). Thus, we measured the adipocyte size. Data confirmed the hypertrophy as expected from previous work (Camastra et al. 2017) (**Figure 7**).
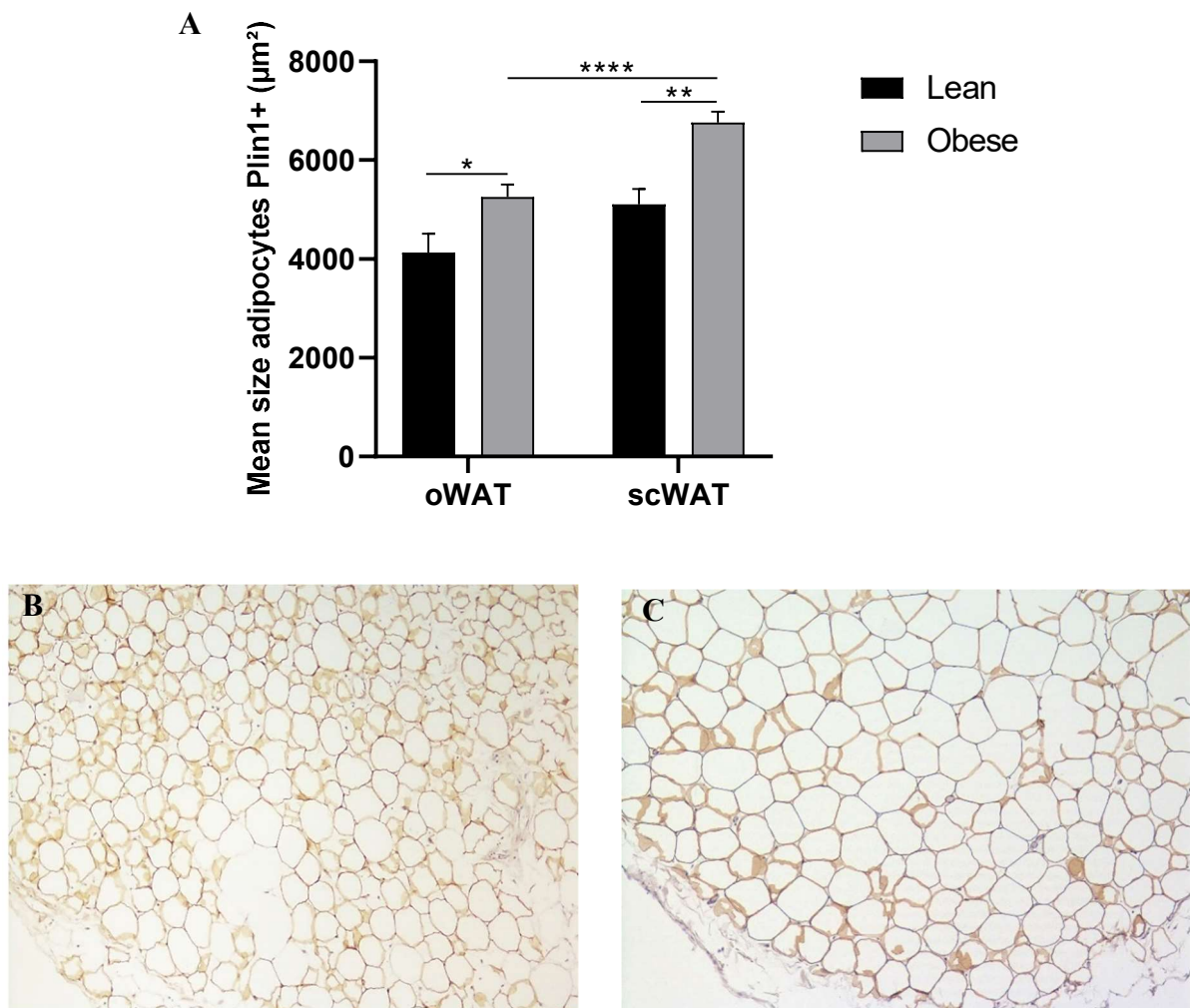

Figure 7: A) Comparison of white adipocytes, Plin1 positive, area in oWAT and scWAT from obese and lean patients; B) Representative microscopy pictures (IHC with Plin1) of adipocyte of omental adipose depot from control subject and C) obese patient. Data are means ± SEM; *p < 0.05.

As shown in **Figure 8** we found a new observation: several dead adipocytes (PLIN1 negative) were present not only in CLS but also without any apparent relationship with macrophages (Cinti et al. 2005, Murano et al. 2013).
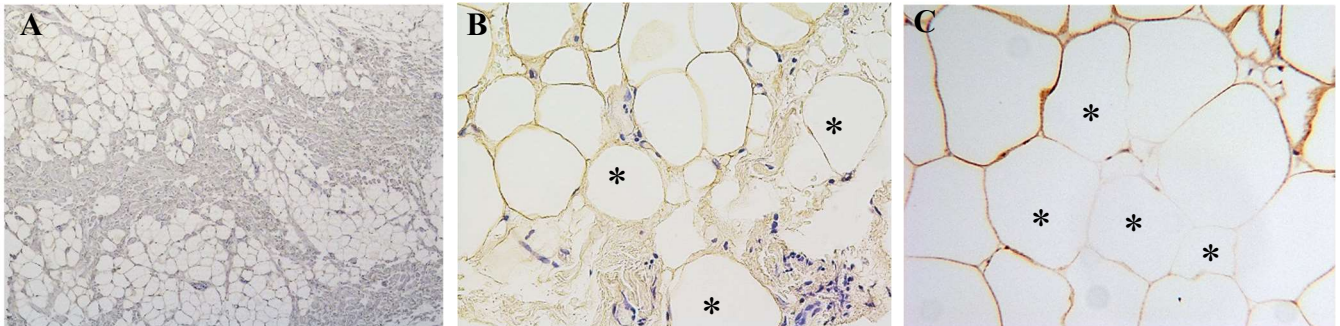
Figure 8: Pictures by light microscopy of Plin1-immunostained section tissues in which entire adipocytic areas (A) or single adipocytes (B and C) appear non-viable, Perilipin-negative (asterisks).

Starting from these first morphological and immunohistochemistry observations, we performed a quantitative measurement of not-immunoreactive areas to Perilipin 1 in every tissue sections. In particular, we performed a morphometric analysis by calculating the percentage of PLIN1negative area in the whole omental and subcutaneous fat samples of all patients. Data showed that non-vital adipocytes occupy about 13% and 16% respectively of the whole oWAT and scWAT examined in all obese patients. Thus, the percentage of non-vital adipocytes was about 4-6 times larger than that of control group (**Figure 9**).
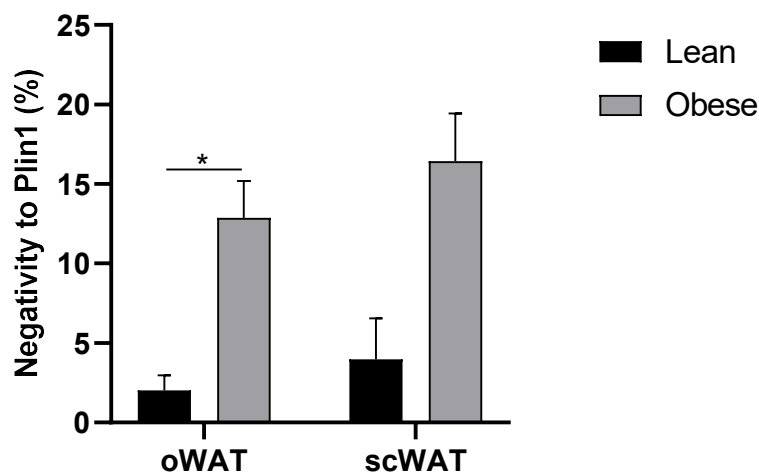

Figure 9: Comparison of percentages relative to metabolically not vital white adipocytes (Plin1-negative: dead adipocytes), in oWAT and scWAT in obese and lean patients. Data are presented as mean ± SEM.

Furthermore, a significant positive correlation (***Figure 10***) was found between Plin1-negative adipocytes in the visceral and subcutaneous fat of obese subjects, which allows us to rule out that, in obese people, adipocytic death a casual tissue event and related to a single adipose depot.
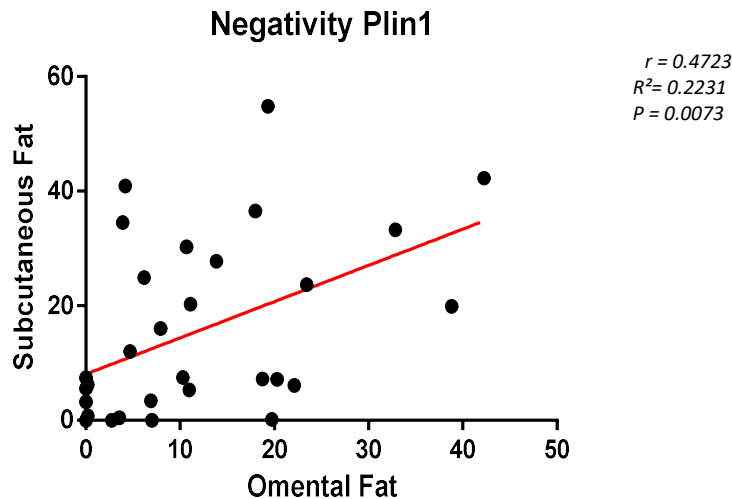
Negativity Plin1

r = 0.4723
R²= 0.2231
P = 0.0073

Figure 10: *Positive significant association between percentage of negativity to Plin1 in the oWAT and scWAT from all obese patients. Pearson correlation coefficient and P-value are shown.*

2. Plin1-negative adipose tissue areas correspond to fibrotic areas

In order to try to understand why a large amount of dead adipose cells are found in both the omental and subcutaneous compartments in obese people, we carefully studied by light microscopy the general tissue organization of adipose tissue sections. This study allowed us to realize that groups of cells or individual adipose cells are surrounded by fibrosis. Indeed, as shown in ***Figure 11*** it was quite evident, with different histochemical techniques, such as Hematoxylin-Eosin, Sirius Red staining and Toluidine blue, that fibrotic bundles surrounding the obese adipocytes.
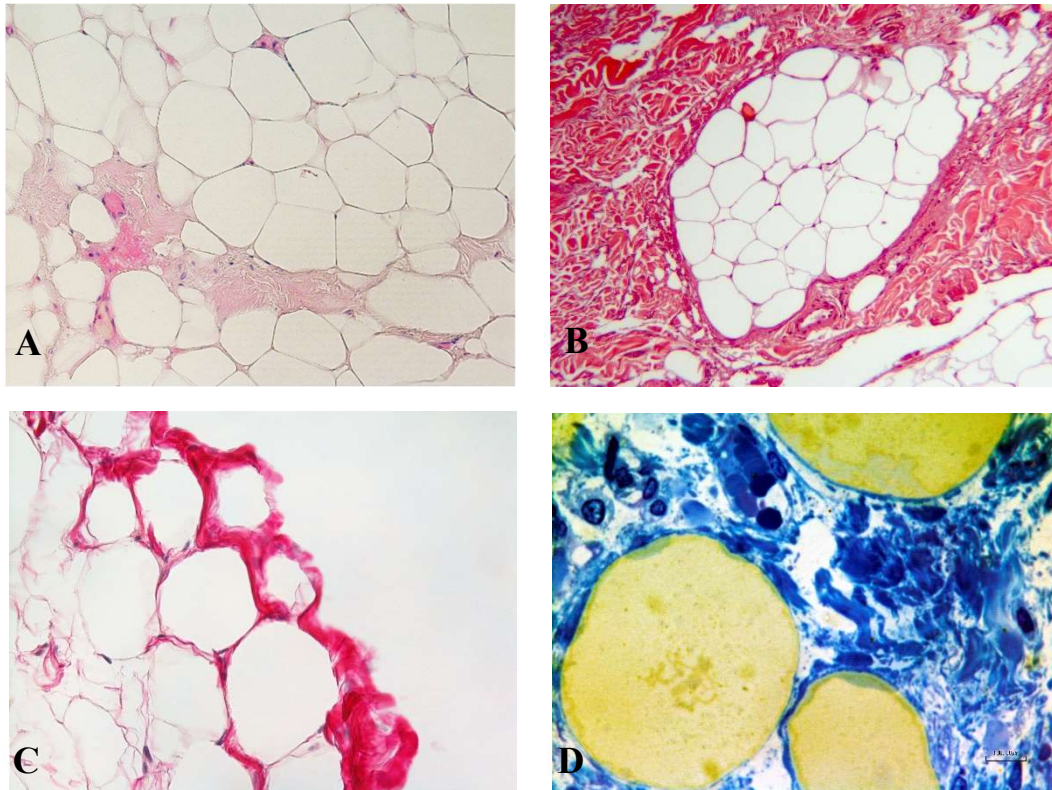
Figure 11: *Representative light microscopy images showing that obese adipose tissues are infiltrated by fibrosis with various histochemical techniques, including hematoxylin-eosin (A), sirius red staining (B and C) and toluidine blue (D).*

Subsequently, it has been found that most non-viable adipocytes localize in fibrotic areas. In fact, comparing serial sections of adipose tissue, immunostained for Plin1 (vital staining) and Sirius Red (fibrosis), it was clear that the adipocytic areas negative to Perilipin 1 corresponded to the areas infiltrated by fibrosis (*Figure 12*). These observations raised the question of a possible link between fibrosis and the loss of immunoreactivity to Perilipin 1. In other words, fibrosis could play an important role in adipocytes death.
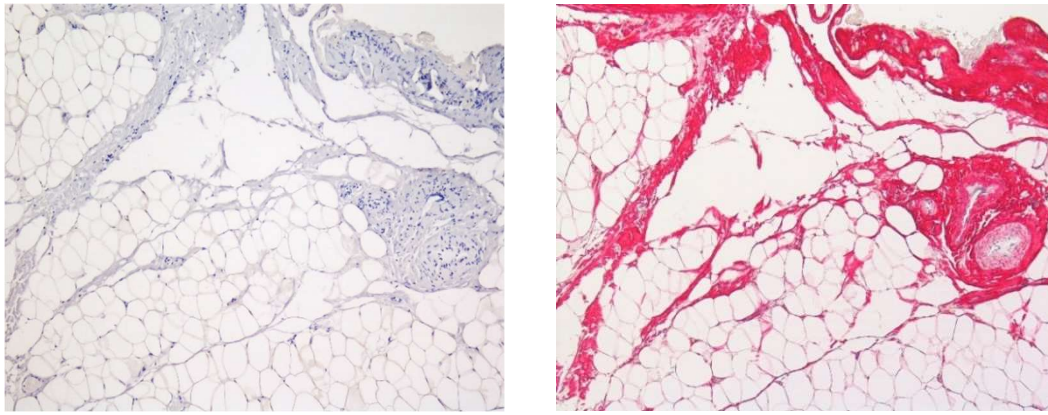
Figure 12: oWAT serial sections of an obese woman showing that a region of tissue with adipose cells not immunoreactive to Perilipin1 (left) corresponds to the same area of fibrosis stained with Sirius Red (right).

To assess the total amount of fibrosis, detected with Sirius Red, we performed a morphometric analysis for each adipose depot both in obese than in lean individuals. As expected, the degree of fibrosis is higher in obese subjects compared to control group, but it resulted significant only in the visceral adipose tissue as shown in ***Figure 13***. In order to evaluate if excessive deposition of fibrosis affects the vitality of the adipose cells, it was considered useful to relate the degree of total fibrosis to the amount of dead adipocytes for each patient and a significant positive correlation was observed in both adipose depots considered (***Figure 14***).
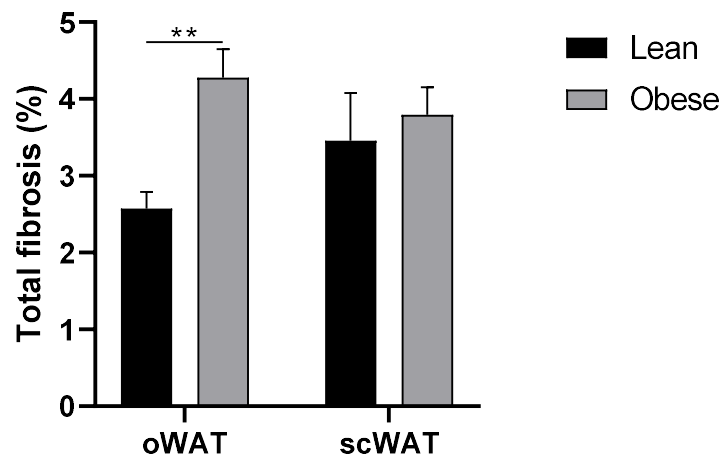

Figure 13: *Comparison of total fibrosis in oWAT and scWAT of lean and obese patients. Data are presented as mean ± SEM.*
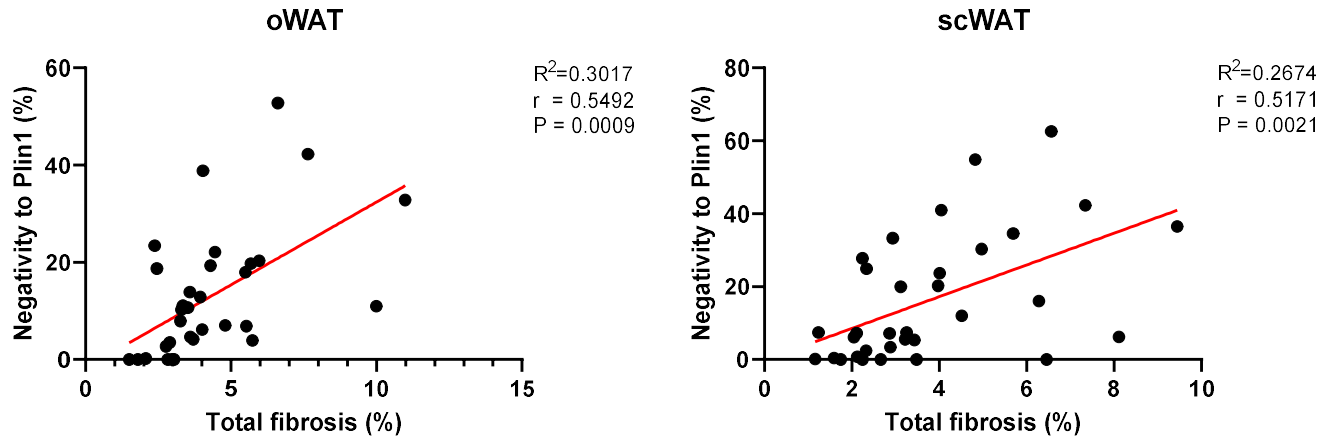
Figure 14: *Positive significant correlation between the degree of fibrosis and the amount of negative-Plin1 adipose cells among all obese patients, in visceral (left) and in subcutaneous adipose tissue (right). Pearson correlation coefficients and P-values are shown.*

In order to evaluate the oxygenation of the obese hypertrophic adipose tissue, infiltrated by fibrosis, we examined the level of gene and protein expression of HIF-1α, main regulator of cellular and systemic homeostatic response to hypoxia. As expected, qRT-PCR (***Figure 15***) and Western Blotting (***Figure 16***) analysis showed that both mRNA and protein levels increase in obese compared to lean subjects in both depots, although gene expression did not reach statistical significance.
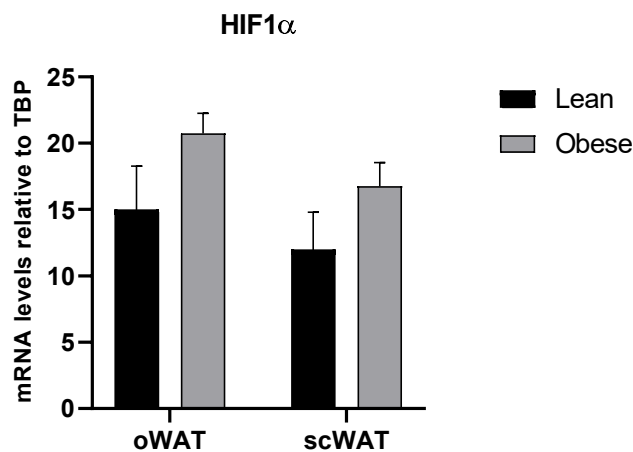

Figure 15*: qRT-PCR analysis of HIF1α mRNA level in omental and subcutaneous adipose tissue from obese and lean patients. Data are presented as mean ± SEM.*
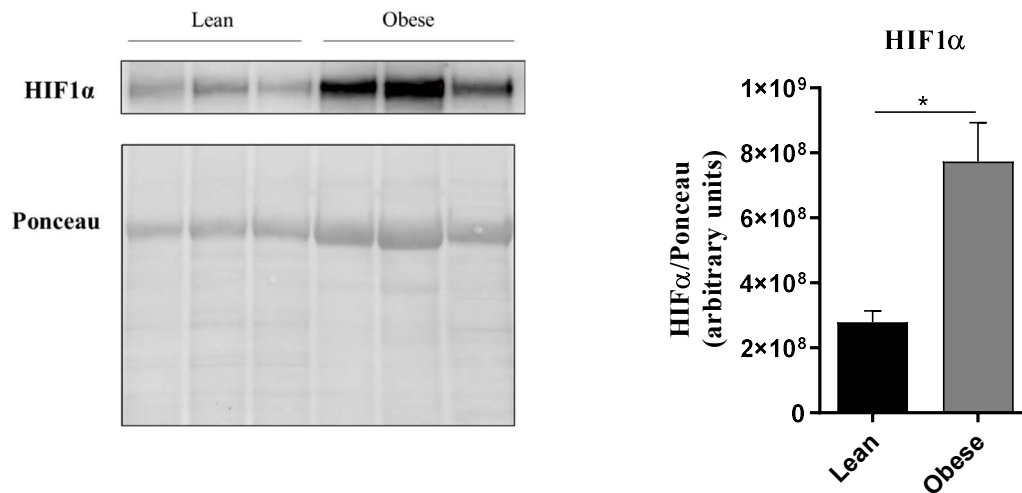
Figure 16: *Representative immunoblot and relative HIF1α quantification in omental adipose tissue from lean and obese patients. Data are presented as mean ± SEM.*

3. Inflammation of human obese adipose tissue is only partially related to CLS

Generally, obese adipose tissues are characterized by an accentuated inflammation compared to adipose tissues of control subjects. To assess the inflammatory state of the adipose tissue in obese patients, we first examined the expression levels of a specific inflammatory gene, MCP1, encoding for the protein involved in macrophage infiltration, in oWAT and scWAT. Indeed, as expected, qPCR analyses showed that the mRNA levels related to the gene, were higher in both visceral and subcutaneous depot in obese compared to controls (***Figure 17***).
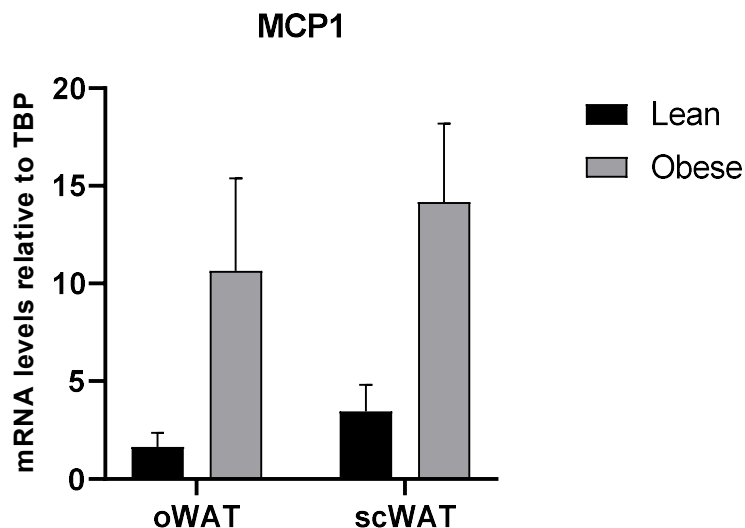

Figure 17*: qRT-PCR analysis of MCP1 mRNA level in omental and subcutaneous adipose tissue from obese and lean patients. Data are presented as mean ± SEM.*

Then to better characterize the inflammatory state of the adipose tissue in obese patients, we studied morphology of tissue sections and we observed that inflammatory cells are present both in relation to dead adipocytes, as shown in ***Figure 18,*** and in other locations. For this reason, immunohistochemical analysis with the antibody CD68, general marker of macrophages, in all samples from obese patients was performed. Subsequently, to see where macrophages localize in adipose tissues obese, we classified and quantified inflammation into five categories: CD68 macrophages located around the dead adipocytes (arranged in CLS), those within fibrotic areas, in the parenchyma, inside the vessels and around the vessels (***Figure 19***).
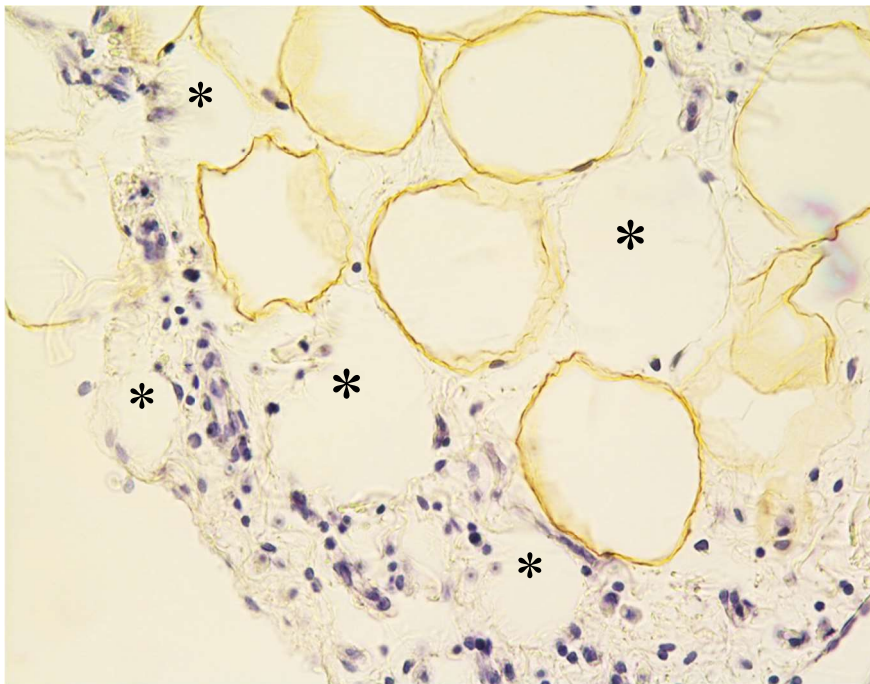

Figure 18*: Representative microscopy picture of immunohistochemical staining of Perilipin1, from an obese man, showing inflammatory cells in relation to Perilipin1-negative adipocytes not vital, (marked with asterisk).*
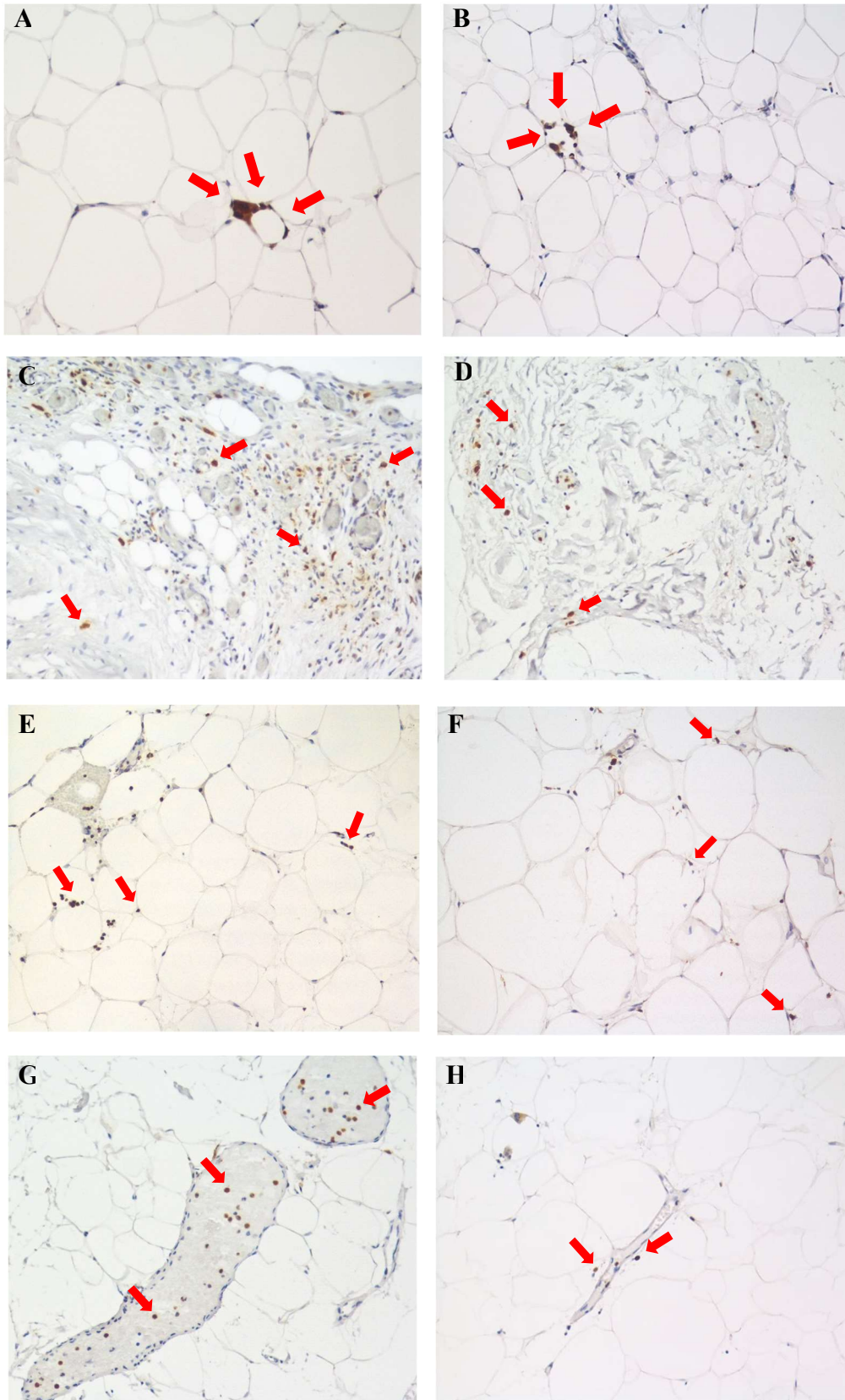
Figure 19*: Representative detection of immunohistochemical staining of CD68 from four obese patients showing macrophages CD68+ (arrows) arranged in CLS (A and B), in fibrotic areas (C and D), into parenchyma (E and F), inside the vessels (G) and around (H) the vessels.*

From the morphometric analysis, we found that macrophages density in the visceral depot was positively correlated with that of macrophages in the subcutaneous (***Figure 20***). Furthermore, the quantitative analisys of anatomical distribution of CD68 immunoreactive macrophages suggested a similar type of inflammatory status in both depots. In particular, most macrophages appeared to be widespread in the adipose parenchyma (***Figure 21***), unlike murine obesity in which more than 90% of macrophages were found associated in CLS (Cinti et al. 2005)**.** Thus, contrary to what we expected, the inflammation due to CLS is only a small fraction of all the inflammation present in the tissue. Moreover, from these results, it emerges that the density of CLS is significantly greater in the subcutaneous than in the omental fat (***Figure 22***).
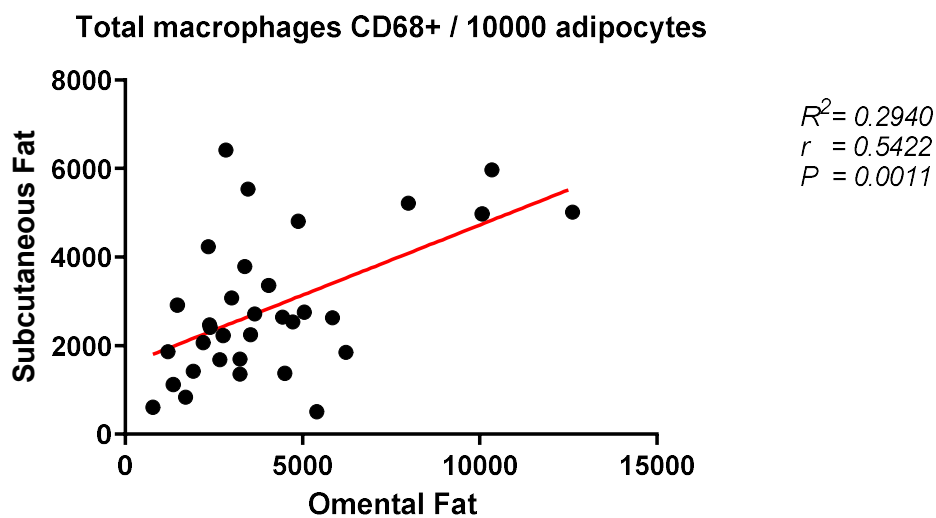

Figure 20*: Positive significant association between total CD68-positive macrophages present in oWAT and in scWAT from 33 obese patients. Pearson correlation coefficient and P value is shown.*
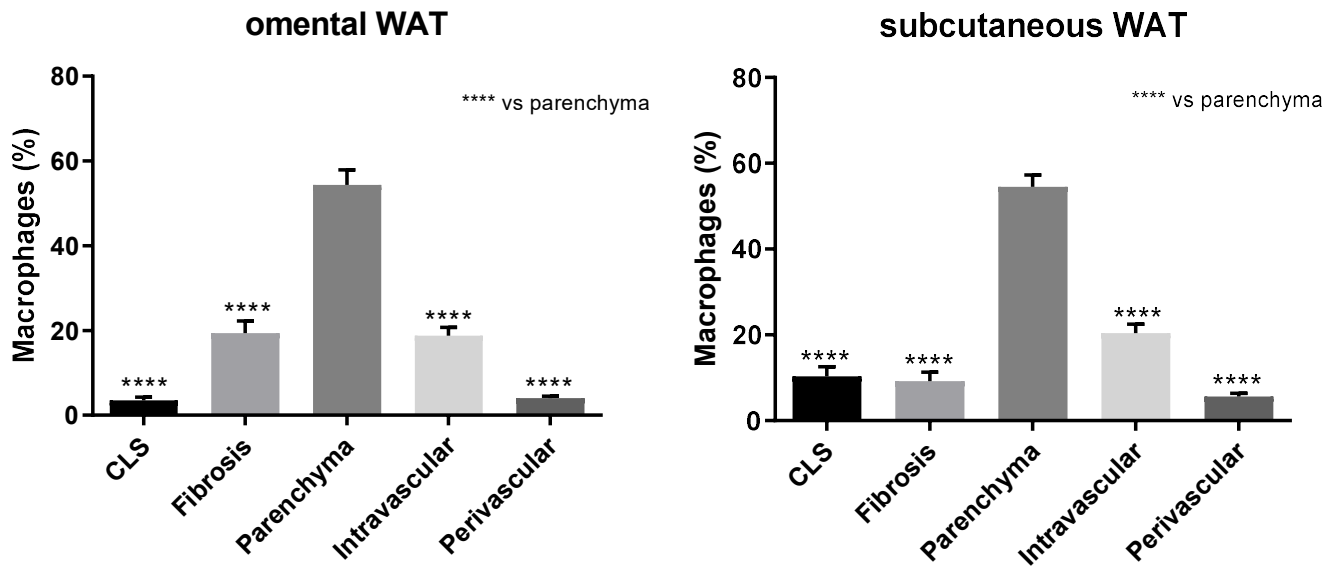
omental WAT

subcutaneous WAT

Figure 21: Morphometric analysis of distribution of CD68-positive macrophages in visceral (left) and subcutaneous (right) adipose tissue from 33 obese patients. The graphs show that most of macrophages are related to parenchyma, unlike the mouse model in which most of them are organized to form CLS. Data are presented as mean ± SEM.
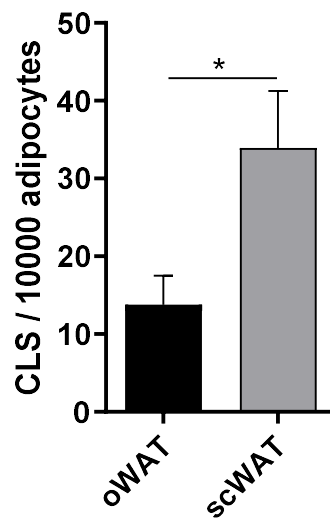

Figure 22: Comparison of CLS density in omental and subcutaneous adipose depot from 33 obese patients. The data is presented as mean ± SEM.

In order to see if the fibrosis is correlated to the amount of dead adipocytes and therefore to inflammation in each single patient considering the strong association between the amount of total fibrosis and the percentage of dead adipocytes (***Figure 14***), we correlated for each patient the level of fibrosis with the degree of inflammation, expressed as the total number of CD68 macrophages per 10^4 adipocytes. Positive significant correlations were found in both fat compartments (***Figure 23***).
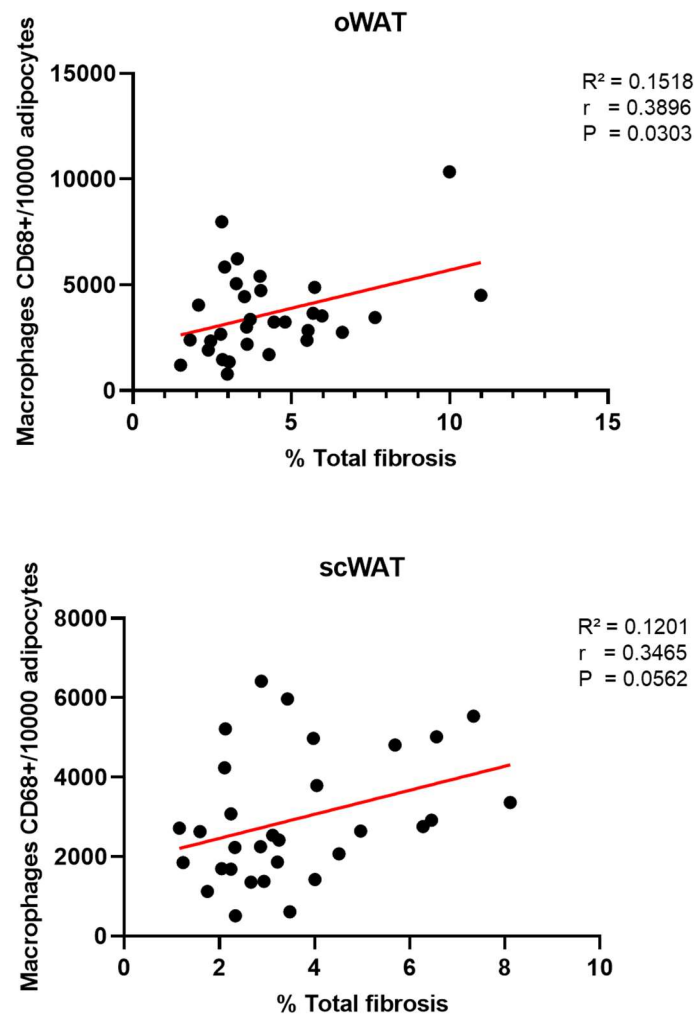

Figure 23*: Positive correlation between the degree of fibrosis and CD68 macrophages density from 33 obese patients, in omental (left) and in subcutaneous adipose tissue (right). Pearson correlation coefficients and P-values are shown.*

4. Hypertrophic adipocytes dead of pyroptosis are not related to fibrosis

In order to investigate the mechanism responsible for death of adipose cells in obese individuals, we evaluated if human obese adipocytes die of pyroptosis, as it occurs in murine obesity (Giordano et al. 2013). We performed immunohistochemical staining of Caspase-1, the main marker of pyroptosis death (Bergsbaken et al. 2009). Data showed Casp-1 positive adipocytes and positive macrophages in CLS. However, immunostaining does not appear to be related to fibrosis, because in the fibrotic regions of adipose tissue no positivity to the marker was detected (***Figure 24***). Therefore, as described above (***Figure 12***), most of the adipose cells surrounded by fibrosis resulted to be not vital, but we can exclude pyroptosis as the cause of death because of Casp-1 immuno-negativity. For these reasons, we confirmed that some hypertrophic adipocytes die for pyroptosis, but we also hypothesize that they die also for another type of death strongly related to fibrosis.
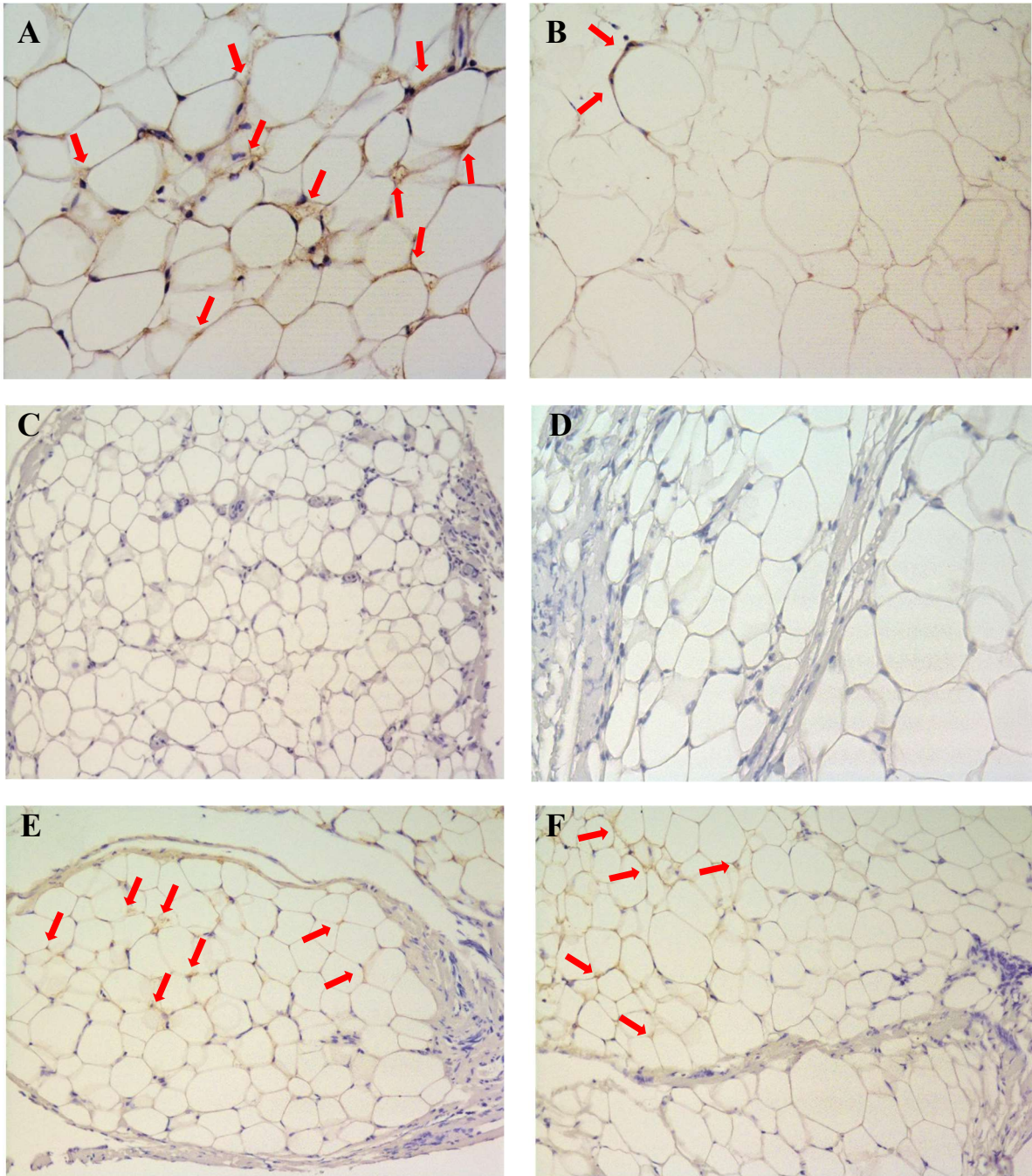
Figure 24*: Representative immunohistochemical staining of Caspase-1 from two obese patients. It is shown that some adipocytes (A) and macrophages, arranged in CLS (B) are Casp-1 positive (arrows highlight brownish precipitate). Instead, in C and in D it is possible to observe fibrotic areas in which no positivity to Casp-1 is present. E and F reveal, at the same time, adipose tissue with positivity for the marker Casp-1 in the parenchyma (arrows) but not near fibrotic tissue areas.*

5. Ultrastructural features show stress signs in human obese adipose cells

In order to further investigate the link between fibrosis and adipocytes, we studied the ultrastructural aspects of human adipose tissue samples. Considering the ultrastructure of normal adipose tissue (Cinti 1999) it must be outlined that only few collagen fibrils are present in tight relationship with the external surface of the basal membrane surrounding any single adipocyte. In murine obesity the amount of collagen fibrils surrounding every single hypertrophic adipocyte is a very common finding and SEM analyses revealed that every hypertrophic adipocyte is surrounded by an increased amount of fibrils (Giordano et al. 2013). Considering all together these data strongly suggest the cell responsible for the collagen production is the adipocyte itself. Data in vitro are in line with this hypothesis (Iyengar et al. 2005). Electron microscopy data strongly suggest that adipocytes produce a relevant amount of collagen fibrils that often surround every single adipocyte (*Figure 25*). The arrangement of collagen fibrils, closely associated with the external surface of adipose cells, resulted very similar to that observed in murine obese adipocytes (Giordano et al. 2013). In particular, it is evident the increased amount of collagen fibrils, in close contact with the external surface of basal lamina of hypertrophic adipocytes often surrounding the whole single adipocyte or only a part of it. Furthermore, these hypertrophic obese adipose cells exhibit ultrastructural features of stress and cellular degeneration, such as the infiltration of small lipid droplets into the cytoplasm, dilatation of RER, increase density and irregularity of the basal membrane (*Figure 26*). Therefore, our data seem to suggest that the pathology process described in murine obesity is present also in obese humans. However, in lean patients, less fibrils collagen were observed outside the basal membrane of the adipocytes than in obese adipose cells, as shown in *Figure 27*.
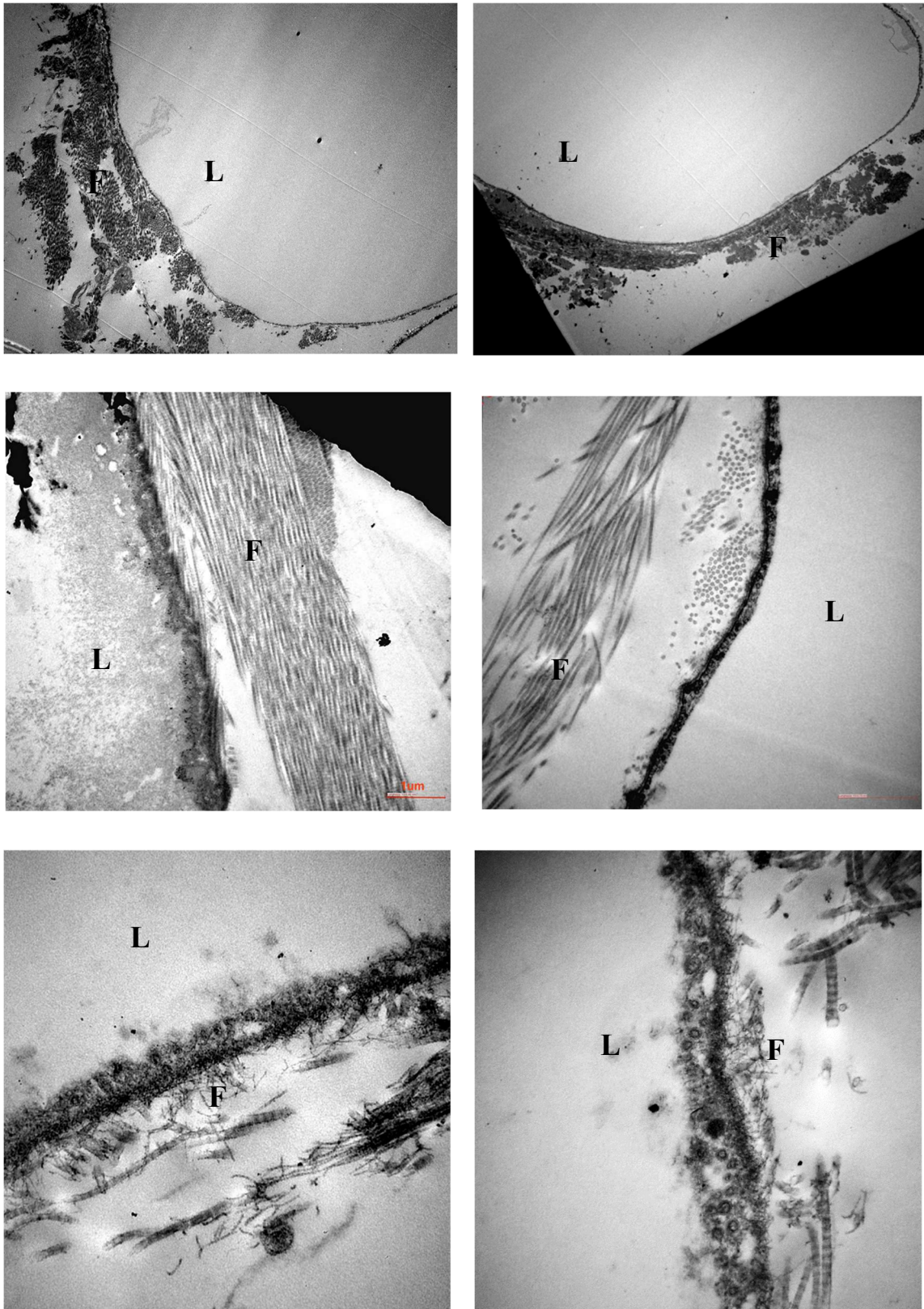
Figure 25*: Transmission Electron Microscopy showing fibrosis (F) in close contact with adipose cells from three obese patients. Numerous collagen fibrils surrounding hypertrophic adipocytes were observed. L = lipid droplet.*
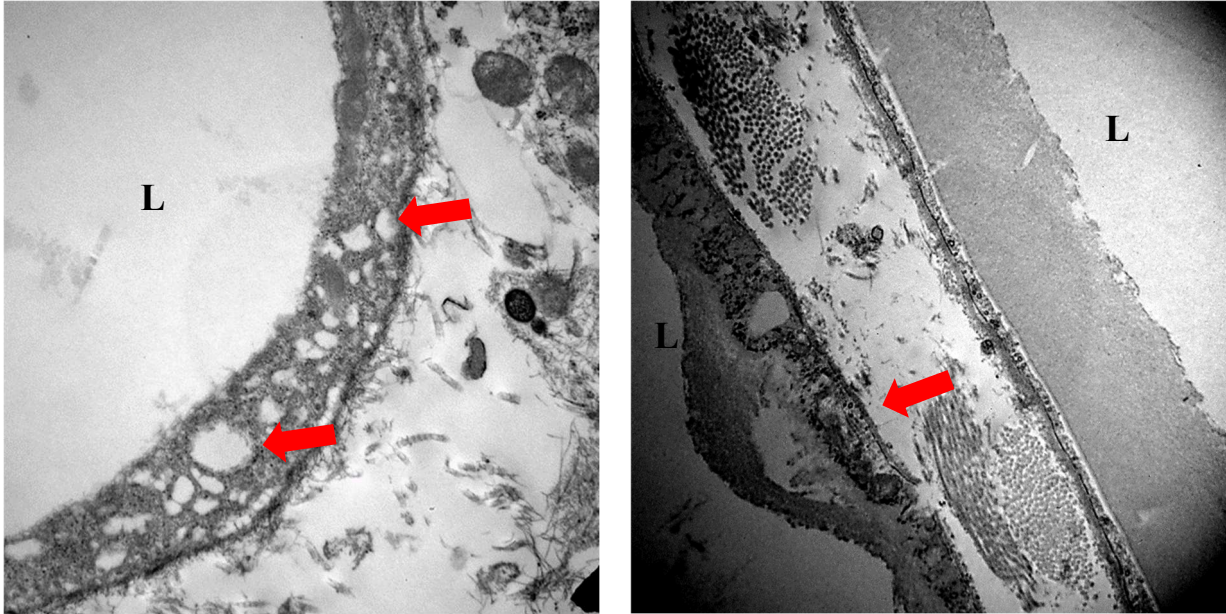
Figure 26*: Transmission electron microscopic images of hypertrophic adipocytes with classical features of stress and cellular degeneration, such as infiltration of lipid droplets into cytoplasm (arrows) and degeneration of the basal membrane (left). Picture showing a degenerating adipocyte (arrow) next to a perfectly preserved one (right). This allows us to exclude that it is an artifact. L= lipid droplet.*

Figure 27*: Different ultrastructural pictures by TEM from omental adipose tissue of two lean control patients. A small number of collagen fibrils (arrows) on the external surface of the adipocyte basal lamina, compared to obese subjects, were observed. L = lipid droplet.*

6. Collagen VI gene expression decreases in obese people

Data reported from literature support that obese adipose tissue is particularly rich of collagen types I, III and VI. The source of these collagens is unknown even if our SEM data seem to suggest that adipoc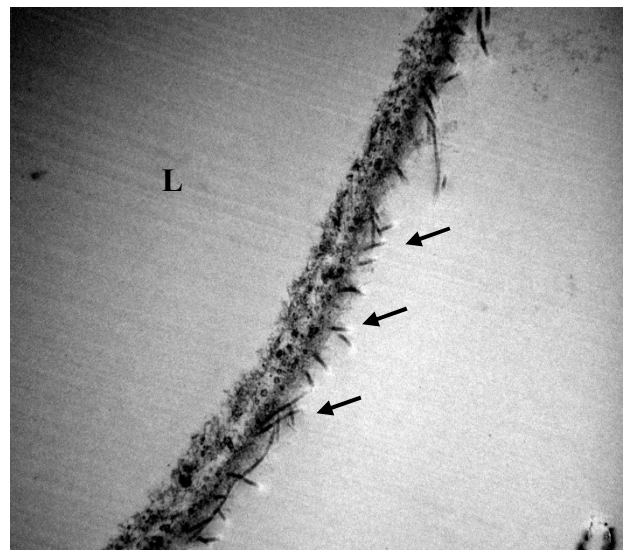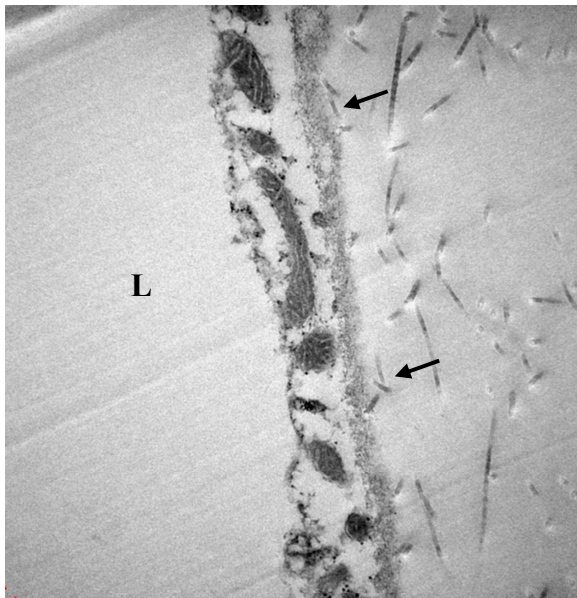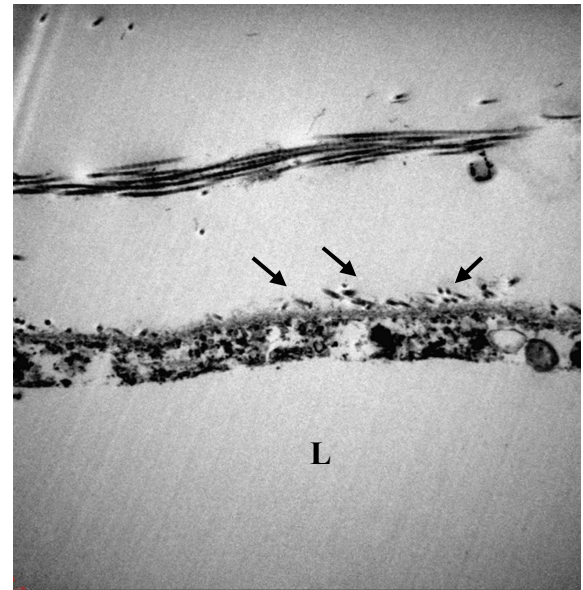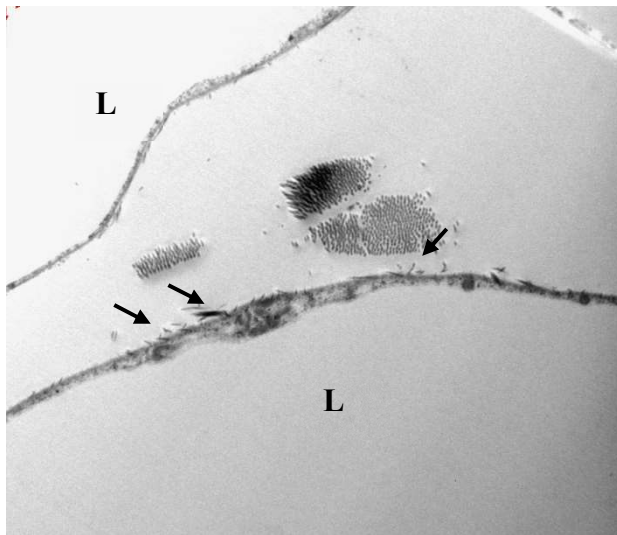ytes can be the source (Giordano et al. 2013). Additionally, most scientific works claim that Col VI increases as obesity progresses (Pasarica et al. 2009, Khan et al. 2009). Collagen VI functional role is still debated, therefore we evaluated the gene expression of Collagen VI both in visceral than subcutaneous adipose tissues. Surprisingly, we found that gene expression decreased in obese patients compared to lean patients in both adipose depots (***Figure 28***).
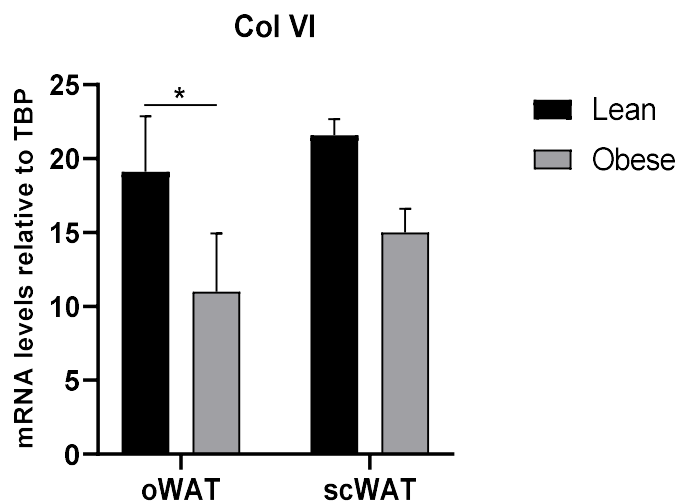
Figure 28*: qRT-PCR analysis of Collagen VI mRNA level in omental and subcutaneous adipose tissue from obese and lean patients. Data are presented as mean ± SEM.*

In order to add new data that could highlight the function of Collagen VI, we examined a rare adipose biopsy of subcutaneous adipose tissue from 15 years old boy, very lean, with Col VI gene mutation and affected by Ullrich Congenital Muscular dystrophy (UCMD), in collaboration with Dr. Patrizia Sabatelli and Prof Luciano Merlini (CNR, Institute Molecular Genetics, I. O. Rizzoli, Bologna). Importantly, this mutation induces the production of inefficient Col VI.

Paraffin sections of scWAT were stained with Sirius Red and it was immediately evident, by light microscopy, that the degree of fibrosis was high. Indeed, from morphometric analysis it was found that 16% of tissue is occupied by fibrosis (***Figure 29***). Moreover, we performed immunostaining of Perilipin 1, and data revealed a high percentage of dead adipose cells (about 6%) (***Figure 30***), quite elevated considering the patient age and his leaness.

Ultrastructural analysis by TEM, in addition to Sirius Red staining and Toluidine blue, confirmed that a large amount of collagen fibrils surrounded groups or individual adipose cells (***Figure 31***). Therefore, the general aspect of the biopsy was very similar to that found in obese patients.

Figure 29: *Sirius Red staining. Light microscopy pictures of scWAT sections from a young lean patient affected by Collagen VI myopathy stained with Sirius Red.*
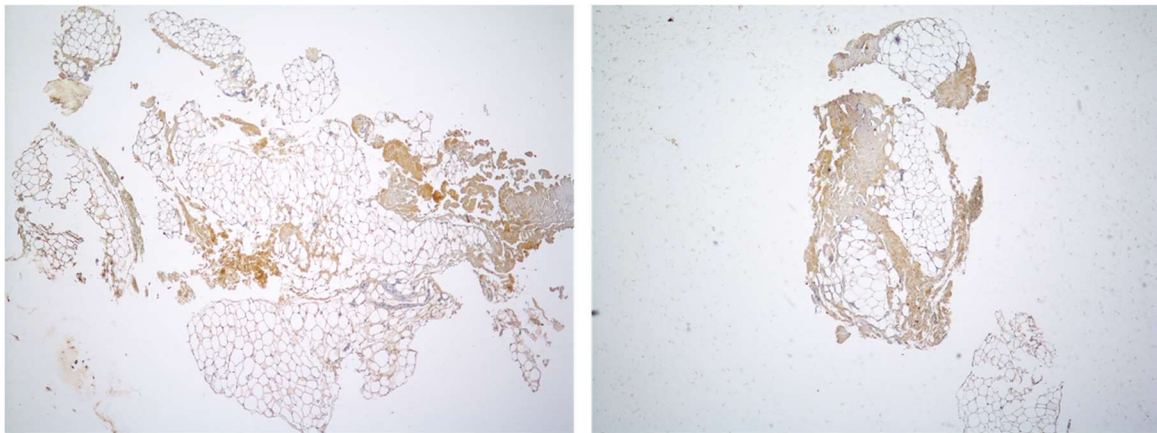
Figure 30: *Light microscopy pictures of scWAT sections from a young lean patient affected by Collagen VI myopathy immunostained for Perilipin 1.*
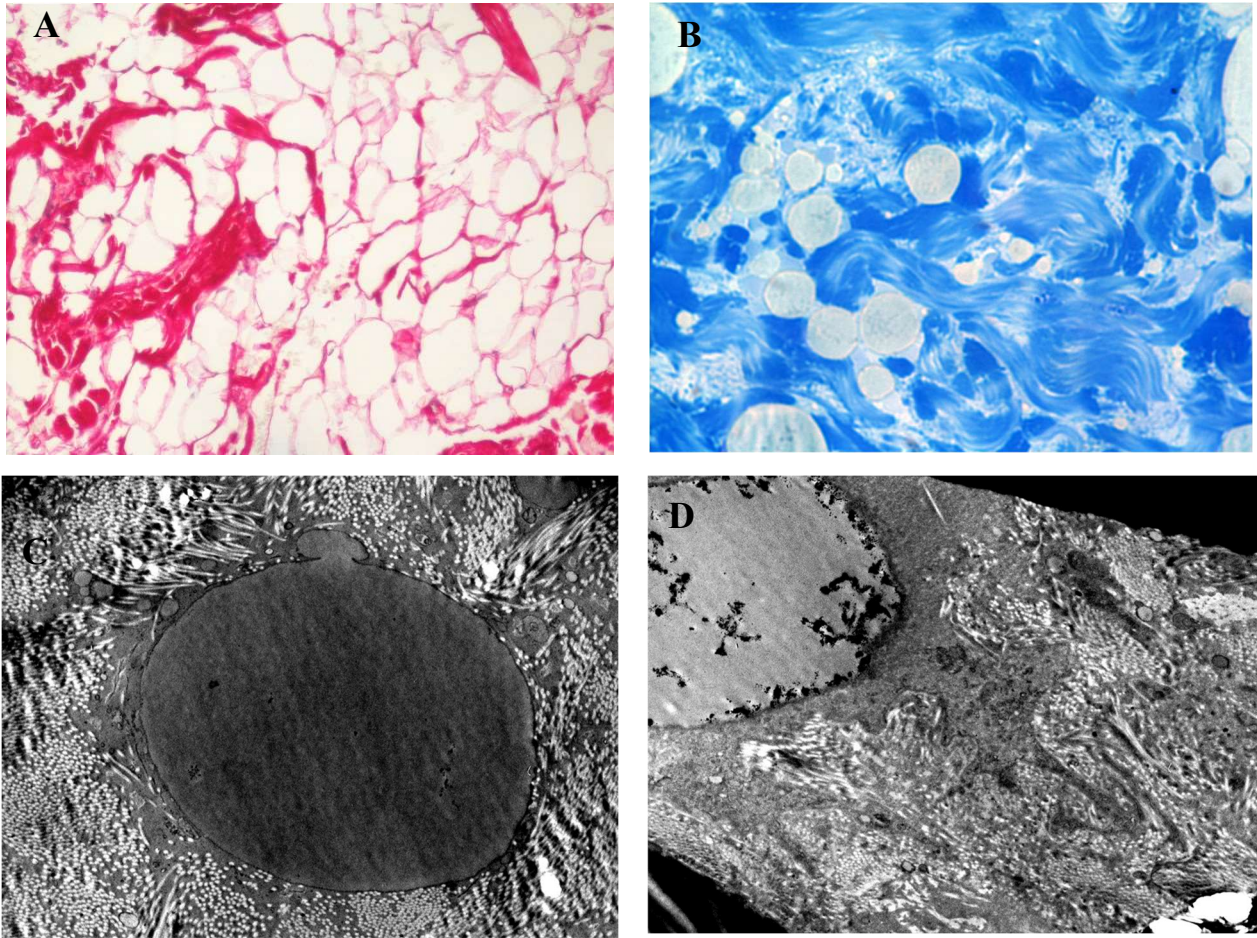
Figure 31: *Sirius Red (collagen red) staining and Toluidine blue (collagen blue) staining showing the high level of fibrosis surrounding groups or single adipocytes from scWAT of young lean patient with Collagen VI gene mutation. (A) Sirius Red staining of a paraffin section, (B) Semithin section stained with Toluidine blue, (C) and (D) TEM showing adipocytes completely surrounded by collagen fibrils.*

DISCUSSION

Obesity and its main related disorder, Type 2 Diabetes Mellitus, are currently two of the world's greater public health problems. 90% of diabetic subjects are also obese and the term "diabesity" has been coined (Zimmet et al. 2001, Chadt et al. 2000).

In periods of positive energy balance White Adipose Tissue (WAT) expands, becomes dysfunctional and undergoes a real pathological remodeling including a whole series of morphological and functional alterations. WAT expansion, in obesity, is accompanied to a massive infiltration of macrophages giving rise, over time, to a chronic low-grade inflammatory state, which it turns induces insulin resistance and, consequently, type 2 diabetes mellitus (Weisberg et al. 2003, Xu et al. 2003). We showed that the cause of inflammation (mainly due to macrophages) is due to death of hypertrophic adipocytes (Cinti et al. 2005). Using a HSL (hormone sensitive lipase) mouse knockout model we also showed that lean animals with hypertrophic adipocytes had the same inflammatory reaction typical of obese animals. These data point to hypertrophy per se as a cause of adipocyte death and consequent inflammation. In line with this concept is the positive correlation between the number of macrophages and size of adipocytes in adipose tissue (Weisberg et al. 2003, Xu et al. 2003).

Thus, hypertrophic adipocytes die, and the remnants must be removed by macrophages. The main remnant is the big lipid droplet that require a long period of time to be reabsorbed by macrophages that surround the lipid droplet forming the characteristic crown-like structures (CLS). Time course experiments fully confirmed the sequence of events: 1-positive energy balance 2-hypertrophy of adipocytes 3-macrophages infiltration of fat mainly due to chemoattractant such as MCP-1 and haptoglobin (produced by stressed hypertrophic adipocytes) 4-loss of the vital protein Perilipin 1 (PLIN1) 5-death of adipocytes 6-CLS formation 7-lipid remnants reabsorption (Strissel et al. 2007, Murano et al. 2008, Murano et al. 2013). Of note, the density of CLS in adipose tissue was positively correlated to the adipocytes size (Murano et al. 2008). Several factors produced by macrophages during this chronic process of lipid reabsorption seem to be responsible for the insulin receptor functional alteration and consequent insulin resistance, that is coincident with the onset of chronic inflammation (Hotamisligil 2006). Since long time insulin resistance has been claimed to play a fundamental role to induce T2 diabetes (Kahn et al. 2006). The main mechanism was considered the functional exhaustion after a long period of insulin hypersecretion by the pancreatic beta cells (Ferrannini 2009, Roden et al. 2019, Petersen et al. 2018).

This concept has been recently questioned by the evidence that obese patients suffering for T2 diabetes and treated by sleeve gastrectomy or intestinal bypass rescued from their diabetic conditions before weigh loss (Greve et al. 2008, Morales-Marroquin et al. 2020, Kahn et al. 2006).

These data seemed to suggest that pancreatic beta cells were not disrupted or at least were not completely disrupted by the chronic hyperactivity.

Interestingly, it has been recently showed that both in murine models and in humans the progressive impairment of insulin secretion in obese subjects could be due to a progressive increase of noradrenergic parenchymal innervation of Langerhans islets with a direct contact of noradrenergic synaptic endings and insulin secreting beta cells (Giannulis et al. 2014, Cinti et al. 2016, Cinti et al. 2021).

Furthermore, a recent paper claims a role for gastric Ghrelin cells. The hormone produced in the gastric mucosae, together with its orexigenic role acting on the central nervous system (arcuate nucleus of the hypothalamus) has a potent inhibitory action on pancreatic beta cells. Gastric Ghrelin cells in obese patients are hyperactive, thus potentially contributing to the beta cells inhibition and offering an explanation to the recovery from diabetes of obese patients treated by sleeve gastrectomy (Castorina et al. 2021).

Together with these last new aspects the role of chronic low-grade inflammation of adipose tissues seems to be of primary importance.

The predominant role of visceral versus subcutaneous fat accumulation in the onset of T2 diabetes was known since many years, but the reason why visceral fat is more important than subcutaneous fat was unknown (Bjorntorp 2000).

A quite recent work offered an explanation (Murano et al. 2008). In this paper it was evident that the critical death size inducing death of adipocytes is lower in visceral fat. In other words, during the hypertrophy process induced by positive energy balance, subcutaneous adipocytes are able to expand much more than visceral adipocytes, that rich their maximal dimension that trigger their death before that inducing death in subcutaneous fat. As a consequence, inflammation is higher in visceral fat and visceral fat accumulation induce easier the metabolic consequence of T2 diabetes.

But the reason for a lower critical death size in visceral fat was not known.

A large part of visceral fat is composed by brown adipose tissue BAT especially in young animals and humans and aging is accompanied by a conversion of BAT into WAT (Giordano et al. 2016), thus the visceral BAT have a different origin of subcutaneous WAT. Thus, the hypothesis is that the lower critical death size is due to this different origin (Cinti 2009a). Recent data support this hypothesis: using genetically modified animals, lacking ATGL, an enzyme indispensable for adipocytes lipolysis, Kotzbeck et al. showed that enlarged hypertrophic adipocytes die and form CLS, bur their density in the adipose tissues is much higher in those depots in which WAT derive from conversion of BAT (Kotzbeck et al. 2018). Thus, the adipocyte death due to its hypertrophy and with the differences in critical death size described above is the central phenomenon of obese fat

histopathology. But the molecular mechanism responsible for obese adipocyte death was unknown and Giordano et al. discovered that this mechanism is pyroptosis, i.e. a specific type of death induced by DAMPS (damage associated molecular patterns) (Giordano et al. 2013).

Considering all together, thus, obese fat inflammation seems to be a very important phenomenon linking this histopathology to important related disorders such as T2 diabetes.

Recently another histopathology aspect drives the attention of researchers in this field: fibrosis (Sun et al. 2011).

Fibrosis is present in obese fat and its abundance correlates with dysmetabolic aspects of obese people, but the reason for this correlation is unknown (Sun et al. 2014). Thus, we studied visceral and subcutaneous WAT biopsies in order to try to understand the mechanisms underlying this correlation. Our main unexpected result was that a large number of obese adipocytes resulted negative for PLIN1 (12-16% in both visceral and subcutaneous fat). The positive correlation between data from the two adipose depots allowed us to exclude any artefactual. Then we looked at the relationship between this death population of obese adipocytes and fibrosis and found a tight correlation suggesting that fibrosis was implicated in determining obese fat massive death.

Considering that the 33 obese patients considered in this study overall have an average BMI of 43 kg/m^2, according to Gallagher et al. the mean percentage of fat in each patient would be around 40-50% of the body weight (Gallagher et al. 2000), and since on average an obese individual weighs at least 100 kg, it means that each patient would have about 4-5 kg of dead adipose cells (taking into consideration only 10% of dead adipocytes).

In order to assess if there is a real correlation between the degree of fibrosis and the percentage of dead adipocytes in obese individuals, we measured the amount of total fibrosis in each adipose depot for all patients. The amount of total fibrosis appeared to be significantly increased in the oWAT of obese subjects versus lean patients, instead no particular differences were found in the scWAT.

Considering the strong positive correlation found between total fibrosis and the percentage of non-viable adipose cells both in omental and subcutaneous fat, we hypothesized that fibrosis itself could be the cause of adipose cell death.

Furthermore, gene and protein expression analysis of HIF1α (key protein marker of hypoxia) showed that obese adipose tissues are more hypoxic compared to those of control subjects.

In line with the general assumption that adipose tissues of obese patients are very inflamed, we confirmed an increase in gene expression of MCP1, which is a protein chemoattractant of macrophages both in oWAT and in scWAT. Interestingly, the quantitative analyses of CD68 immunoreactive macrophages revealed that their distribution was only in minimal part linked to CLS

(Cinti et al. 2005) and showed that macrophages were mainly randomly distributed among adipocytes (parenchymal macrophages).

This result further supports our hypothesis that there is another type of cell death, probably related to fibrosis.

Moreover, it turns out that there is a positive significant correlation between the amount of total fibrosis and the degree of inflammation for all obese individuals, thus reinforcing the concept that fibrosis per se could cause inflammation.

The absence of pyroptosis markers in fibrotic areas containing PLIN1 negative adipocytes (metabolically non-viable) further reinforce the idea that an alternative cause of death should exist in this type of pathologic adipose tissue.

Previous data from our laboratory showed, by TEM and HRSEM (High Resolution Scanning Electron Microscopy), that murine obese adipocytes were often surrounded by dense collagen fibrils in close apposition to the external surface of basal membrane and that they exhibited ultrastructural features of stress and cell degeneration (Giordano et al. 2013). Electron Microscope data from the present study confirm in humans that hypertrophic adipose cells from obese patients have features very similar to those found in murine models. In fact, we found ultrastructural signs of stress and degeneration. Furthermore, it is evident the frequent presence of abundant collagen fibrils in close apposition to the outer side of the basal membrane, especially if compared with the few fibrils found in adipose tissues from lean patients, in line with fibrosis quantitative data.

 Therefore, considering all together, it seems that in the condition of obesity an overproduction of collagen fibrils occurs, probably due to the adipose cells themselves, perhaps as a mechanism of resistance to the increase in cell size.

Finally, in order to deepen on the predominant type of collagen in fat biopsies of obese patients we evaluated the gene expression of Collagen VI and unexpectedly we found that it decreases in obese patients versus lean patients, both in oWAT than in scWAT. These results are in contrast with data from other Authors (Sun, Tordjman, et al. 2013, Divoux et al. 2010, Khan et al. 2009).

In order to try to understand these discrepancies we found the opportunity to study what happens when the Collagen VI gene is not functional in humans. In collaboration with Prof. Sabatelli and Merlini (Institute of Molecular Genetics, CNR Ist Rizzoli, Bologna), we studied a biopsy of subcutaneous fat from a lean child with a gene mutation of Collagen VI. Data obtained showed a very high degree of fibrosis, higher than that present in obese subjects, infiltrating adipose tissue formed by small adipocytes. Furthermore, single or grouped adipocytes were observed completely surrounded by fibrotic bundles, and a certain percentage of Perilipin-negative dead adipose cells was

also found, just as has been documented in obese patients. Therefore, these results quite perfectly reproduce what happens in the condition of obesity.

Considering all together, our final hypothesis is that adipocytes produce Collagen VI and at least fibrillar Collagen III. Collagen VI could play a role limiting the fibrillar production. This hypothesis allows to explain why the absence of Collagen VI (due to gene mutation) induces fibrosis in an adipose tissue composed by small adipocytes (lean and young patient). Furthermore this hypothesis is also in line with the reduced Collagen VI gene expression found in adipose tissues of our obese patients series, but in contrast to data obtained from other Authors (Sun, Tordjman, et al. 2013, Divoux et al. 2010, Khan et al. 2009).

Thus, the final picture could be that the reduced Col VI production induces pericellular and peri-adipose tissue fibrosis, consequent hypoxia and massive cell death. Interstitial massive necrotic material must be removed, inducing, together with the chemoattractant MCP1 produced by stressed adipocytes, a massive macrophages infiltration.

Thus, together with the known cause of death for obese adipocytes we can now propose the new type of death due to excess of collagen fibrils production: the self-choking death.

In conclusion, preliminary data obtained from this thesis allow us to formulate the following pathogenetic hypothesis that may have relevant perspectives of interpretation of link obesity-type 2 diabetes. Our data suggest that in the condition of hypertrophy, the visceral and subcutaneous adipose cells produce a greater quantity of collagen fibrils, presumably due to a loss of inhibition in the production of fibrillar collagen (mainly type 3), which are arranged around the adipocyte itself. This cellular activity could be related to attempt of the cell itself to resist to an excessive expansion, with organular damage and consequent death of pyroptosis. At the same time the accumulation of collagen around the adipose cell could lead to a condition of hypoxia resulting in loss of PLIN1 and accumulation of necrotic material. This material would recruit inflammatory cells, mainly macrophages, in an attempt to clean up the tissue. The massive inflammation would produce insulin resistance for known molecular mechanisms (Hotamisligil 2017), recently reinforced by new findings (Ying et al. 2017) and this could explain one of the links between obesity and type 2 diabetes.

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