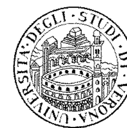

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TITLE OF THE DOCTORAL THESIS

Characterisation of epicardial adipose tissue and myocardial fat infiltration in humans

S.S.D. MED/09

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ABSTRACT

Obesity and type 2 diabetes are characterized by mild systemic inflammation, enlargement of fat depots, and uncontrolled release of free fatty acids into the circulation; they are both strongly associated with metabolic and cardiovascular disorders, such as dyslipidemia, coronary heart disease, high blood pressure and myocardial infarct.
White adipose tissue has been widely accepted to be much more than a static fuel storage organ. Its capability of regulating homeostatic and metabolic mechanisms has been underlined during the last 20 years; several studies have proposed adipose tissue as an endocrine organ, secreting hormones, adipokines and other biologically active agents acting locally or in a systemic manner.
Adipose tissue depots are not uniform; their characteristics change in different areas of the body, displaying distinct structural and functional properties and having different putative roles in pathologies.

Epicardial adipose tissue (EAT) is a peculiar adipose tissue depot, which has recently been the center of many studies. It's located predominantly on the right free wall of the heart, surrounding coronary arteries and being directly in contact with the myocardial layer. EAT has been shown to secrete many different adipokines and is supposed to have a role in the generation and progression of coronary artery diseases.

The aim of this thesis was to better characterize EAT and the underlying myocardial layer.

In paper 1 we investigated adipocyte cell size and adiponectin secretion comparing EAT, visceral AT (VAT) and subcutaneous AT (SAT). EAT resulted to have smaller adipocytes and lower adiponectin secretion levels. Adipocyte size, both in EAT and in SAT, is positively related with insulin resistance, shows negative association with local adiponectin gene expression, and bigger in subjects with coronary artery disease. Adiponectin gene expression is significantly lower in EAT than in SAT.

In paper 2 we focused our attention on the effect of diabetic state on EAT, showing higher MCP-1, CD-68, lower adiponectin level, and bigger adipocytes in subjects with than those without diabetes.

We also analyzed thorough immunohistochemistry and present as unpublished data, the characteristics of fat and macrophagic infiltration of the myocardium of the same cohort of patients.

Additionally, the result of a one year internship conducted at Lipid Laboratory in KI Sweden, has been included in the thesis, as paper 3. Leaving the subject of EAT, the paper is focused on adipose tissue and it's molecular and genetic aspects in obesity. Since the gene Niemann-Pick C1 (NPC-1)

has recently being implicated in susceptibility to obesity, through a genome wide association study, we dig into the relationship between NPC-1 and obesity in humans. The analysis of NPC-1 mRNA ad protein in obesity, showed that NPC1 mRNA was significantly increased in obese individuals in SAT and VAT and down-regulated by weight loss. NPC-1 mRNA was enriched in isolated fat cells of WAT, in SAT versus VAT, but not modified during adipocyte differentiation. NPC-1 protein mirrored expression of mRNA in lean and obese individuals

LIST OF PUBLICATIONS

1. Adiponectin gene expression and adipocyte diameter: a comparison between epicardial and subcutaneous adipose tissue in men.

Bambace C, Telesca M, Zoico E, Sepe A, Olioso D, Rossi A, Corzato F, Di Francesco V, Mazzucco A, Santini F, Zamboni M.
Cardiovasc Pathol. 2011 Sep-Oct;20(5):e153-6.

2. Inflammatory profile in subcutaneous and epicardial adipose tissue in men with and without diabetes.

Bambace C, Sepe A, Zoico E, Telesca M, Olioso D, Venturi S, Rossi A, Corzato F, Faccioli S, Cominacini L, Santini F, Zamboni M.
Heart Vessels. 2013 Jan 8.

3. NPC1 in human white adipose tissue and obesity.

Bambace C, Dahlman I, Arner P, Kulyté A.
BMC Endocr Disord. 2013 Jan 30;13(1):5.

4. Effect of moderate weight loss on hepatic, pancreatic and visceral lipids in obese subjects.

Rossi AP, Fantin F, Zamboni GA, Mazzali G, Zoico E, Bambace C, Antonioli A, Pozzi Mucelli R, Zamboni M.
Nutr Diabetes. 2012 Mar 5;2:e32.

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LIST OF ABBREVIATIONS

AMP adenosine monophosphate
AT adipose tissue
ATP adenosine triphosphate
BAT brown adipose tissue
BMI body mass index
CAD cardiovascular disease
CD Cluster of Differentiation
CNS central nervous system
DM diabetes mellitus type 2
EAT epicardial adipose tissue
ER endoplasmic reticulum
FABP4 Fatty acid–binding protein 4
FFA free fatty acids
HOMA homeostatic model assessment
IL interleuchin
LD lipid droplet
MCP-1 Monocyte chemoattractant protein-1
MESCs mesenchymal stem cells
MG macrophages
mRNA messenger RNA
NO nitric oxide
NPC1 Niemann-Pick C1
omWAT omental white adipose tissue
PAI1 Plasminogen Activator Inhibitor 1
PAT peritoneal adipose tissue
PPAR-α peroxisome proliferator-activated receptors-alfa
PVAT perivascular adipose tissue
RT-qPCR quantitative Reverse transcriptase-polymerase chain reaction
SAT subcutaneous adipose tissue
scWAT subcutaneous withe adipose tissue
TG trigliceride, triacylglycerides
TNF-α Tumor necrosis factor-alfa
UCP1 uncoupling protein 1
VAT visceral adipose tissue
WAT white adipose tissue

1. INTRODUCTION

1.1 **Obesity**

Obesity (defined as a body mass index (BMI) > 30 kg/m2) is the result of a prolonged imbalance between caloric intake and energy expenditure.

In recent years, the prevalence of overweight and obesity has increased in almost all developed countries (Prentice 2006). Recent estimates for the prevalence of obesity among adults are 36% in the United States (Flegal 2012) and 24% in Canada (Public Health Agency of Canada 2012). European Countries compared to the United States show a lower prevalence of obesity; recent data registered a prevalence of obesity of 9% for females and 10.7% for males in Italy (Micciolo 2010). The World Health Organization has shown that obesity levels have reached epidemic proportions worldwide with approximately 2·3 billion adults predicted to be overweight or obese by the year 2015 (WHO 2005).

The interest of research in obesity is easily explained by a recent meta-analysis, which confirms the association of obesity with all-cause mortality. (Flegal 2013)

1.2 **Adipose tissue as an endocrine organ**

Adipose tissue is composed by many different cell types. Main component are the adipocytes, which derive from mesenchymal stem cells (MESCs), common precursors for adipocytes, osteoblasts, myocytes and chondrocytes (Rosen 2006). MESCs initially develop into pre-adipocytes, committed precursors to the two main adipocytes types: white (WAT) and brown (BAT) adipocytes (Rosen 2006). These two types of adipocytes are different in both morphology and function: white adipocytes are spherical cells with ~90% of their volume made up of a single lipid droplet and store energy for the metabolic needs of the organism; whereas brown adipocytes are polygonal cells with a roundish nucleus and several cytoplasmic lipid droplets, with many packed mitochondria expressing of uncoupling protein 1 (UCP1) and they are specialized in burning energy for thermogenesis.

WAT is the main triacylglycerides (TG) storage depot of the body and tightly regulates TG mobilization. In conditions of excessive energy, fatty acid (FFA) hydrolysis occurs. Once within the cell, FFAs are hydrolysed by acyl-CoA-synthase to form acyl-Co-A, which may be metabolized in the TG synthesis pathway. With negative energy balance the process of lipolysis takes place, resulting in mobilisation of lipids from adipocytes under the influence of hormonal factors such as insulin (Vàzquez-Vela 2008).

Besides the role of energy storage, WAT is known to be a complex organ capable of producing and secreting a huge amount of molecules, called adipokines. Through the secretion of adipokines, AT is capable of modulating its own metabolic activity in an autocrine manner, and also to interact with other tissues both at local and systemic levels in a paracrine and endocrine manner (Vàzquez-Vela 2008).

The number of discovered hormones and cytokines secreted by adipocytes has expanded rapidly through the recent years. A simplified model of adipocyte activities is shown in Figure 1.

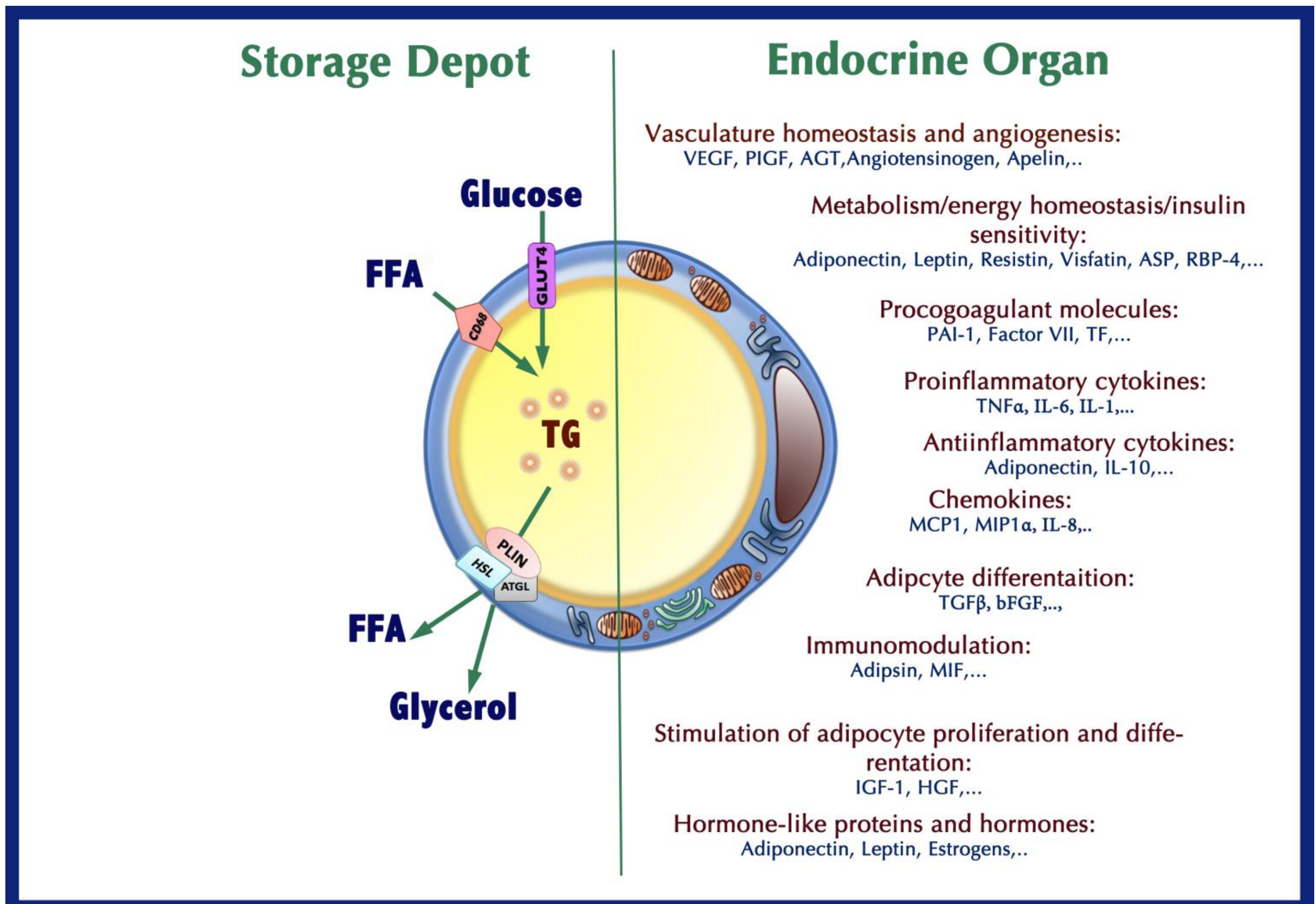


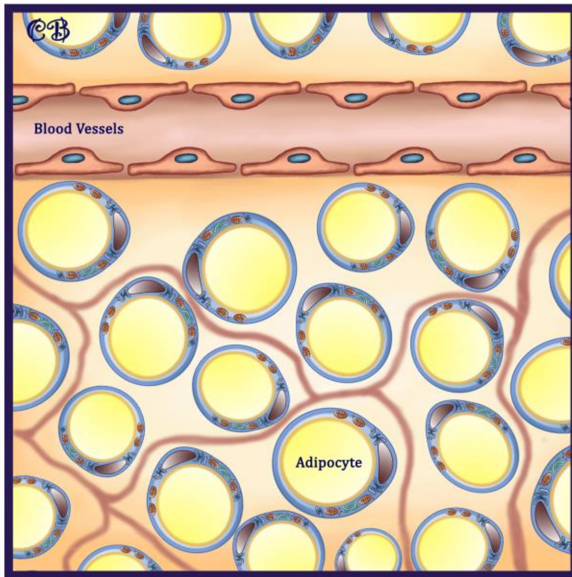
Figure 1. Simplified model of adipocytes functions

Besides adipocytes, AT contains a stromal-vascular matrix (SVM), including fibroblastic connective tissue cells, leukocytes, macrophages (MG), and pre-adipocytes, which may have a role in the secretion of many cytokines. The release of chemoattracting molecules (such as Monocyte chemoattractant protein-1 (MCP-1)), from the adipocytes, stimulates MG migration to the depot, in

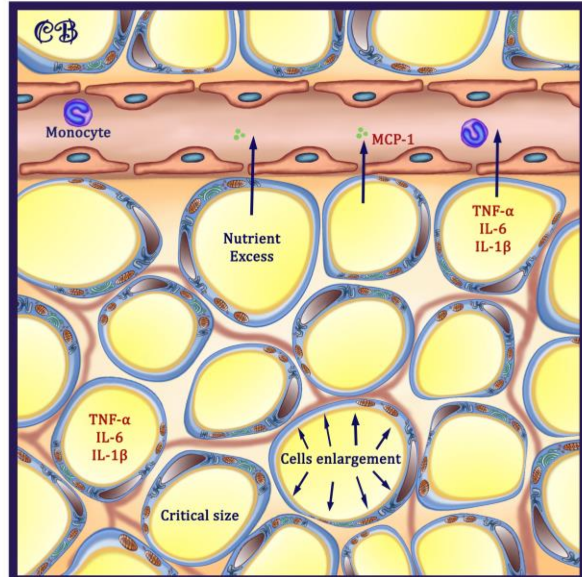
a process called chemotaxis, further exacerbating inflammation and adipocyte dysfunction. MG infiltration of AT is positively correlated to the size of adipocytes and to insulin resistance.

In situations of nutrient excess, AT can respond rapidly with adipocyte hypertrophy and hyperplasia. This process leads to the production of higher amounts of inflammatory adipocytes and the arrival to a “critical size”, specific and different in each AT depot. When reaching the “critical size” endoplasmic reticulum (ER) stress and subsequent cells death are triggered. It has been shown that a large number of MG infiltrate AT to remove remnants of dead adipocytes. It’s possible to identify MG surrounding dead adipocytes, forming characteristic structures called “crown-like structures” (CLS) (Smorlesi 2012). Figure 2 and 3.

Lean Adipose Tissue



Obese Adipose Tissue



Obese Inflamed Adipose Tissue

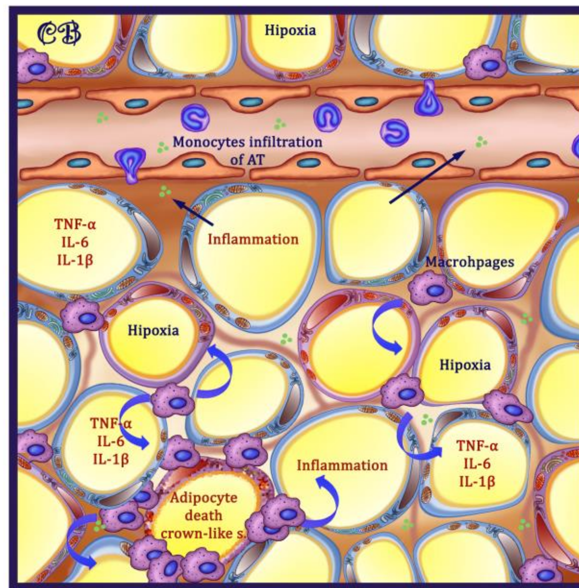


Figure 2. Simplified model of adipose tissue hypertrophy, inflammation and recruitment of macrophages.

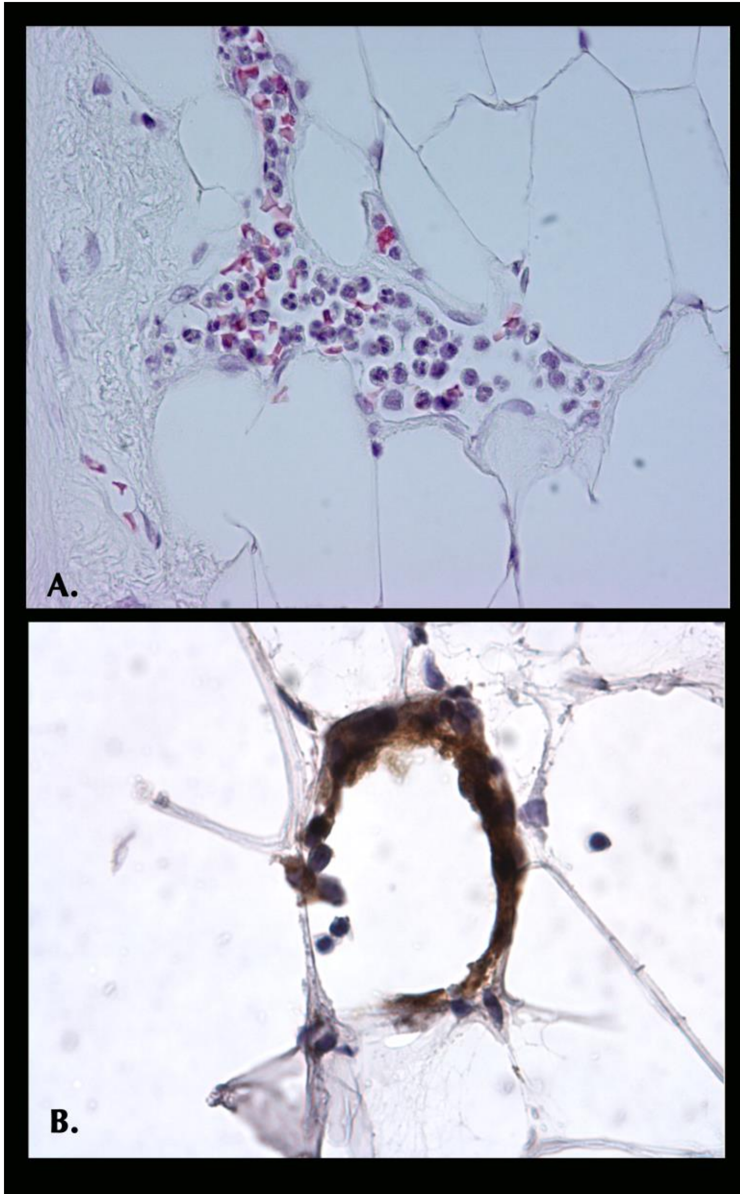


Figure 3. Himmunohistochemistry details of human epicardial adipose tissue:

A. Vase packed with neutrophiles; 40x hematoxylin eosin;

B. Crown-like structures macrophages surrounding an adipocyte:400x anti-CD68 and hematoxylin

MGs are heterogenic in both function and surface markers; they have a highly plastic phenotype in response to cytokines and microbial products of microenvironmental stimuli. Main classifications are: M1 classically activated MGs and M2 alternatively activated MGs. M1 are induced by LPS and interferon-gamma and produce proinflammatory cytokines and reactive oxygen species, and are capable of inducing Th1-polarized T-cell responses. M2, which can be induced by IL-4 and IL-13, are generally supportive of antiinflammatory processes driven by IL-10, playing a role in tissue repair. Lumeng et al reported that in mice, diet-induced obesity leads to a shift in the activation state

of AT MGs from M2 to M1 state, contributing to insulin resistance (Lumeng 2007). Furthermore it has been reported that adiponectin is a regulator of MG polarization (Fujisaka 2009).

Although there is a clear association of obesity and insulin resistance with MG infiltration of AT, the dominant phenotype of AT MG is still an open debate.

1.3 **Adipose tissue depot-specific characteristics and their impact on the association with diseases**

Since 1956 (Vague 1956) the notion that different distribution of AT can influence the predisposition of metabolic diseases, has raised great interest. Different depots are characterized by differences in their structure, expression profiles, responsiveness to endocrine and nervous stimuli, as well as local supply of oxygen, nutrients and hormones, resulting in different putative roles in pathologies. Preadipocytes isolated from different depots and cultured under the same conditions, resulted in mature cells maintaining fat depot-specific characteristics (Tchkonia et al. 2002, 2005).

The two main subgroups of WAT are subcutaneous (SAT) and visceral AT (VAT).

SAT is the AT under the skin, located in the hypoderms. It's further divided into superficial and deep SAT (Smith et al. 2001, Miyazaki et al. 2002, Walker et al. 2007).

VAT on the contrary, surrounds inner organs in the abdominal cavity and mediastinum. Main localization of VAT is the abdominopelvic cavity. Intrabdominopelvic AT includes intraperitoneal (further divided into omental and mesenteric AT) and extraperitoneal (further divided into intraabdominal (retroperitoneal and preperitoneal) and intrapelvic) AT. VAT includes also smaller amounts of fat localized in the mediastinum (intrathoracic or paracardial fat) and around specific organs, such as the heart (epicardial AT), stomach (epigastric fat tissue) and blood vessels (perivascular adipose tissue). (Shen 2003) Figure 4.

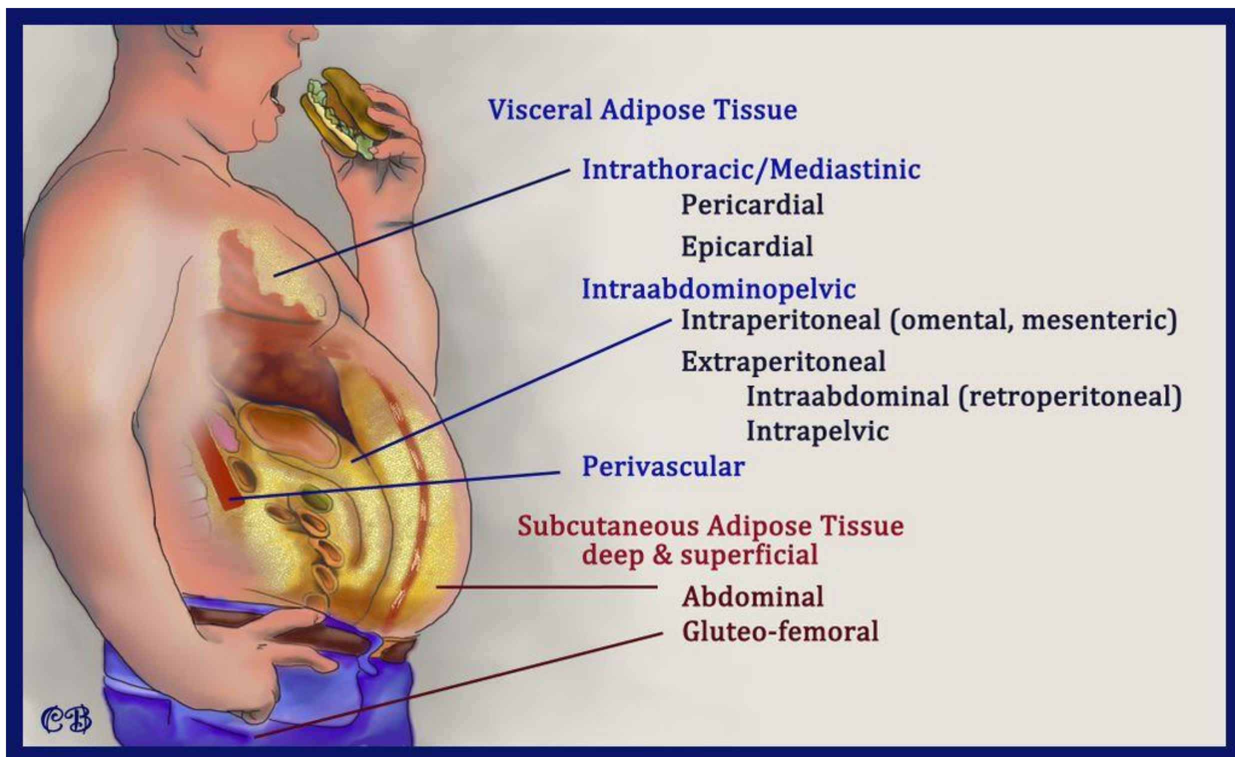


Figure 4. Adipose tissue depots classifications

Main differences between VAT and SAT have been studied in obese individuals. Subcutaneous adipocytes seems to have a higher differentiation potential (Walker et al. 2007), whereas visceral adipocytes are smaller (Fried 1993) with a higher content of mitochondria, with higher respiratory rates (Kraunsoe 2010); moreover visceral adipocytes are characterized by lower basal and NA-induced lipolysis (Ray 2009; Fried 1993), higher insulin-stimulated glucose uptake (Virtanen 2002) and higher lipolysis rate (Hoffstedt 1997). Visceral adipocytes seem to be more fragile and reach the critical death size earlier than subcutaneous adipocytes. In fact VAT shows higher density of CLS (Smorlesi 2012).

Differences are seen not only in adipocytes, but also in the tissue in toto, in different depots; in particular: SAT seems to have a more dense vascularization due to higher angiogenic potential (Gealekman 2011); Regarding adipokines secretion VAT secretes more pro-inflammatory cytokines and lower level of adiponectin (Fontana 2007; Yang 2008; Van Harmelen 1998), also according to a higher MG infiltration (Cancello 2006).

Due to its characteristics and its anatomical position, VAT seems to be involved in the onset of hyperglycaemia, hyperinsulinemia and eventually insulin resistance. According to the ‘portal hypothesis’ the excess of VAT, due to both its high lipolytic activity and its drainage into the portal system, results in high levels of FFA in blood plasma, leading to impaired insulin responsiveness of the liver (Miyazaki 2002) (Heilbronn 2004).

SAT is indeed considered as less metabolically active than VAT, with gluteofemoral AT mass considered to have even protective features on the metabolic syndrome (Snijder 2005, Buemann 2006).

In normal conditions adipose tissue maintains the balance between storage and release of FFA into circulation. With overnutrition and an imbalance between energy intake and energy expenditure, the capacity of adipose tissue to store lipids and hold balance, reach saturation. Consequently, non-AT are exposed to excessive amount of FFA, which can lead to accumulation of ectopic fat and lipotoxicity, especially in the liver, skeletal muscles, heart and pancreas.

Ectopic fat deposition can lead to endoplasmic reticulum (ER) stress, cells death and lastly, organ disfunction (Borradaile & Schaffer 2005).

1.4 **Fat and the heart**

Recently there has been particular interest in human fat in the heart. The fat of the heart is composed by different kinds of TG storages. Heart and coronaries are surrounded by significant amount of AT. AT around all arteries is called perivascular AT (PVAT). PVAT surrounding the coronaries is part of epicardial AT (EAT); externally to the pericardium there is pericardial AT. Another type of fat is myocardial fat, which refers to the storage of TG droplets within cardiomyocytes.

1.4.1 **Epicardial Adipose Tissue**

The locational, embryological and vascularization differences between EAT and pericardial AT, account for the different interest that those tissues have aroused. EAT is located between the myocardium and the visceral pericardium, and originates from the splanchnopleuric mesoderm; on the contrary, pericardial AT is located on the external surface of the parietal pericardium, originates from the primitive thoracic mesenchyme and is vascularized from non coronary sources

(Iacobellis and Bianco 2011).

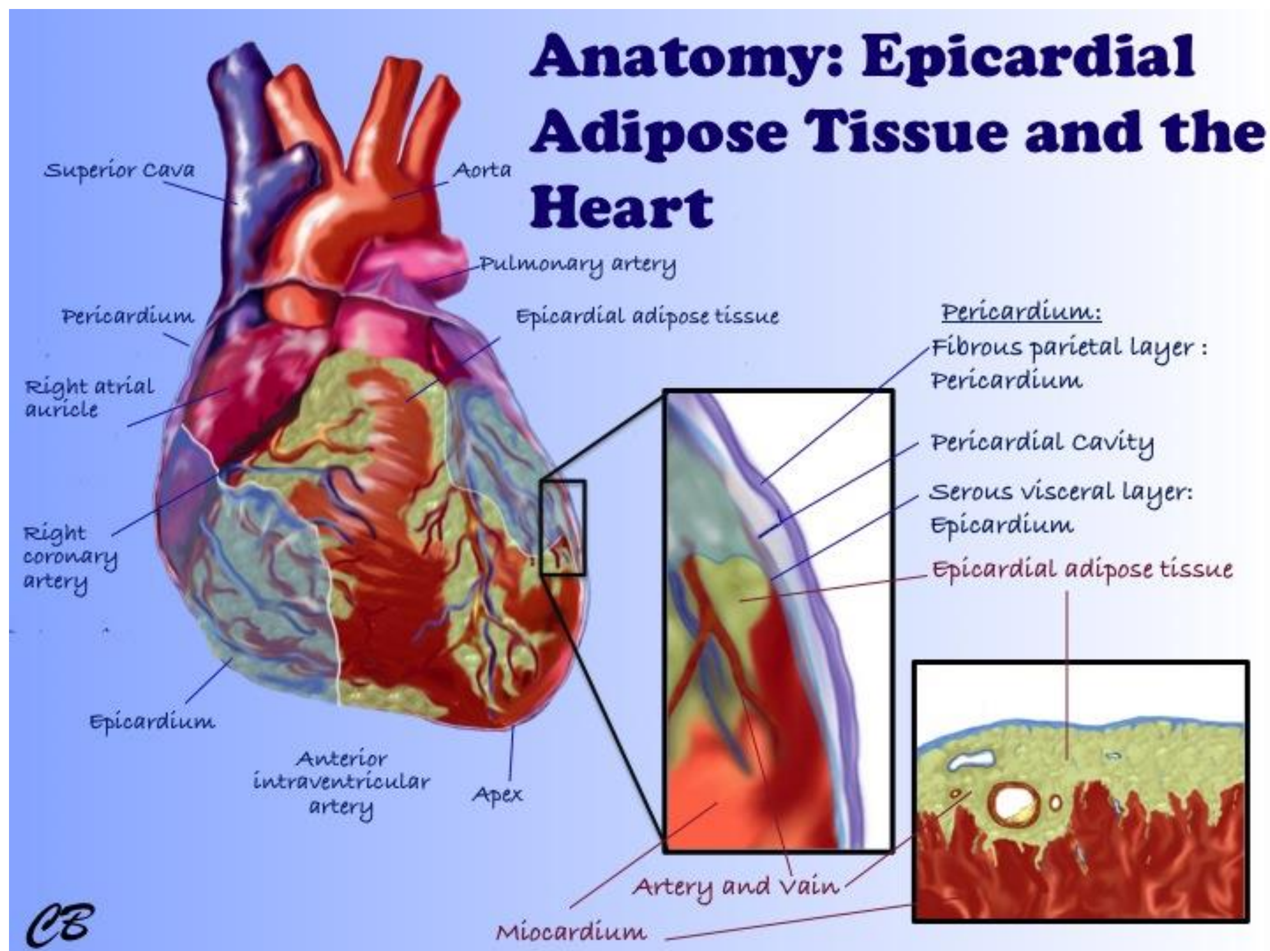


Figure 5. Anatomy of epicardial adipose tissue

EAT covers 80% (range 56-100%) of the surface area of the human heart (Rabkin 2007); it may have a supportive mechanical purpose, attenuating vascular tension and torsion and participating in coronary remodeling. EAT is predominantly located on the right-ventricular free wall and on the left ventricular apex, so that the coronary arteries and their main epicardial branches are embedded in it (Rabkin 2007, Iacobellis 2008). EAT adipocytes have a higher rate of FFA uptake and secretion and may act as a local energy supplier and buffer for preventing toxic levels of FFA in the myocardium (Marchington 1990). Recent data suggest that EAT thickness may directly associate with coronary artery disease (CAD), hypertrophy and also left ventricular mass in healthy subjects and visceral fat, independently of BMI (Iacobellis 2011, Corradi 2004). A recent study analyzed the effect of weight loss through very-low-calorie diet, exercise or bariatric surgery on EAT thickness; EAT reduction was quicker and higher than the decrease in BMI, waist circumference and body weight, resulting in an independent relation with the changes in systolic blood pressure, insulin sensitivity and left ventricular mass and function (Izzo 2011).

Around the coronaries the distance between PVAT and adventitia measures less than 0,1 mm with no anatomic barrier between the two tissues. Adipocytes have been reported even to encroach the outer adventitial region (Chatterjee 2009, Iacobellis 2007) and it has been postulated that PVAT might promote an inflammatory environment on the adventital layer. Interestingly atherosclerosis appears to be virtually suppressed in intramyocardial (buried) segments of coronary arteries (Arslan 2009). It has been recently observed a correlation of adventitial inflammatory lymphocytic and macrophage infiltration with severity of intimal disease (Tavora 2009), more common in lesions with unstable plaque and necrotic core.

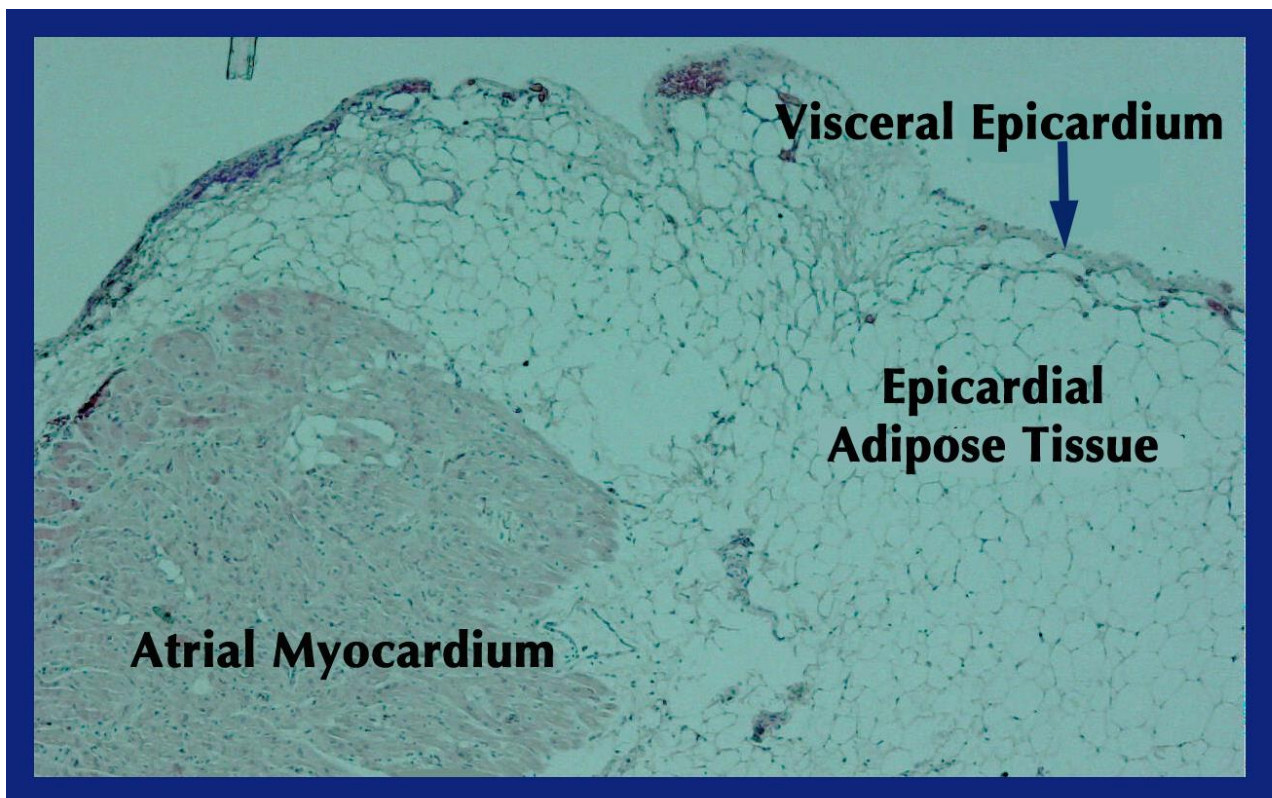


Figure 6. Himmunohistochemistry, structure of human epicardial adipose tissue: 10x hematoxylin eosin;

Higher expression and secretion of inflammatory adipokines and chemokines (TNF-alfa, IL-8, MCP-1, serum amyloid A, IL-1beta and IL-6) and infiltration of chronic inflammatory cell (machrophage, lymphocytes and basophilis), have been reported in EAT when compared to SAT from obese cardiovascular patients (Mazurek 2003, Fain 2010, Chen 2010 e Beker).
Several studies have evaluated alterations in adipokines expression in EAT in pathological states as CAD, MS and DM2 (Table 1).

Adipokine/CD	Protein/mRA	Phatological state	Expression	References
ActivinA	mRNA	DM2	↑	(1).
Adiponectin	mRNA	CAD	↓	(2, 3, 4, 16).
	Protein	CAD	↓	(5).
	mRNA	CAD	=	(6, 7, 8, 15).
	mRNA	DM2	=	(14).
	mRNA	MS	↓	(14).
Adrenomedullin	mRNA/Protein	CAD	↑	(8, 9).
Angiopoietin-2	mRNA	DM2	↑	(1).
CD14	mRNA	DM2	↑	(1).
CD68	mRNA	CAD	↑	(15).
CD150	mRNA	CAD	↑	(15).
CD163	mRNA	CAD	↑	(15).
CD206	mRNA	CAD	↑	(15).
CCR2	mRNA	CAD	↑	(15).
Chemerin	mRNA/Protein	CAD	↑	(2).
GLUT4	mRNA	CAD	↓	(9).
IL-1Ra	mRNA	CAD		(15, 16).
	mRNA	DM2	↑	(10).
	mRNA	MS	↑	(10).
IL-6	mRNA	CAD	↑	(3, 4, 6, 7, 15).
	mRNA	CAD	=	(6).
IL-10	mRNA	CAD	↑	(6, 15).
	mRNA	DM2	↑	(10).
	mRNA	MS	↑	(10).
IL-18	mRNA	CAD	↑	(11).
IKK-β	Protein/mRNA	CAD	↑	(12).
JNK	Protein/mRNA	CAD	↑	(12).
Leptin	mRNA	CAD	↑	(7, 3, 15).
	mRNA	DM2	=	(14).
MCP-1	mRNA	CAD	↑	(7, 6, 15).
	mRNA	CAD	=	(6).
NGF	mRNA	CAD	↑	(15).
NFkB	Protein/mRNA	CAD	↑	(12, 15).
NPR-A	mRNA	CAD	=	(7).
NPR-C	mRNA	CAD	↑	(7).
PPAR-γ	mRNA	DM2	=	(10).
	mRNA	MS	=	(10).
	mRNA	CAD	=	(7).
RANTES	mRNA	CAD	↑	(16).
RPB4	mRNA	CAD	↑	(9).
Tpl2	mRNA	CAD		(15).
TNF-alfa	mRNA/Protein	CAD	↑	(2, 3, 6, 15).
	mRNA	CAD	=	(6).
Visfatin	mRNA	CAD	↑	(3).

Adipokine/CD	Protein/mRA	Phatological state	Expression	References
Reactive oxygen/redox genes				
GPX-3	mRNA	CAD	↑	(15).
P47phox	mRNA	CAD	↑	(15).
eNOS	mRNA	CAD	↑	(15).
HMOX-1	mRNA	CAD	↑	(15).
SOD-2	mRNA	CAD	↑	(15).

Table I: Main literature on the subject; References: 1: Greulich 2011; 2: Gao 2011; 3: Chen 2010; 4: Eiras 2008; 5: Iacobellis 2005; 6: Hirata 2011; 7: Skabal 2011; 7: Shibasaki 2010; 8: Silaghi 2005; 9: Salgado-Somoza 2011; 10: Sacks 2011; 11: Dozio 2012; 12: Baker 2008; 13: Teijeira-Fernandez 2009; 14: Teijeira-Fernandez 2010; 15. Sacks 2011; 16. Karastergiou 2010

Form the recent leterature we can then generally deduce that EAT expands and becomes hypoxic and dysfunctional in metabolic and CAD states, starting to recruit phagocytic cells. This process reduce the production of protective in favor of inflammatory adipocytokines such as IL-6, TNFα. Adipokines produced by the PVAT component of EAT might interact with cells in each of the artery wall layers by diffusion, representing an “outside-in” component, in addition to the traditional endothelial and intimal layers “inside-out” view to atherogenesis. Moreover the vasa vasorum, which proliferates during vascular inflammation, are interspersed in perivascular AT. Adipokines produced by PVAT can access directly into the vasa vasorum, and be delivered into the intima.

PVAT seems to contribute to smooth muscle cell proliferation and migration. Takaoka et al in mouse and rat models of vascular injury, showed a rapid phenotypic modification of perivascular AT associated with infiltration of inflammatory cells and foam cells, induced by endovascular injury (Takaoka 2010).

In a porcine model of coronary artery balloon angioplasty, Jabs et al. identify pervascular tissue as a main source of inflammatory response, leucocyte chemoattractant factors and macrophage activation (Jabs 2007).

PVAT can modulate also vasoreactivity, in a mechanism that appears to be both endothelium dependent, via relase of nitric oxide (NO), and endothelium-independent, via generation of hydrogen peroxide (Greenstein 2009). Greenstein et al. in a recent study in human, underlined obesity-realted changes in PVAT vasoactive properties. The inhibition of vascular contraction in healthy subjects, was lost in obese patients with metabolic syndrome. The same lost of the dilator effect of PVAT was observed from Hosogai et al in (Hosogai 2007) a model of low oxygen or hypoxia.

Besides their possible role on the coronary vessels, EAT adipokines have been proposed to interact directly with cardiomyocytes. The absence of any fascia (seen on the contrary in skeletal muscle) separating the adipocytes and the myocardial layer, allows a direct paracrine crosstalk between the tissues.

The effects of adipocytes derived factors on cardiomyocytes have been evaluated in a series of in vitro studies (Lamounier-Zepter 2006; Lamounier-Zepter 2009; Look 2011; Look 2008; Greulich 2012): adipocyte-derived resulted to have a negative inotropic effect on isolated cardiomyocytes by reducing sarcomere shortening and intracellular Ca^{2+} fluxes (Lamounier-Zepter 2006; Greulich 2012); adipocyte conditioned medium on isolated perfused rats heart showed direct and acute suppression of heart contraction due to adipocyte derived factors, that was completely reversed after washout (Look 2008 and 2011). The effects of some well known proinflammatory cytokines were tested at the same concentrations detected in AT conditioned medium: TNF-α, IL-6, IL-1beta resulted to be not responsible for the cardiodepressant effect.

Adiponectin, a cytokine secreted mainly by adipocytes, with anti-inflammatory, insulin-sensitizing and anti-atherogenic properties has been lately proposed to exert protective actions also on

cardiomyocytes . In vitro studies demonstrated an antiapoptotic effect of full-length adiponectin on cardiomyocytes, largely via an AMP-kinase-mediated signaling pathway (Shinmura 2007). Adiponectin seems also to exerts its beneficial metabolic effects through a lowering of cellular ceramide in left ventricular of mice (Holland 2011). Ceramide is considered an enhancer of apoptotic susceptibility in the heart (Holland 2011, Park 2009).

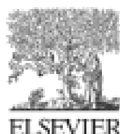
1.4.2 **Myocardial fat infiltration**

Intrinsic cardiac metabolism of the adult heart depends primarily (50-70%) on the utilization of fatty acids for oxidative phosphorylation and generation of ATP, while the remainder (w30%) is principally obtained via metabolism of glucose. Although the myocardium presents an extremely high rate of lipolytic turnover and abundantly express PPAR-α, a key regulator of the genes involved in fatty acid oxidation, the healthy heart has a relatively limited capacity for storing lipids (Paul 2008). A decrease in glucose transport, glycolysis, and glucose oxidation, together with an increase in fatty acid uptake and oxidation, is typically observed in the heart pathological state such as obesity and diabetes (Stanley 2005, Abel 2008).

In the human aging heart, myocardial fatty acid oxidation is reduced, with an increase of TG storage which correlates to the age-related decline in diastolic left ventricular function (van der Meer 2008). A dose dependent increase in LD storage has been reported during fasting (Suzuki 2001) or in pathological conditions, such as ischaemia (Jodalen 1985; Greve 1990) diabetes, obesity (McGavock 2006) and metabolic syndrome (Marfella 2009).

Little is know about the characteristics of myocardial fat infiltration in CAD and in relation to EAT. As in the liver, pancreas and other organs, also accumulation of TG in the heart is supposed to lead organ disfunction.

2. Adiponectin gene expression and adipocyte diameter: a comparison between epicardial and subcutaneous adipose tissue in men (paper1)



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Cardiovascular Pathology 20 (2011) e153–e156

CARDIOVASCULAR PATHOLOGY

Original Article

Adiponectin gene expression and adipocyte diameter: a comparison between epicardial and subcutaneous adipose tissue in men

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Abstract

Introduction: Interest has recently focused on epicardial fat, but little is known about epicardial adipocyte size and its relation with insulin resistance and adipokines. **Methods:** Biopsies were collected from subcutaneous, epicardial-, and peritoneal fat from 21 males undergoing elective cardiac surgery either for coronary artery bypass grafting (n=11) or for valve replacement (n=10). We assessed epicardial adipocyte size, comparing it with that from subcutaneous fat and peritoneal fat. The adipocyte size was determined by using collagenase digestion of adipose tissue, separation of adipocytes by centrifugation, methylene blue staining of the nuclei, and measurement of the cell diameter. Patient's weight, height, body mass index, waist, as well as glucose, insulin, homeostatic model assessment index, adiponectin, and leptin serum levels were determined. Adiponectin mRNA levels were determined by real-time polymerase chain reaction on subcutaneous fat and epicardial fat biopsies. **Results:** Adipocytes in epicardial fat were significantly smaller than those in subcutaneous and peritoneal fat. The adipocyte size in epicardial fat correlated positively with insulin resistance and serum leptin, and correlated negatively with serum and mRNA expression of adiponectin. Adiponectin mRNA expression in epicardial fat was significantly lower than in subcutaneous fat. Adipocyte size in epicardial fat was significantly smaller in valve-replacement patients than in coronary artery bypass graft patients. Adiponectin gene expression was lower in the latter than in the former, although not significantly. **Conclusions:** Adipocytes in epicardial fat are smaller than those in peritoneal and subcutaneous fat. Adipocyte size, both in epicardial and in subcutaneous fat, is positively related with insulin resistance, shows negative association with local adiponectin gene expression, and is decreased in subjects with coronary artery disease. Adiponectin gene expression is significantly lower in epicardial- than in subcutaneous fat. © 2011 Elsevier Inc. All rights reserved.

Keywords: Epicardial adipose tissue; Adipocytes; Adiponectin; Leptin; Insulin resistance

1. Introduction

Adipose tissue (AT) is known to express and secrete a variety of bioactive peptides, known as adipokines, which act at both local (autocrine/paracrine) and systemic (endocrine) levels [1]. In obese patients, a dysregulation of the pro- and anti-inflammatory adipocytokine functions and production leads to a state of chronic low-grade inflammation which may contribute to the development of atherosclerosis and cardiovascular diseases. The risk of metabolic complication is increased not only by the amount but also by the location of AT [1].

Potential conflict of interest: nothing to declare.

Disclosure: None of the authors had a financial interest or professional or personal affiliation that compromises the scientific integrity of this work.

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The contributions of the authors were as follows: MZ and CB conceived the overall study; CB, AR, EZ, FS, and MZ drafted the manuscript; CB, MT, AS, and FC performed the subjects' screening and data collection; DO performed the RT-qPCR; CB and VDF performed the statistical analysis; AM, FS, EZ, and VDF contributed to the conclusion of the manuscript.

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Particular interest has recently focused on epicardial fat (EAT), a visceral AT depot, situated predominantly on the right-ventricular free wall, due to its proximity to coronary arteries and the lack of any fascia between the adipocytes and the myocardial layer, suggesting the possibility of the paracrine effect of epicardial adipocytokines on myocardial metabolism and their potential role in the pathogenesis of coronary artery disease [2].

It has recently been shown that enlargement of the adipocytes is an independent marker of insulin resistance [1]. Larger adipocytes release higher amounts of proinflammatory mediators such as leptin and resistin, and lower amounts of adiponectin [1]. Clinical studies reported differences in adipocyte sizes among different AT depots in the same patient, indicating that visceral adipocytes are significantly smaller than subcutaneous adipocytes [1].

Little is known about EAT cellularity. We investigated EAT adipocyte size and its relationship with insulin resistance, circulating levels of adiponectin and leptin, and EAT expression of adiponectin, in order to achieve a better insight into the physiology of epicardial adiposity.

2. Research methods and procedures

We collected data from 21 males (mean age 62.67±9.08 years), undergoing elective cardiac surgery either for coronary bypass grafting (CAD, n=11) or for valve replacement (non-CAD, n=10). Clinical records were prospectively collected. In the CAD group, three patients had type 2 diabetes, eight had hypertension, and seven had dyslipidemia. In the non-CAD group, two patients had type 2 diabetes, five had hypertension, and seven had dyslipidemia.

Written informed consent was obtained from each participant and the study was approved by the Ethical Committee of the University of Verona. Anthropometric parameters (weight, height, waist circumference) were determined, and body mass index (BMI 27.3±4.41) was computed as weight in kilograms divided by height in square meters. Glucose (5.9±1.24 mmol/l) and insulin (5.07±3.21 mU/l) serum levels were evaluated in all subjects. We used the homeostatic model assessment (HOMA) index (1.45±1.23) as an indirect index of insulin resistance, calculated as the product of the fasting plasma insulin level (mU/l) and the fasting plasma glucose level (mmol/l), divided by the constant 22.5.

Serum leptin and adiponectin were measured using specific ELISA kits (respectively from DBC-Diagnostic Biochem Canada, Inc., London, Ontario, Canada, and Linco Research, Inc., St. Charles, MO, USA).

Fat biopsies (0.2–1 g) were obtained prior to initiating cardiopulmonary bypass, within 1 h after induction of general anesthesia, from (A) subcutaneous fat (SAT) at the manubrium sterni level, (B) EAT as close as possible to the origin of the right coronary artery, and (C) peritoneal fat (PAT) via a subxifoideal approach.

Biopsies were immediately frozen at −80°C.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) method. For quantitative RT-qPCR, 15 ng of total RNA was reversed transcribed into cDNA in 20-µl reactions with the IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Aliquots of the reversed transcriptase, or water only (negative control), were PCR amplified with Quanti-Tect SYBR Green PCR kit (Qiagen) and with Quantitect Primer Assays (Qiagen) in the iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The Entrez Gene ID for adiponectin was 9370. Thermal cycling conditions were 95°C for 15 min followed by 40 cycles of 94°C for 15 min, 55°C for 39 min, and 72°C for 39 min. The melting curve analysis for the control of aspecific amplifications was done. The values were normalized against the reference gene, β-actin, for each sample. mRNA quantification was performed in triplicate for each well, and the gene expression (calculated by $\Delta\Delta C_T$) algorithm analysis was conducted using the Bio-Rad iQ5 software.

The biopsies of SAT, PAT, and EAT were temporarily stored in phosphate buffered saline and processed in order to determine adipocyte size. AT samples were digested with collagenase, adipocytes were separated by centrifugation (2000 rpm), and the nuclei were stained with methylene blue (as previously described) [3].

Cell diameters were calculated as mean cell diameter on optical measure of 100 cells by two independent readers (CB, AS).

Differences in the adipocyte sizes and adiponectin gene expression were estimated by ANOVA. Differences in adipocyte size and adiponectin gene expression in EAT, between CAD and non-CAD subjects, were tested after adjustment for HOMA index (ANCOVA test). Pearson correlation coefficients were calculated to evaluate the association between anthropometric and metabolic parameters, gene expression, and mean adipocyte size. All analyses were performed using SPSS software (version 17.0). Data were presented as mean±S.D. and P<.05 denoted statistical significance.

3. Results

Adipocyte size was assessed in the whole population (SAT 92.19±7.97 µm, PAT 82.50±10.69 µm, and EAT 73.65±8.25 µm) (Fig. 1). Adipocytes in SAT were significantly bigger than those in PAT, and those in the latter were significantly bigger than those in EAT.

The adipocyte size of SAT correlated positively with BMI (r=0.44; P<.05), HOMA index (r=0.68; P<.001), and serum leptin (r=0.66; P<.01), and correlated negatively with age (r=−0.51; P<.05), serum adiponectin (r=−0.66; P<.001), and mRNA expression of adiponectin (r=−0.56; P<.005).

The adipocyte size of EAT was positively correlated with HOMA index (r=0.69; P<.001), serum leptin (r=0.49; P<.05), and negatively with serum adiponectin

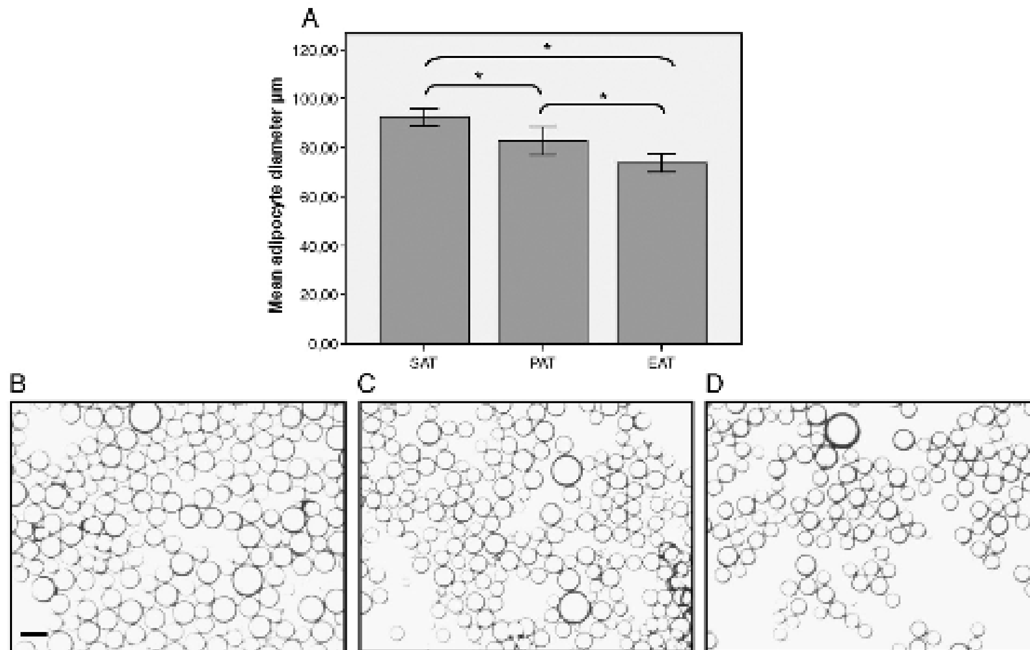


Fig. 1. Mean adipocyte diameters (µm) of subcutaneous (SAT), peritoneal (PAT), and epicardial (EAT) adipose tissue. (Top, A) Statistical analysis of the mean adipocyte diameter in the tissues (*$P<.05$). (Bottom) Light microscopic appearance of human fat after collagenase digestion and methylene blue staining to identify the nuclei (magnification 4×); tissues were harvested from the same patient. (B) SAT; (C) PAT; (D) EAT. Scale bar=10 µm.

($r=-0.60$; $P<.01$) and mRNA expression of adiponectin ($r=-0.49$; $P<.05$).

The adiponectin mRNA expression was significantly higher in SAT than in EAT (10.33±6.97 vs. 6.25±2.20 relative expression, respectively; $P<.01$).

No statistical difference in BMI, age, and serum leptin was found between CAD and non-CAD patients. CAD patients had significantly higher HOMA-index levels than non-CAD patients (2.00±1.48 vs. 0.84±0.43, respectively; $P=.025$) and lower serum adiponectin (4.31±1.32 vs. 9.03±4.76 µg/ml; $P=.002$).

EAT adipocytes of CAD patients were significantly bigger than those in non-CAD patients (79.49±7.73 vs. 68.62±6.13 µm, respectively; $P<.01$). A higher EAT adiponectin gene expression, although not statistically significant ($P=.09$), was observed in non-CAD patients compared to CAD patients (7.15±2.58 vs. 5.51±1.59, respectively).

After adjustment for HOMA index, the significant difference in adipocyte size between CAD and non-CAD patients disappeared.

4. Discussion

Our study shows that EAT adipocytes are smaller than those in PAT and SAT and that adipocyte size, both in EAT and in SAT, is positively correlated with insulin resistance and negatively associated with adiponectin gene expression.

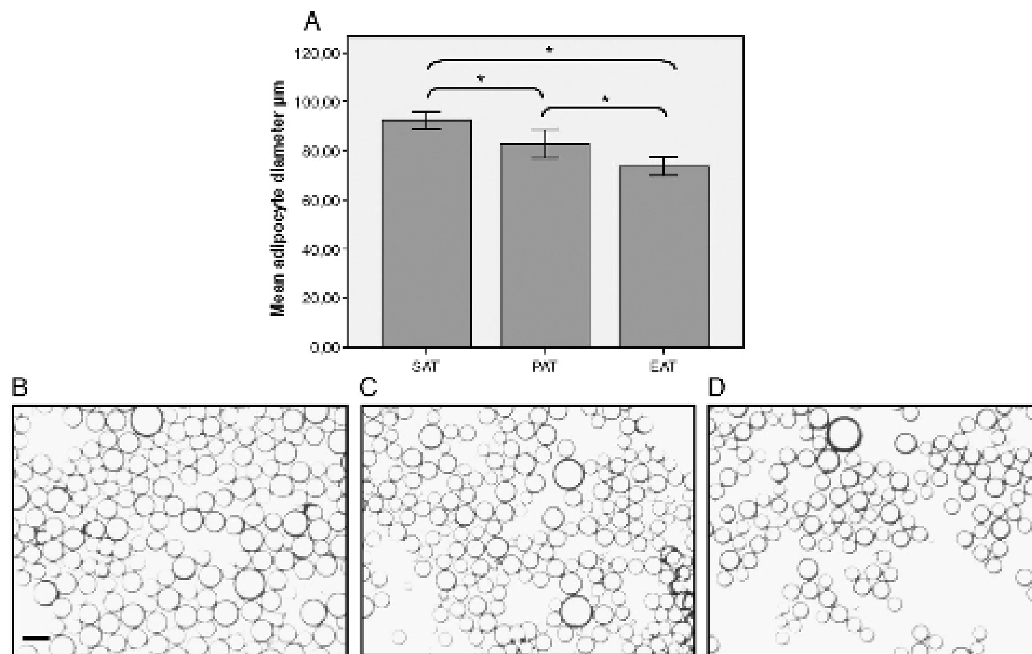
Particular interest has recently focused on human perivascular AT surrounding the coronary arteries, usually referred to as EAT, which covers about 80% (range 56–100%) of the surface area of the human heart [2]. EAT is situated predominantly on the right-ventricular free wall and on the left ventricular apex, so that the coronary arteries and their main epicardial branches are embedded in it [2].

Although it has been shown that as adipocytes become larger, their secretory profile evolves toward a more inflammatory phenotype, our data seem to support the hypothesis that adipocyte growth in size is indeed relatively different in different fat depots.

Actually, this is well known in PAT, where visceral adipocytes, despite being smaller than those in SAT, show higher secretion of inflammatory cytokines.

The different size of adipocytes in EAT compared to PAT is also interesting because EAT is usually considered as just a subset of PAT.

Our data seem also to support the potential clinical significance of inflammation in EAT and its relation with cardiovascular diseases. Interestingly enough, in our patient's cohort, adipocytes size, wherever located (EAT, PAT, SAT), appeared related to insulin resistance.



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3. **Inflammatory profile in subcutaneous and epicardial adipose tissue in men with and without diabetes. (paper 2)**

Heart Vessels
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ORIGINAL ARTICLE

Inflammatory profile in subcutaneous and epicardial adipose tissue in men with and without diabetes

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Abstract In recent years, evidence has emerged indicating that insulin resistance and diabetes mellitus type 2 are associated with inflammation of adipose tissue (AT). Interest has been focused on epicardial AT (EAT) because of its possible involvement with atherosclerosis and cardiovascular diseases. The aim of this study was to characterize adipocyte size and inflammatory profile in subcutaneous (SAT) and EAT among subjects with or without diabetes. Biopsies were collected from SAT and EAT in 34 men undergoing elective cardiac surgery. Weight, height, body mass index, waist circumference, as well as serum levels of glucose, insulin, lipids, adiponectin, and leptin were determined in all subjects. Adiponectin, MCP-1, and CD68 mRNA levels present within cells from AT biopsies were determined by real-time polymerase chain reaction. Adipocyte size was determined by optic microscopy and morphometry. Regarding the experimental group as a whole, gene-expression levels within EAT were significantly lower for adiponectin and higher, albeit not significantly, for MCP-1, when compared with that of SAT. In addition, adipocytes in EAT were significantly smaller than those in SAT. Subjects with diabetes showed lower adiponectin gene-expression levels in both SAT and EAT when compared with subjects without diabetes. By contrast, MCP-1 and CD68 gene-expression levels were higher in both tissue types of diabetic subjects. Adipocyte size in EAT was significantly larger in diabetic subjects than in nondiabetic subjects. Our data revealed a predominantly inflammatory profile in both SAT and EAT in subjects with diabetes in comparison with those without diabetes.

Keywords Epicardial adipose tissue · Subcutaneous adipose tissue · Adipocyte · Inflammation · Diabetes

Introduction

Adipose tissue (AT), the primary location of energy storage, is known to be a complex and highly active metabolic and endocrine organ [1]. Adipocytes, of which AT is composed, provide not only a flexible storage depot for excess nutrients but are also endocrine cells that secrete the hormones responsible for regulation of energy intake and expenditure throughout the body [2]. With excess nutrition, however, adipocytes are pushed to the limits of their ability to store lipids and to regulate nutrient metabolism. Along with obesity, such changes in the homeostatic and secretory profile of AT lead to an increase in the expression of inflammatory markers [2]. A higher level of proinflammatory cytokines in serum has been linked to endothelial dysfunction [3].

Particular interest has recently focused on human perivascular AT surrounding coronary arteries, usually referred to as epicardial AT (EAT), which covers 80 % (range 56 %–100 %) of the surface area of the human heart [4]. EAT is predominantly located on the right-ventricular free wall

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and on the left ventricular apex, such that the coronary arteries and their main epicardial branches are embedded in it [4, 5]. Because of the proximity of EAT to coronary arteries and the lack of any fascia between the adipocytes and the myocardial layer, the paracrine effect of EAT has been suggested to play a role in the pathogenesis of ventricular dysfunction and coronary artery disease [6]. In a previous study we analyzed subcutaneous AT (SAT) and EAT, focusing our attention on the differences between patients with and without cardiovascular diseases (i.e., coronary artery disease (CAD)) [7]. In this paper we expanded the previous cohort to include patients undergoing treatment with oral antidiabetic agents or insulin therapy.

In recent studies, it has also been highlighted that EAT thickness is significantly higher in subjects with metabolic syndrome, obesity [8], and type 2 diabetes [9]. However, studies regarding EAT adipokine gene expression in subjects with diabetes are limited.

The aim of the present study was to compare adipocyte size and inflammatory profile of subcutaneous AT (SAT) and EAT from a study sample of subjects who underwent elective cardiac surgery. In addition, we tested the association between diabetes, adipocyte size, and inflammatory profile.

Research methods and procedures

Study population

A total of 34 men, aged between 51 and 80 years (65.76 ± 9.03 years), with body mass index (BMI) ranging from 19.9 to 37.1 kg/m^2 (27.51 ± 4.06 kg/m^2), undergoing elective cardiac surgery, either for coronary bypass grafting or valve replacement, were studied. A group of 14 patients was diabetic according to the American Diabetes Association [10], and either on diet or drug therapy. A group of 20 subjects was nondiabetic. In this paper we extended the cohort (21 subjects, of whom 11 with CAD) of a previously published article [7], including 13 new subjects (8 with CAD). In the previous cohort we included only five diabetic subjects undergoing diet treatment, since we considered the Homeostasis Model Assessment (HOMA) index as an insulin resistance index. In this new study, deciding to focus our attention on diabetes, we collected and included nine subjects undergoing treatment with oral antidiabetic agents and/or insulin therapy (7 with CAD). We added also four newly collected subjects without diabetes (1 with CAD) to extend the cohort.

Herein we present some results involving the extended cohort, in line with our previous reported data (adipocyte size and adiponectin levels in the whole population compared between SAT and EAT) for reasons of clarity and completeness.

Patients with liver disease or neoplastic diseases were excluded from the protocol study. Written informed consent was obtained from each participant, and the study was approved by the Ethical Committee of the University of Verona.

Blood collection

Venous blood samples for all metabolic assessments were obtained after overnight fasting.

Immune-reactive insulin within plasma samples was evaluated using a direct chemiluminescent-based two-site sandwich immunoassay (ADVIA Centaur Insulin assay; Siemens, Erlangen, Germany). The sensitivity of the assay was 0.5 mU/l.

Serum leptin and adiponectin were measured using specific enzyme-linked immunoassay kits (respectively from DBC-Diagnostic Biochem Canada, London, ON, Canada and B-Bridge, Cupertino, CA, USA). The sensitivity of the assays was 0.5 ng/ml for leptin and 0.02 ng/ml for adiponectin.

Adipose tissue collection

Fat biopsies (1 g) were obtained prior to initiating cardiopulmonary bypass, approximately 1 h after induction of anesthesia, from (a) SAT from the sternotomy incision at the manubrium sterni level, and (b) EAT from the right ventricle.

RNA extraction and quantitative real-time polymerase chain reaction

Biopsies (1 g) were immediately frozen at −80 °C. RNA was extracted by the RNeasy Mini Kit (Qiagen, Hilden, Germany) method. Quantitative real-time polymerase chain reaction (qPCR) of adiponectin, MCP-1, and CD68 was performed. Total RNA was extracted with Qiazol (Qiagen) and chloroform, and the aqueous phase purified using an RNeasy Mini or Micro Kit (Qiagen). For real-time qPCR, 15 ng of total RNA was reversed transcribed into cDNA within 20-μl reactions using the IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Aliquots of the reverse transcriptase, or water only (negative control), were PCR-amplified with the QuantiTect SYBR Green PCR kit (Qiagen) and with QuantiTect primer Assays (Qiagen) for each gene in the iCycler thermocycler (Bio-Rad). The Entrez gene IDs were 9370 for adiponectin, 6347 for MCP-1, and 968 for CD68. Thermal cycling conditions for PCR reactions were 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The adiponectin, MCP-1, and CD68 values were normalized against β-actin RNA for each sample.

Quantification of mRNA was performed in triplicate for each well, and gene-expression analysis was performed using Bio-Rad iQ5 software.

Optic microscopy and morphometry

Small fragments of SAT and EAT tissue were fixed by immersion in 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4 °C, then dehydrated, cleared, and embedded in paraffin.

Tissue samples were stained with hematoxylin–eosin and observed with a Nikon Eclipse E800 light microscope using a 20× objective, and digital images were captured with a DXM 1200 camera. Adipocyte diameter was determined using a drawing tablet and the Nikon Lucia IMAGE version 4.61 morphometric program (Nikon, Tokyo, Japan) [11].

Statistical analysis

Data are shown as mean ± standard deviation. Log transformation was performed before analysis for abnormally distributed variables (adipocyte diameters, serum adiponectin, leptin, glycemia, insulinemia, and lipids as well as all gene expressions in AT). To compare the prevalence of CAD, and different drugs between the two groups, the Chi-Quadrate test was used. Differences in adipokine gene expression and adipocyte diameters were estimated by analysis of variance, and evaluated after adjustment for BMI or waist circumference, age, and CAD by analysis of covariance (ANCOVA). Pearson correlation was used to determine association between variables. All analyses were performed using SPSS (version 17.0) software (SPSS, Chicago, IL, USA). Statistical significance was indicated by $P < 0.05$.

Results

Specific health-related characteristics differed significantly between diabetic and nondiabetic patients (Table 1). Specifically, diabetic subjects had significantly higher BMI, waist circumference, glycemia, insulinemia, and lower levels of high-density lipoprotein cholesterol. Leptin serum levels were higher and adiponectin serum levels were lower in subjects with diabetes (respectively 9.04 ± 8.09 vs 3.77 ± 2.2, $P < 0.05$; 5.65 ± 3.12 vs 8.14 ± 4.07, $P < 0.05$). Age, total cholesterol, and triglyceride levels were not significantly different in these two groups.

Ten of 14 (71.4 %) diabetic patients had coronary artery disease compared with 9 of 20 (45 %) diabetic subjects ($P = 0.119$).

Table 1 Clinical and metabolic characteristics at baseline and between diabetic and nondiabetic patients

Variables	Total mean ± SD (n = 34)	DM mean ± SD (n = 14)	No DM mean ± SD (n = 20)	P
Age (years)	65.76 ± 9.03	63.43 ± 7.13	67.4 ± 10	0.21
CAD	19 (55.8 %)	10 (71.4 %)	9 (45 %)	0.119
Height (cm)	171.03 ± 7.47	173.28 ± 6.28	169.45 ± 7.93	0.14
Waist (cm)	100.06 ± 11.13	104.79 ± 13.25	96.75 ± 8.18	0.36
BMI (kg/m^2)	27.51 ± 4.06	29.63 ± 4.66	26.02 ± 2.86	0.008
Serum/plasma assessments				
Total cholesterol (mmol/l)	168.52 ± 41.64	159 ± 29.87	174.7 ± 47.48	0.3
HDL cholesterol (mmol/l)	44.31 ± 10.78	39.11 ± 9.79	47.05 ± 10.52	0.073
Triglycerides (mmol/l)	122.91 ± 63.3	130.31 ± 69.38	118.1 ± 60.39	0.59
Glycemia (mg/dl)	108.48 ± 22.56	126.36 ± 23.29	94.85 ± 9.77	<0.001
Insulinemia (mU/l)	6.2 ± 4.95	9.59 ± 5.74	3.36 ± 1.29	<0.001
Adiponectin (μg/ml)	7.08 ± 3.85	5.65 ± 3.12	8.14 ± 4.07	0.039
Leptin (ng/ml)	5.94 ± 5.95	9.04 ± 8.09	3.77 ± 2.2	0.015
Drugs				
Statins	22 (64.7 %)	11 (78.6 %)	11 (55 %)	0.147
ACE-I	16 (47.1 %)	5 (35.7 %)	11 (55 %)	0.224
Sartanics	8 (23.5 %)	3 (21.4 %)	5 (25 %)	0.440
Insulin	4 (11.8 %)	4 (28.6 %)	–	–
Oral antidiabetics	6 (17.6 %)	6 (42.9 %)	–	–

Values are expressed as mean value ± standard deviation

DM diabetes mellitus subjects, *CAD* coronary artery disease, *BMI* body mass index, *HDL* high-density lipoprotein, *ACE-I* angiotensin-converting enzyme inhibitor

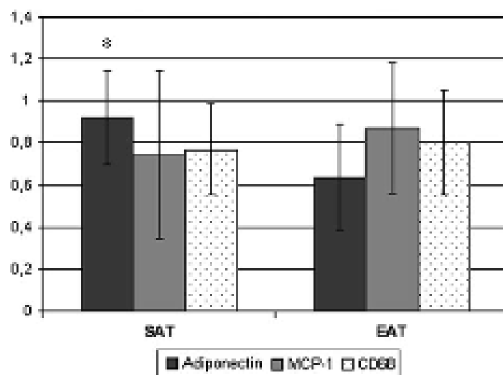

Fig. 1 Comparison of adiponectin, MCP-1, and CD68 gene expression in subcutaneous (*SAT*) and epicardial (*EAT*) adipose tissue in the whole population. *$P < 0.05$. Values are expressed as logarithmic transformation

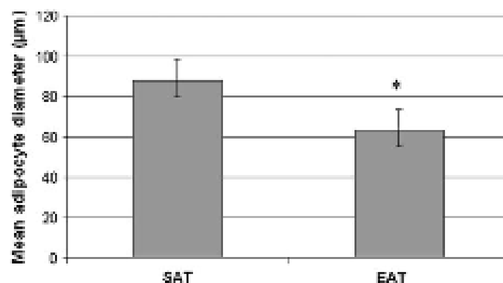

Fig. 2 Comparison of mean adipocyte diameter (μm) among subcutaneous (*SAT*) and epicardial (*EAT*) adipose tissue in the whole population

Considering the study population as a whole, adiponectin gene expression was significantly higher in SAT than in EAT (log value 0.92 ± 0.21 vs 0.64 ± 0.25; $P < 0.005$). Higher MCP-1 gene expression, although not statistically significant (log value SAT 0.73 ± 0.41 vs EAT 0.87 ± 0.31; $P = 0.12$), was observed in then EAT patients. No difference was found in CD68 gene expression between SAT and EAT (Fig. 1).

In line with our previous results [7], adipocyte size was significantly greater in SAT than in EAT (88.15 ± 10.32 vs 63.37 ± 8.3 μm; $P < 0.01$) (Fig. 2).

Table 2 shows the correlations between BMI, waist circumference, serum adiponectin, and leptin, as well as gene expression of adiponectin, MCP-1, and CD68 in SAT and EAT. BMI and waist circumference were both significantly associated with serum leptin and gene-expression levels of both MCP-1 and CD68 in EAT. Serum adiponectin was significantly related to its gene expression in both SAT and EAT. More interestingly, adiponectin gene expression in SAT was negatively related to gene expression of MCP-1 and CD68 in SAT, and adiponectin gene expression in EAT was negatively related to gene expression of CD68.

Subjects with diabetes had significantly lower adiponectin gene expression within both SAT and EAT when compared with nondiabetic subjects (SAT log value 0.80 ± 0.17 vs 1.0 ± 0.20; $P < 0.01$; EAT log value 0.46 ± 0.25 vs 0.75 ± 0.21; $P < 0.01$). By contrast, higher MCP-1 and CD68 gene expression was observed in SAT and EAT of diabetic subjects when compared with nondiabetic subjects (MPC-1 SAT log value 0.98 ± 0.38 vs 0.56 ± 0.33, $P < 0.05$; EAT log value 0.58 ± 0.36 vs 0.37 ± 0.20, $P < 0.05$;

Table 2 Correlations between BMI, waist circumference, serum adiponectin and leptin, and gene expression of adiponectin, MCP-1, and CD68 in subcutaneous (SAT) and epicardial (EAT) adipose tissue in the whole population

	BMI (n = 34)	WC (n = 34)	Serum adiponectin (n = 33)	Serum leptin (n = 34)	Adiponectin SAT (n = 34)	Adiponectin EAT (n = 34)	MCP-1 SAT (n = 34)	MCP-1 EAT (n = 34)	CD68 SAT (n = 34)	CD68 EAT (n = 34)
BMI	1	0.581**	−0.109	0.745**	−0.254	−0.257	0.320	0.569**	0.523**	0.364*
WC	0.581**	1	−0.350*	0.696**	−0.103	−0.229	0.206	0.445**	0.512**	0.384*
Serum adiponectin	−0.109	−0.350*	1	−0.182	0.554**	0.576**	−0.421*	−0.233	−0.234	−0.257
Serum leptin	0.745**	0.696**	−0.182	1	−0.233	−0.237	0.100	0.549**	0.491**	0.295
Adiponectin SAT	−0.254	−0.103	0.554**	−0.233	1	0.316	−0.675**	−0.118	−0.445**	−0.252
Adiponectin EAT	−0.257	−0.229	0.576**	−0.237	0.316	1	−0.418*	−0.299	−0.392*	−0.596**
MCP-1 SAT	0.320	0.206	−0.421*	0.100	−0.675**	−0.418*	1	0.230	0.528**	0.277
MCP-1 EAT	0.569**	0.445**	−0.233	0.549**	−0.118	−0.299	0.230	1	0.250	0.219
CD68 SAT	0.523**	0.512**	−0.234	0.491**	−0.445**	−0.392*	0.528**	0.250	1	0.523**
CD68 EAT	0.364*	0.384*	−0.257	0.295	−0.252	−0.596**	0.277	0.219	0.523**	1

BMI body mass index, *WC* waist circumference

* $P < 0.05$; ** $P < 0.01$

CD68 SAT log value 0.61 ± 0.19 vs 0.44 ± 0.22, $P < 0.05$; EAT log value 0.94 ± 0.23 vs 0.50 ± 0.29, $P < 0.01$) (Fig. 3).

After adjustment for waist circumference, age, and CAD with an ANCOVA test, the difference between subjects with and without diabetes regarding adiponectin gene expression in SAT and EAT were still significant (SAT: without diabetes (noDM) 0.99 ± 0.044 mean ± standard error, with diabetes (DM) 0.816 ± 0.055, $P < 0.05$; EAT: noDM 0.73 ± 0.056, DM 0.486 ± 0.068, $P < 0.05$). Differences in MCP-1 gene expression after the same adjustments were still significant in SAT (noDM 0.596 ± 0.085, DM 0.937 ± 0.104, $P > 0.05$) but lost in EAT. Finally, differences in CD68 gene expression following adjustments were lost in SAT but still significant in EAT (noDM 0.497 ± 0.058, DM 0.935 ± 0.071, $P < 0.001$). Similar results were obtained if BMI instead of waist circumference was considered.

SAT adipocyte size was not statistically different between diabetic and nondiabetic subjects while EAT adipocytes were significantly larger in diabetic subjects (66.85 ± 7.09 vs 60.76 ± 8.38 μm; $P < 0.05$).

After adjustment for waist circumference or BMI, age, and CAD, the difference between adipocyte size in EAT of subjects with and without diabetes was lost (if waist circumference was considered, $P = 0.086$; if BMI was considered, $P = 0.062$).

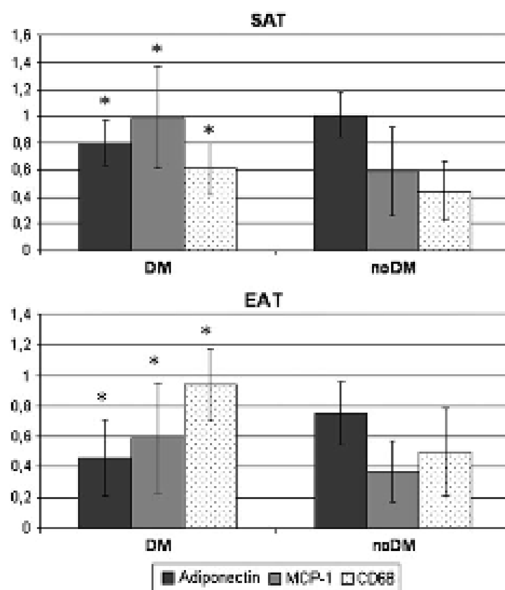

Fig. 3 Comparison of adipocytokine gene expression between diabetic and nondiabetic subjects. Adiponectin, MCP-1, and CD68 gene expression in a subcutaneous (*SAT*) and b epicardial (*EAT*) adipose tissue. **P* < 0.05. Values are expressed as logarithmic transformation. *DM* with diabetes mellitus, *noDM* without diabetes mellitus

Discussion

An interesting debate has recently developed regarding the possible link between perivascular AT inflammation and atherosclerotic lesions [12]. Attention has been particularly focused on EAT, given its proximity to heart and coronary arteries [6, 13–15]. It has been established that EAT exhibits peculiar biochemical properties, with differences in fatty acid composition, release, and uptake in comparison with SAT [16]. In addition, EAT is a source of several bioactive molecules [13, 14].

The inflammation of adventitia has recently emerged as a mechanism involved in the atherosclerosis process within the artery wall [17]. Many potential adipocyte signals seem to be relevant to this process.

Around the coronaries and the aorta, the distance between periadventitial fat and adventitia measures <0.1 mm [15]. AT can secrete adipokines directly into the bloodstream, affecting cells in each of the artery wall layers in a paracrine manner. Alternatively, AT releases these adipokines directly into the vasa vasorum. Inflammatory cytokines and nonesterified fatty acids released by perivascular AT could indeed modulate insulin sensitivity and cellular function.

Yudkin et al. [18] hypothesized that perivascular AT in cases of overnutrition might also act in a paracrine and vasocrine manner, altering the action of insulin-mediated balance between nitric oxide and endothelin-1, effectively reducing insulin-induced vasodilation. Moreover, in a recent paper Derici et al. [19] showed a possible direct effect of nitric oxide on the contraction of the right atrium in a rat heart model.

Besides adipocytes, AT contains a stromal-vascular matrix (SVM), which may have a role in the secretion of many cytokines [1]. In a related study, obesity in mice was demonstrated to lead to increased macrophage infiltration and cytokine expression in perivascular AT surrounding the abdominal aorta [20]. Moreover, the release of chemoattracting molecules from adipocytes stimulates macrophage migration to the depot, further exacerbating inflammation and adipocyte dysfunction.

In 2003 Mazurek et al. [14] pointed out the importance of EAT as a source of inflammatory markers and EAT infiltration by inflammatory cells. Following these findings different authors have addressed this issue, obtaining conflicting results [21]. Recently, Fain et al. [22] compared the gene-expression levels of 45 genes in obese subjects, reporting no differences in the expression of inflammatory

adipokines in EAT when compared with substernal SAT. With the aim of evaluating the inflammatory profile and macrophage infiltration in EAT, we measured gene expression of MCP-1 and CD68 in both EAT and SAT. MCP-1 is a chemotactic factor that promotes macrophages homing within AT, and is produced both by adipocytes and SVM. CD68 is a standard macrophage marker.

In line with Fain et al. [22], we did not observe statistical differences in gene expression of MCP-1 and CD68 when comparing EAT with SAT. By contrast, a significant difference was observed in expression of the anti-inflammatory factor adiponectin in EAT when compared with SAT. In contrast to Fain et al. [22], we observed that both BMI and waist circumference were significantly related to both MCP-1 and CD68 expression in EAT. More interestingly, we observed that serum adiponectin levels were related to adiponectin gene expression in both SAT and EAT.

We further analyzed the inflammatory characteristics of EAT in diabetics in comparison with nondiabetic subjects. EAT thickness has been associated with type 2 diabetes and insulin resistance [8, 23], but studies concerning alterations to adipokine expression within EAT in relation to diabetes are limited [24]. Teijeira-Fernandez et al. [25] recently reported similar adiponectin and leptin mRNA expression in EAT and SAT between subjects with and without diabetes. By contrast, we identified higher gene expression of both MCP-1 and CD68, and lower adiponectin gene expression in diabetic subjects when compared with nondiabetic subjects. In addition, diabetic subjects were determined to have larger adipocytes.

This difference between our findings and the those of Teijeira-Fernandez et al. could be partially explained by highlighting differences between the two studied populations. Our diabetic subjects were younger than the subjects considered by Teijeira-Fernandez et al. (mean age 63.4 vs 70.4 years). They were also more overweight and presented with a tendency toward visceral obesity compared with the nondiabetic subjects, whereas the groups analyzed in Teijeira-Fernandez et al. were more homogeneous [25].

It has been suggested that enlargement of adipocytes, usually referred to as hypertrophy, is strongly related to insulin resistance [1, 26]. Larger adipocytes have been shown to have increased lipolytic capacity, an observation that is independent of the specific donor [27]. Both rodent and human in vitro studies have demonstrated that enlarged adipocytes are insulin resistant with respect to glucose uptake [28]. Enlargement of adipocytes is frequently observed in prediabetic individuals and in type 2 diabetics [28]. Fat cells pushed to the limits of their ability to store lipids reach a genetically determined "critical size," which leads to hypoxic stress and the release of higher levels of proinflammatory mediators, such as leptin and resistin, and lower amounts of adiponectin [1, 29, 30]. However, clinical studies reported differences in adipocyte size among different AT depots in the same patient, showing, for example, that visceral adipocytes are significantly smaller than subcutaneous adipocytes [1], both in normal-weight and obese subjects [30].

Insulin resistance and type 2 diabetes, as well as obesity, have been linked to a chronic subclinical state of inflammation [31]. It is tempting to speculate that obesity may lead to an increase in adipocyte size within the EAT depot and that insulin resistance and inflammation may be related to a critical adipocyte size. Owing to the location of EAT, this phenomenon may be particularly relevant from a clinical point of view and may represent a link between diabetes and CAD. In fact, in our study population, as expected, more than 70 % of subjects with diabetes had CAD whereas 40 % of those without diabetes had CAD.

Some limitations of our study should be recognized. First, the study was designed only to show an association between variables and cannot provide any cause–effect relationships. Thus, our results are somewhat preliminary. Further in vitro and/or in vivo studies will help to clarify other pending issues. Second, the role of other cells, besides adipocytes and macrophages, has not been investigated. However, it appears likely that other inflammatory cells may be involved in the inflammatory process.

In conclusion, this study shows a significant relationship between diabetes and both adipocyte size and inflammation within AT. Our data indicate that EAT has a lower anti-inflammatory profile and smaller adipocytes than SAT. Data also suggest a link between diabetes and a more inflammatory profile within EAT. These findings stress the role of perivascular fat as a potential contributor to cardiovascular diseases, and emphasize the multiple characteristics of different AT depots.

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Conflict of interest None of the authors had a financial interest, nor professional or personal affiliation that compromises the scientific integrity of this work.

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4. **Characterization of myocardial fat and macrophages infiltration in human**

4.1 Material and Methods

A number of 45 subjects were included in the study.

All participants were selected as described in paper number 1 and 2.

Serum collection and analysis have been previously described in paper 1 and 2.

Atrial fragments (1 gr) were collected and stored for immunohistochemistry during cardiosurgery.

Immunohistochemistry:

Freshly isolated atrial tissue was fixed in 4% formaldeid 0.1M sodium PB, pH7.4, overnight at 4°C, and later embedded in paraffin block. The paraffin-embedded tissue block were sectioned at 4 µm thickness on a microtome and transferred onto glass slides for immunohistochemistry.

Deparaffinization of the slides was performed trough 2 changes of xylene for 10 minutes. Slides were transferred to 100% alcohol, for 2 changes, 2 min each, and then transferred once through 95%, 80% alcohols respectively for 2 min each, followed by a quick passage into pure distilled water. Antigen retrieval microwave heating (750W) technique with citrate buffer solution (pH 6.00) was applied. Saturation of non specific binding sites was performed with Universal Protein Blocking Reagent (150 um for each glass slide 30 minutes, room temperature).

Evaluation of mitochondial and endoplasmic stress: Grp75 and Grp78.

Primary antibody: Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA goat polyclonal anti-Grp78 (sc-1050) and rabbit polyclonal anti-Grp75 (sc- 13967) (dilution 1:100 in PBS).

Secondary antibody: kit rabbit ABC-peroxidase staining system (Santa Cruz Biotechnology Inc) and DPX;

Analysis: Intensity of Grp75 and Grp78 has been evaluated through an optic microscope Olympus BX50 and Image Pro Plus 4.5.1, Immagini e Computer, Milano, Italy, and has been quantitatively

analized. Optical density (IOD) has been calculated for arbitrary areas measuring 10 areas for each sample 40x.

Evaluation of Macrophage infiltration: CD68 and CD 163.

Primary antibody: Invitrogen Human Anti CD 68 Clone KP1 (no dilution; 100 microliter for slide); Leica Novocastra Human Anti CD163, clone 10D6 (room temperature incubation for 60 minutes; dilution 1:1000 in PBS buffer 0.1M pH7.4, 5% normal goat serum; 100 microliter for slide);

Secondary antibody: SignalStain Boost IHC detection reagent HRP Mouse from Cell Signaling (30 minutes at room temperature);

Analysis: macrophage count has been conducted manually, through an optic microscope Olympus BX50, and images have been acquired through photo camera Imagin Qicam fast 1394, Image Pro Plus softwer version 7.0.

Evaluation of adipocytes myocardial infiltration: Perilipin1;

Evaluation of intra myocyte LD: Perlipin2;

Primary antibody: Cell Signaling Human Anti Perilipin1 (overnight 4°C incubation; dilution 1:1000 SignalStain Antibody Diluent Cell Signaling; 100 microliter for slide); SBio LifeSpan BioSciences Human Anti Perilipin2 (overnight 4°C incubation; dilution 1:1000 in PBS buffer 0.1M pH7.4, 5% normal goat serum; 100 microliter for slide);

Secondary antibody: DAB substrate from Vector Labs (5 minutes at room temperature; 200 microliter per slide);

Analysis: Intensity of PLIN1 and PLIN2 has been evaluated through an optic microscope Olympus BX50, and images have been acquired through photo camera Imagin Qicam fast 1394, Image Pro Plus softwer version 7.0, red green blue channels, with conversion to 8 bit images. IOD has been calculated for arbitrary areas measuring 10 areas for each sample 40x.

Statistic analysis

All results are reported as mean ± SD. Logarithmic transformation was performed on serum adiponectin, leptin, mRNA levels to normalize the distribution. Comparisons between the 2 groups of patients were performed using Student's 2-tailed unpaired test. Relation between variables were evaluated by simple correlation coefficients and by stepwise multiple regression analysis.

4.2 Results

Characteristics of the subjects are summarized in Table II. There was no difference in age and height between CAD and nonCAD, whereas the CAD group had significantly higher percentage of diabetic subjects, higher waist and BMI. Circulating levels of adiponectin were significantly lower and circulating levels of leptin were significantly higher in CAD subjects than in nonCAD. Regarding drugs, Statins (CAD 23 (85%) vs nonCAD 5(28%)), Insulin (CAD 7 (26%) vs nonCAD 0) and oral anti-diabetics (CAD 10 (37%) vs nonCAD 0) were significantly more common in CAD subjects then in nonCAD. No difference was found between CAD and nonCAD regarding ACE-I and Statins.

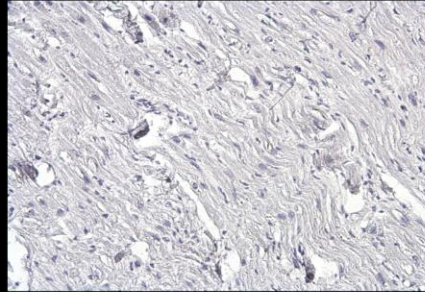
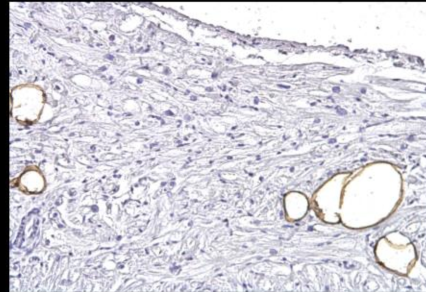
Variables	**CAD M±SD (n 27)**	**nonCAD M±SD (n 18)**	**total M±SD (n 45)**	p
Age	65.6 ± 8.1	64.9 ± 9.5	65.4 ± 8.6	0.797
DM2	17 (63%)	3 (17%)	20 (22.5%)	0.002
Height (cm)	170.6 ± 5.6	171.9 ± 8.7	171.1 ± 6.9	0.55
Waist (cm)	102.2 ± 12.7	94.5 ± 12.4	99.3 ± 13	0.06
BMI (kg/m^2) .	28.6 ± 5.5	25.6 ± 3.3	27.3 ± 4.9	0.045
Adiponectin (ug/mL)	11.4 ± 12.6	24.3 ± 25	16.7 ± 20.1	0.014
Leptin (ng/mL)	7.8 ± 7.9	3.4 ± 2.1	6 ± 6.6	0.010

Table II.

Both PLIN1 and PLIN2 resulted to be significantly higher in CAD subjects (respectively: 0.32±0.04 vs 0.28±0.05; $p<0.01$; 0.27±0.05 vs 0.22±0.05; $p<0.01$); as well as Grp 78, CD68 and CD163 (respectively: 1.48±0.51 vs 1.1±0.38; $p<0.01$; 1.43±0.51 vs 0.94±0.51; $p<0.01$). Grp75 showed a tendency, but was not significant (Figure 7). Even after adjusting the model for DM2 or BMI Grp78, the difference in PLIN1 and PLIN2 between CAD and nonCAD remained significant, whereas the difference in CD68 and CD163 between the two groups did not. In Table III are shown the correlations between the variables. Circulating levels of adiponectin were significantly negatively correlated to Grp78, Grp75, PLIN1 and PLIN2. PLIN2 resulted correlated to BMI. Stepwise regression analysis was performed using PLIN1 as dependent variable, and BMI, age, DM, serum adiponectin, serum leptin, PLIN2, Grp75, Grp78, CD68, CD163 as independent variables for all subjects. Only serum adiponectin entered the regression ($r=.540$, $p<0.05$). Stepwise regression analysis was performed using PLIN2 as dependent variable, and BMI, age, DM, serum adiponectin, serum leptin, PLIN1, Grp75, Grp78, CD68, CD163 as independent variables for all subjects. Only serum DM entered the regression ($r=.483$, $p<0.05$)

Table III

	Serum Adiponectin	**BMI**	**Età**	**Grp 78**	**Grp 75**	**PLIN 1**	**PLIN 2**	**CD68**	**CD163**
Serum Adiponectin	1 n45								
BMI	-.054 .727 n45	1 n47							
Età	.002 .991 n45	-.060 .686 n47	1 n48						
Grp 78	-637** .000 n27	.225 .249 n28	.129 .514 n28	1 n28					
Grp 75	-.544** .003 n27	-.011 .957 n28	.015 .938 n28	.810** .000 n28	1 n28				
PLIN 1	-.609** .002 n23	.343 .101 n24	.081 .702 n25	.252 .324 n18	.344 .162 n18	1 n25			
PLIN 2	-.480** .003 n36	-.408* .011 n38	-.165 .315 n39	.393 .052 n25	.115 .585 n25	.334 .120 n23	1 n39		
CD68	-.059 .716 n41	-.008 .959 n43	.215 .162 n44	.105 .602 n27	-.019 .927 n27	.137 .523 n24	.083 .620 38	1 n44	
CD163	-.226 .155 41	.036 .821 n43	.219 .154 n44	.205 .304 n27	.077 .702 n27	.298 .158 n24	.193 .247 n38	.945** .000 n44	1 n44

nonCAD
CAD
Plin-1


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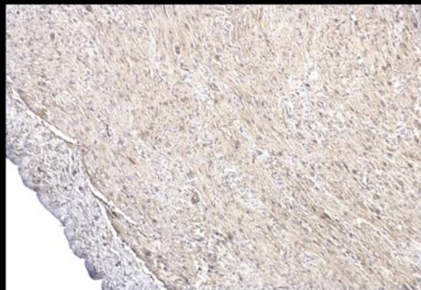
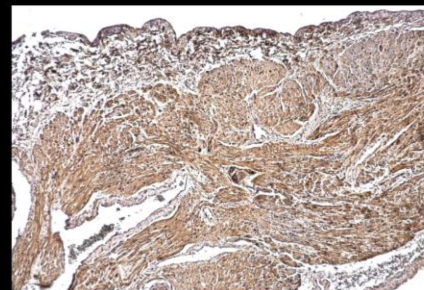
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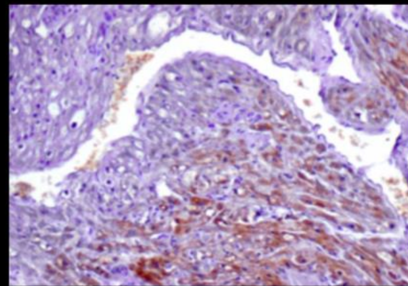
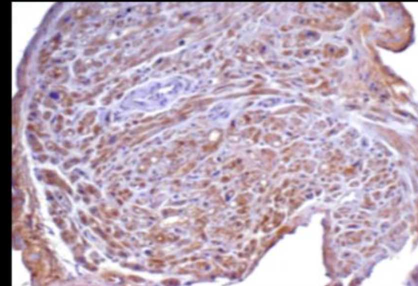
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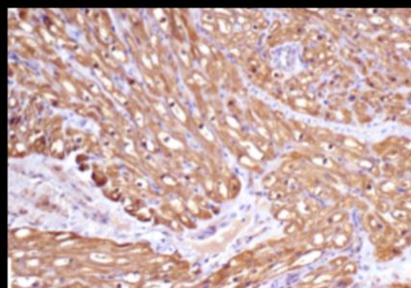
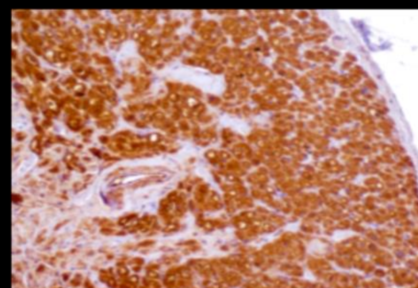
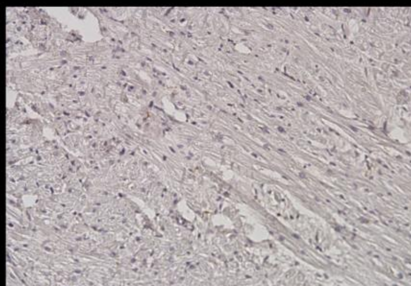
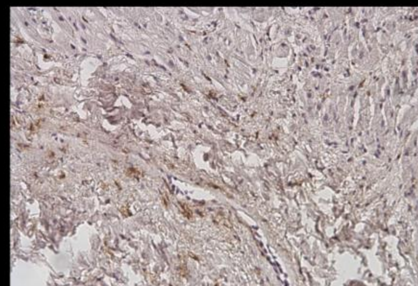


CD68



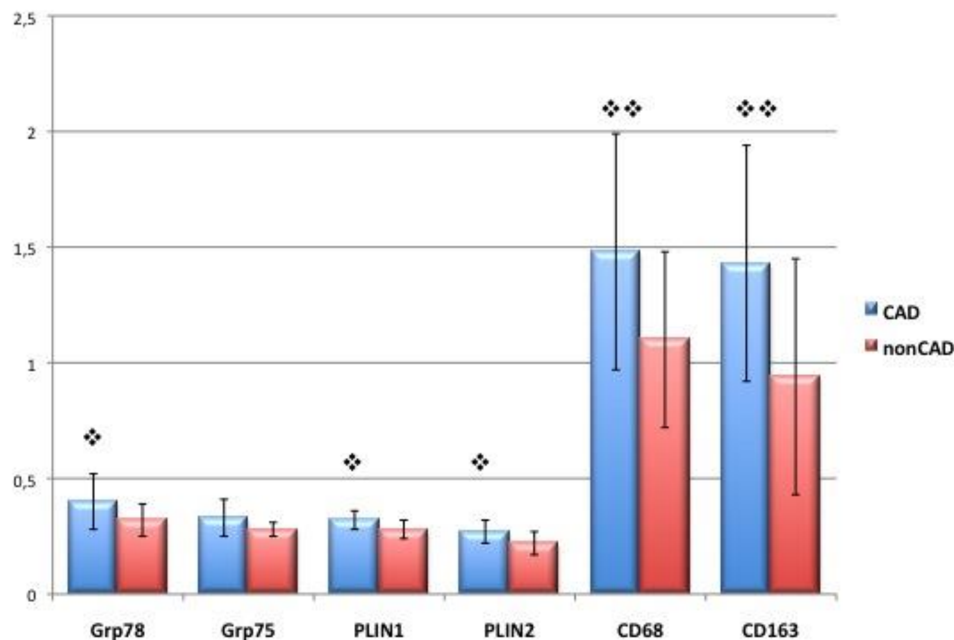


Figure 7. Immunohistochemistry of Myocardium in nonCAD and CAD subjects (10x); and statistical analysis.

4.3 Discussion

In paper 1 and 2 we evaluated the characteristics of EAT. The recent results are focused on the characterization of atrial myocardium in patient with and without CAD, through immunohysochemistry analysis.

Our evaluation of myocardial fat infiltration was focused on the identification of two different kind of TG accumulation: adipocyte infiltration among myocardiocites, as well as intramyocardioctes lipid droplets (LDs) infiltration.

We found that CAD subjects compared to nonCAD, have significantly higher adipocyte infiltration and LDs infiltration, as well as significantly higher markers of ER stress and MGs infiltration.

TG are stored in multiple minute LDs, with a core of lipid and a monolayer of phospholipids, free cholesterol (FC), and proteins. The most abundant proteins in LDs are the PAT family proteins, which take the name from the founding member, Perilipin (Paul 2008), involved in the hormone-stimulated lipolysis and regulated by peroxisome proliferator-activated receptors (PPARs). Perilipin1 (PLIN1) is selectively expressed by adipocytes and steroidogenic cells, whereas Perilipin2 (PLIN2) has been described ubiquitously on mammary tissues on myocardial LDs and not on adipocytes (Yamaguchi 2006). Analyzing muscular biopsies, it has been seen that PLIN1 is located among myofiber on the LD membrane of the intramuscular adipocytes, whereas PLIN2 is located within myofibers with high lipid content. (Gandolfi 2011) PLIN1 is indeed considered a marker of vital and mature adipocytes, whereas PLIN 2 is considered a marker of LD (Gandolfi 2011, Yamaguchi 2006). According to our results CAD subjects had a significant higher amount of both PLIN-1 and PLIN-2.

The consequent accumulation of lipotoxic lipid products, leads to lipotoxicity, that can mediate both apoptosis and oncotic cell death in cardiomyocytes (Kong 2003), impairing contractile function and organ dysfunction. In animal models of obesity and diabetes mellitus, intramyocardial lipid accumulation has been demonstrated to be related to decreased myocardial function, primarily associated with diastolic dysfunction (Christoffersen 2003).

Given the fact that the induction of apoptosis in just 0.02% of myocytes is sufficient to cause a lethal, dilated cardiomyopathy (Wencker 2003), it’s easy to understand the growing interest in understanding the relationship between the fat and the heart.
Mitochondrial and ER dysfunctions seem to be kay mechanism leading to cell death in myocardial lipotoxicity. Obesity is associated with an increment of the number and a change in the morphology of adipocytes mitochondria. This mechanism is supposed to make front to the higher energy request, but these mitochondria don’t express higher genes involved in the oxidative

phosphorylation, showing reduced production of ATP (The mitochondrial pathway seems to be linked to activation of caspase and mitochondrial loss of cytochrome c. The increase in mitochondrial number, supposed to be a supplying mechanism to the higher energy request, occurs without a concomitant increase in the expression of nuclear-encoded genes that encode oxidative phosphorylation subunits. These mitochondria shows reduced oxidative capacity for glucose and ATP generation, resulting in dysfunctional mitochondria (Kim 2006; Wende 2010).

A well known marker of mitochondrial dysfunction is Grp75 (Hayashi 2009). Grp78 is on the other hand altered when incorrect protein folding occurs. When there is a mismatch between the UPR and protein translation, ER stress ensues and may ultimately lead to cell death. Grp78 is indeed used as marker of ER stress (Gregor 2007).

Supporting this thesis our results show that CAD subjects express significantly higher amounts of Grp78 and have a tendency of a higher expression of Grp75.

Cell disfunction and death can lead to MG infiltration.

Only few studies have evaluated EAT MG infiltration. Markers of macrophage infiltration seem to be higher in EAT of CAD patients, with a relevant component of M2-like MG (Sacks 2011). More specificity Hirata et al evaluated cell surface markers and cytokine expression in EAT and SAT in patients with and without CAD (Hirata 2011). CD68 (panMG marker) was significantly higher in CAD, and interestingly no only M1 but also M2 resulted higher in CAD EAT; the ratio M1/M2 was positively correlated with the severity of CAD, showing a relative increase in M1 and a relative decrease in M2 in EAT CAD (Hirata 2011).

Infiltration of MG in the myocardium has been described in advanced stages of myocardial infarction (Akasaka 2006) and in subjects with apoptotic cell death during acute rejection in heart transplants (Ozdemin 200), but it has not been well characterized in CAD.

In our subjects we analyzed CAD68 expression as a marker of panMG infiltration and CD163 as a marker of M2 infiltration. According to our results the myocardium of CAD subjects is

significantly more infiltrated by MG compared to nonCAD subjects. M2 infiltration results to be also significantly higher in CAD.

The role of M2 infiltration could be considered as an attempt of tissue repairmen. Recently it has been discussed about the effective antiinflammatory activity of M2 in different tissues.

Flow citometry of AT MG pointed out how different AT depots express higher amount of CD163 MG; in vitro differentiated AT MG showed a surprising proinflammatory secretion pattern from M2 (Zeyda 2007). This founding drove the researchers to the conclusions that AT MG comprise a particular macrophage type that has M2 surface characteristics but that produces extensive amounts of inflammatory cytokines (Zeyda 2007).
More studies are needed to better elucidate MG infiltration in the myocardium and its significance.

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BMC Endocrine Disorders

RESEARCH ARTICLE Open Access

NPC1 in human white adipose tissue and obesity

Clara Bambace, Ingrid Dahlman, Peter Arner and Agné Kulyté*

Abstract

Background: Genetic studies have implicated the *NPC1* gene (Niemann Pick type C1) in susceptibility to obesity.

Methods: To assess the potential function of NPC1 in obesity, we determined its expression in abdominal white adipose tissue (WAT) in relation to obesity. *NPC1* mRNA was measured by RT-qPCR in lean and obese individuals, paired samples of subcutaneous (sc) and omental (om) WAT, before and after weight loss, in isolated adipocytes and intact adipose pieces, and in primary adipocyte cultures during adipocyte differentiation. NPC1 protein was examined in isolated adipocytes.

Results: *NPC1* mRNA was significantly increased in obese individuals in scWAT and omWAT and downregulated by weight loss. *NPC1* mRNA was enriched in isolated fat cells of WAT, in scWAT versus omWAT but not modified during adipocyte differentiation. NPC1 protein mirrored expression of mRNA in lean and obese individuals.

Conclusions: NPC1 is highly expressed in human WAT adipocytes with increased levels in obese. These results suggest that NPC1 may play a role in adipocyte processes underlying obesity.

Background

Obesity is characterized by an excess of white adipose tissue (WAT) and marked adipocyte dysfunction that increase the risk for insulin resistance, type 2 diabetes mellitus and cardiovascular disease. White adipose tissue is not only energy reserve; it is also an active organ that contributes to whole body energy homeostasis by several different mechanisms. The underlying causes of obesity are complex and involve factors including caloric oversupply, a sedentary life-style, and a genetic predisposition.

A recent genome-wide association study report has indicated that the Niemann-Pick C1 gene (*NPC1*) is associated with early-onset and morbid adult obesity [1]. The NPC1 protein regulates transport of lipoprotein-derived lipid (cholesterol and fatty acids) from late endosomes/lysosomes to other cellular compartments and is responsible for maintaining intracellular cholesterol homeostasis [2-4]. However, the human genome-wide association study did not address how the *NPC1* gene variant promotes weight gain. Interestingly, a recent report showed that *NPC1+/−* male mice, when fed a high fat diet, deposited more fat and were heavier than their wild-type siblings in the absence of hyperphagia. They also developed adipocyte hypertrophy [5,6]. This data suggest that NPC1 has a previously unknown role in maintaining energy and metabolic homeostasis.

Our interest in NPC1 was stimulated by these findings, which imply a role for NPC1 in obesity and potentially adipose metabolism. We have previously reported that *NPC1* is highly expressed in human WAT [7]. In this study we investigated the hypothesis that NPC1 is active within human WAT. We performed a more detailed profiling of NPC1 mRNA and protein levels in relation to obesity and mRNA in relation to regional adipose depots and cellular origin.

Methods

Cohorts and clinical investigation

Investigated cohorts are described in Table 1. Cohorts and clinical investigation were previously described in details [7]. Obesity was defined as a BMI (body mass index) ≥30 kg/m^2, whereas non-obesity was defined as BMI <30 kg/m^2. Some subjects were examined before and 2–3 years after weight loss following either bariatric surgery or behavioral modification when a weight stable non-obese state had been reached. Subjects were recruited by local advertisement for the purpose of studying genes regulating obesity and fat cell function. Informed consent was obtained from all study subjects. The project was conducted in accordance with the guidelines in The

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Results

Adipose *NPC1* expression in obesity

Both obese females and males displayed higher *NPC1* mRNA levels in intact pieces of abdominal scWAT (P=<0.0001 and 0.002, respectively), when compared to non-obese subjects (Cohort 1, Figure 1A). *NPC1* mRNA expression was also increased in omWAT (P=0.013, Cohort 2, Figure 1B) of obese females. We did not have access to male omWAT. *NPC1* mRNA levels were normalized by weight loss following either bariatric surgery or behavioral modification (P=0.0452, Cohort 3, Figure 1C). Age did not influence *NPC1* gene expression (values not shown).

The adipose source of *NPC1* was also studied in more detail. *NPC1* mRNA was higher expressed in scWAT than in omWAT (P=0.0017, Cohort 2, Figure 2A). Moreover, *NPC1* mRNA levels were about two-fold higher in isolated fat cells as compared to intact adipose tissue pieces (Cohort 5, Figure 2B, P=0.018). However, *NPC1* expression was not altered during adipocyte differentiation as evidenced by the mRNA levels in primary adipocyte cultures at day 8 and 12 of differentiation relative to day 4 (Cohort 6, Figure 2C). To investigate if NPC1 protein levels reflect mRNA expression, we assessed NPC1 expression by Western blot in isolated fat cells of obese and lean subjects. Level of NPC1 protein was significantly higher in obese (P=0.0002, Cohort 7, Figure 2D).

To further explore the role of NPC1 in WAT, the associations between scWAT *NPC1* mRNA levels and anthropological measurements, as well as phenotypes reflecting fat cell metabolism was determined (Figure 3). *NPC1* mRNA was positively correlated with BMI (P<0.0001, r=0.473, Figure 3A), waist circumference (P<0.0001, r=0.533, Figure 3B) and fat cell volume (P<0.0001, r=0.429, Figure 3C). There was no association between *NPC1* mRNA and levels of catecholamine-stimulated lipolysis or insulin-stimulated lipogenesis in adipocytes (data not shown).

Discussion

The present detailed study of the obesity-gene *NPC1* revealed that *NPC1* mRNA levels were increased in both subcutaneous and omental fat depots, enriched in subcutaneous fat and isolated fat cells and down-regulated by weight loss. Adipose NPC1 protein mirrored the mRNA levels. Expression of *NPC1* was stable during *in vitro* adipocyte differentiation. There was no gender-specific difference on *NPC1* expression.

The correlation between *NPC1* mRNA levels with BMI and waist circumference in humans clearly fits into the concept of *NPC1* as an obesity gene. In agreement with this, humans affected by Niemann-Pick syndrome, with mutated *NPC1* gene, show impaired gain and maintenance of body weight [15]. However, in Niemann-Pick syndrome we do not know whether the *NPC1* gene has a specific influence on fat accumulation, or whether the impaired weight gain is an unspecific effect due to chronic disease. As regards experimental models, both female and male mice with decreased *NPC1* gene dosage gain weight on a high-fat diet [16]. On the other hand, *NPC1* gene is increased in livers of obese wild-type mice fed a high-fat diet [17]. This upregulation of *NPC1* in liver of obese mice mimic the effect we observe in human adipose tissue and could indicate that upregulation of NPC1 in obese subjects may be part of a pathway to counteract obesity. A similar paradoxical effect is observed for e.g. leptin, which counteracts obesity but still is increased in obese subjects [18]. We demonstrate no gender impact to *NPC1* expression in humans which is in concordance with the mice model.

Until recently, studies of *NPC1* gene function have been mostly focused on the pathogenesis of the CNS

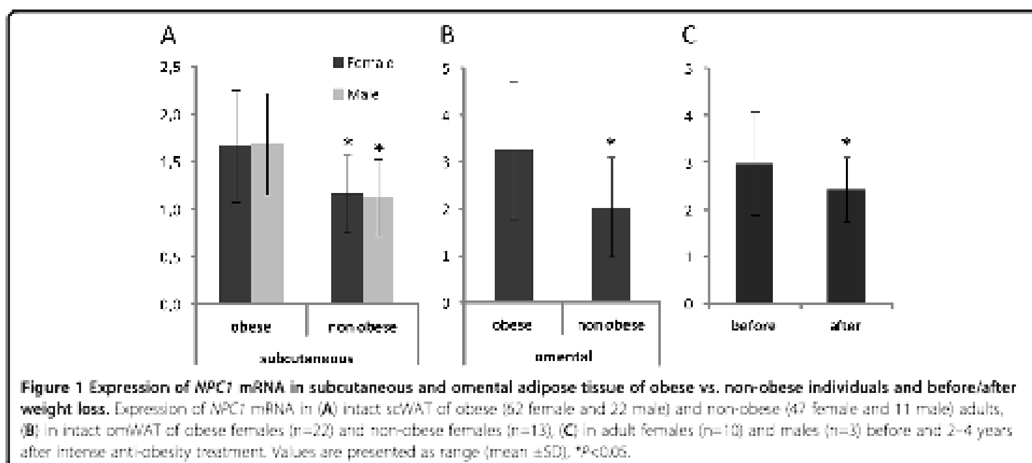

Figure 1 Expression of *NPC1* mRNA in subcutaneous and omental adipose tissue of obese vs. non-obese individuals and before/after weight loss. Expression of *NPC1* mRNA in (**A**) intact scWAT of obese (62 female and 22 male) and non-obese (47 female and 11 male) adults, (**B**) in intact omWAT of obese females (n=22) and non-obese females (n=13), (**C**) in adult females (n=10) and males (n=3) before and 2–4 years after intense anti-obesity treatment. Values are presented as range (mean ±SD). *P<0.05.

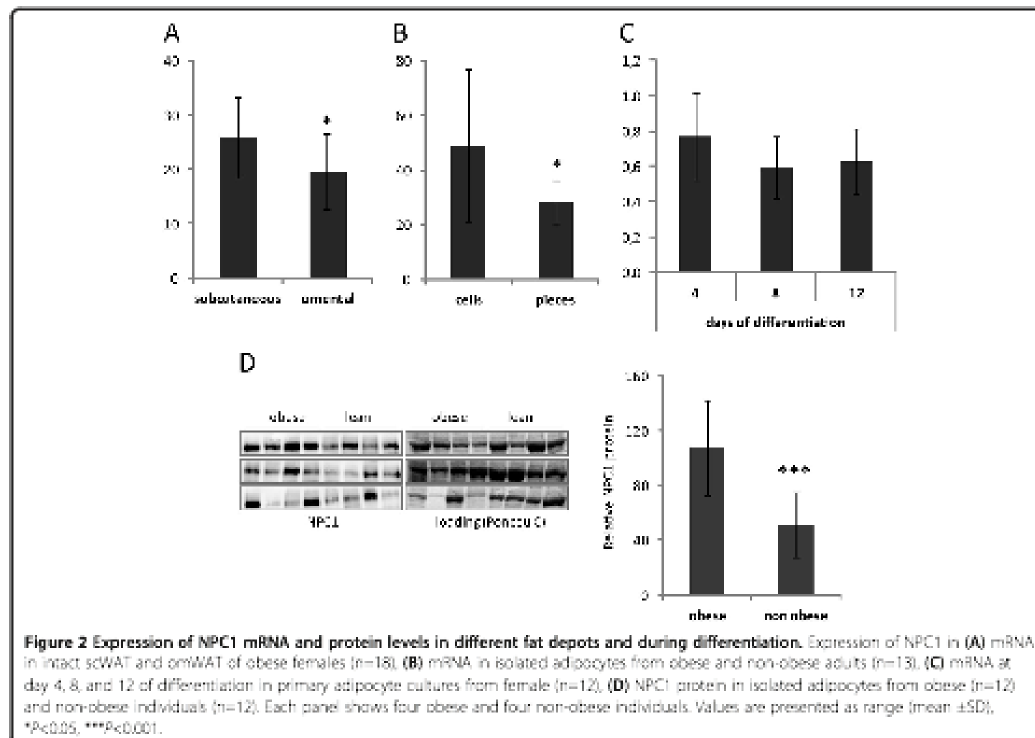

Figure 2 Expression of NPC1 mRNA and protein levels in different fat depots and during differentiation. Expression of NPC1 in **(A)** mRNA in intact scWAT and omWAT of obese females (n=18), **(B)** mRNA in isolated adipocytes from obese and non-obese adults (n=13), **(C)** mRNA at day 4, 8, and 12 of differentiation in primary adipocyte cultures from female (n=12), **(D)** NPC1 protein in isolated adipocytes from obese (n=12) and non-obese individuals (n=12). Each panel shows four obese and four non-obese individuals. Values are presented as range (mean ±SD), *P<0.05, ***P<0.001.

disease even though that is a secondary neurological impairment of lipid accumulation. Our results of increased NPC1 levels in adipose tissue of obese subjects and the consequent normalization after weigh loss, as well as the association between *NPC1* mRNA and fat cell size add impact on NPC1 function on visceral effects and support the concept of a metabolic function of *NPC1* gene in adipose tissue.

Conclusions

NPC1 is enriched in fat cells of human adipose tissue, is elevated in obesity and affected by anti-obesity therapy.

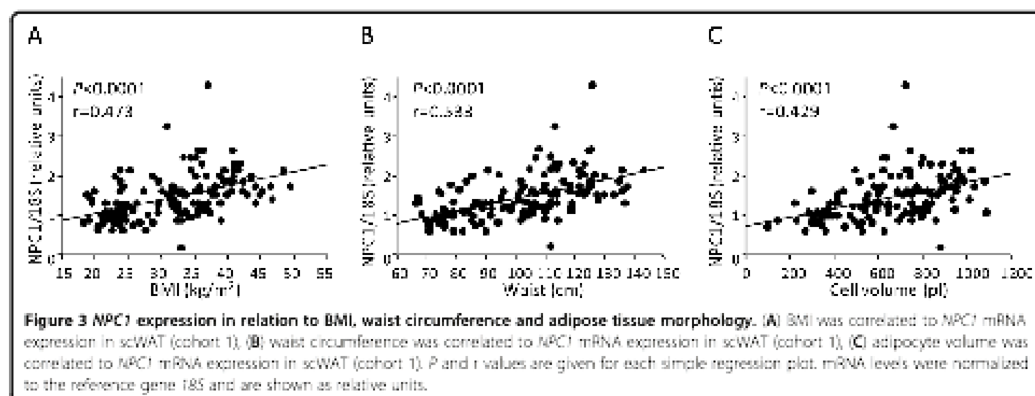

Figure 3 ***NPC1*** **expression in relation to BMI, waist circumference and adipose tissue morphology.** **(A)** BMI was correlated to *NPC1* mRNA expression in scWAT (cohort 1), **(B)** waist circumference was correlated to *NPC1* mRNA expression in scWAT (cohort 1), **(C)** adipocyte volume was correlated to *NPC1* mRNA expression in scWAT (cohort 1). *P* and r values are given for each simple regression plot. mRNA levels were normalized to the reference gene *18S* and are shown as relative units.

NPC1 may partially influence susceptibility to obesity by altering adipocyte function although further studies are needed to decipher its contribution to obesity development.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ID and AK designed the study. CB, ID and AK acquired, analyzed, interpreted the data and drafted the manuscript. ID and PA collected patient material. PA revised the final version of the manuscript. All authors read and approved the final manuscript.

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6. Effect of moderate weight loss on hepatic, pancreatic and visceral lipids in obese subjects.

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ORIGINAL ARTICLE

Effect of moderate weight loss on hepatic, pancreatic and visceral lipids in obese subjects

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OBJECTIVE: To compare the effects of weight loss on visceral and subcutaneous abdominal fat, liver and pancreas lipid content and to test the effects of these changes on metabolic improvement observed after weight loss.
DESIGN: Weight-loss program designed to achieve a loss of 7–10% of the initial weight.
SUBJECTS: 24 obese subjects (13 males and 11 females) with age ranging from 26 to 69 years and body mass index (BMI) 30.2–50.5 kg m^{-2}. Measurements: weight, BMI, waist circumference, body composition as assessed by dual-energy X-ray absorptiometry, metabolic variables, leptin, adiponectin, visceral and subcutaneous abdominal fat, liver and pancreas lipid content as assessed by magnetic resonance were evaluated before and after weight loss achieved by hypocaloric diet.
RESULTS: After a mean body weight decrease of 8.9%, BMI, waist circumference, fat mass, all metabolic variables, homeostasis model assessment of insulin resistance (HOMA), alanine amino transferase, gamma glutamyl transpeptidase, high-sensitivity C-reactive protein (hs-CRP) and leptin, but not adiponectin and high-density lipoprotein-cholesterol, significantly decreased (all $P<0.01$). Visceral and subcutaneos abdominal fat, liver and pancreas lipid content significantly decreased (all $P<0.01$). Percent changes in liver lipid content were greater (84.1 ± 3%) than those in lipid pancreas content (42.3 ± 29%) and visceral abdominal fat (31.9 ± 15.6%). After weight loss, percentage of subjects with liver steatosis decreased from 75 to 12.5%. Insulin resistance improvement was predicted by changes in liver lipid content independently of changes in visceral fat, pancreas lipid content, systemic inflammation, leptin and gender.
CONCLUSION: Moderate weight loss determines significant decline in visceral abdominal fat, lipid content in liver and pancreas. Reduction of liver lipid content was greater than that of pancreas lipid content and visceral fat loss. Liver lipid content is the strongest predictor of insulin resistance improvement after weight loss.

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Keywords: weight loss; visceral fat; ectopic fat; adipokines; insulin resistance

INTRODUCTION

Several studies showed that even moderate weight loss improves metabolic complications of obesity.[1,2] This may be due to a preferential loss of visceral fat compared with subcutaneous[3,4] or may also be due to a reduction in the ectopic fat content.[5–9] In fact, it has been demonstrated that ectopic fat deposition (i.e., liver, pancreas and muscle fat infiltration) significantly contributes to insulin resistance and metabolic alterations observed in obese subjects.[6,7] Recently, in a murine model it has been shown that impairment of glucose homeostasis and insulin resistance are associated with hepatosteatosis[9] and triglyceride overaccumulation in the pancreas,[10] consequent to long-term high-fat feeding. Using computed tomography at the midthigh level, a significant decrease in muscle lipid content after moderate weight loss has been observed in obese sedentary subjects.[5,6,8]

Using magnetic resonance, Colles *et al.*[11] observed a significant decline in liver volume, a proxy of liver fat infiltration, after a mean weight loss of 10% in obese subjects. In eight obese subjects with type 2 diabetes, Peterson *et al.*[12] observed that a weight loss of 8 kg normalized fasting glucose and that this improvement was associated with an 81% decline of intrahepatic lipid content.

To the best of our knowledge, no studies have evaluated the effect of weight loss on pancreas lipid content in obese people or the possible contribution of its decrease to the metabolic improvement observed after weight loss. Furthermore, no studies compared the relative simultaneous decline in liver and pancreas ectopic fat deposition after weight loss.

The aim of this study was to compare the effect of weight loss on visceral and subcutaneous abdominal fat and on liver and pancreas fat content. A further aim was to test the combined and separate effects of these simultaneous declines on metabolic improvement. Magnetic resonance, considered the gold standard for assessment of body composition,[13] was used to quantify visceral and subcutaneous adipose tissue as well as liver and pancreas lipid content before and after weight loss.

MATERIALS AND METHODS

Subjects

A total of 24 subjects (13 men and 11 women) with mean age 46.7 ± 14.3 years and mean body mass index (BMI) 35.4 ± 4.5 kg m^{-2}, were included in the study. All subjects were in good general health, as determined by a complete medical history and physical examination, as well as a normal blood count, chemical screening battery and urine analysis except for obesity, hypercholesterolemia (42.8%), hypertension (35.7%). No patients had previously diagnosed diabetes according to American Diabetes Association,[14] 21.4% were smokers. Subjects with fasting plasma glucose ⩾7 mol l^{-1} (two men and one woman) were excluded from the study as

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well as subjects with daily alcohol consumption, > 30 g for men and > 20 g for women.[15] All women were post-menopausal and not on hormonal replacement therapy. None of the participants was regularly engaged in physical activity or changed his physical activity level during the study. All participants gave their informed consent and the experimental protocol was approved by the Ethical Committee of our University.

Anthropometric measurements

With the subjects wearing light indoor clothes and no shoes, body weight was measured to the nearest 0.1 kg, (Salus scale, Milan, Italy), and height to the nearest 0.5 cm using a stadiometer (Salus stadiometer). BMI was calculated as body weight adjusted by stature squared (kg m $^{-2}$). Waist circumference was obtained with a measuring tape as the minimum circumference between the xyphoid process and the umbilicus.

Body composition assessment

Body composition was measured using dual-energy X-ray absorptiometry (Hologic QDR 4500, Waltham, MA, USA) array beam System Software Version 11.1.

The characteristics and physical concepts of dual-energy X-ray absorptiometry measurement have been described elsewhere.[16] Daily quality-assurance tests were performed according to the manufacturer's directions. All scans were subsequently analyzed by a single trained investigator. Total body fat was expressed in kg (fat mass) and as a percentage of body weight (fat mass %).

Magnetic resonance data acquisition

MRI (magnetic resonance imaging) was performed with a 1.5 T magnet (Symphony, Siemens Medical Systems, Erlangen, Germany) and two body phased-array coils. The imaging protocol included an axial T1-weighted dual-phase gradient-echo sequence (repetition time ms/echo time ms, 96/2.33 (opposed phase), 96/4.85 (in phase); flip angle 70°; matrix 320 × 320; field of view 500 cm) of the upper abdomen and an axial T1-weighted gradient-echo sequence (TR 90; TE 3.46; flip angle 70°; matrix 256 × 256; field of view 500 cm) from L3 to L5. APR and GAZ rated the quality of all MR images on a scale of 1 to 5 (5 being optimal signal to noise ratio, 1 being unreadable) as previously described.[17] The average MR quality was 4.1 (ranging from 3 to 5).

Abdominal fat evaluation

All images were analyzed using Sliceomatic image analysis software (version 4.2, Tomovision, Montreal, Canada) and a single reader measured visceral adipose tissue (AT) and subcutaneous AT on a single slice at L4–L5 for men and women, as previously described.[13]

Abdominal subcutaneous AT area was defined as the area of adipose tissue between the skin and the outermost aspect of the abdominal muscle wall. All the adipose tissue pixels within the abdominal cavity between the innermost aspect of the abdominal and oblique wall musculature and the anterior aspect of the vertebral body were considered visceral abdominal AT. Interclass correlations for intra-reader comparisons in a subgroup of 20 subjects were 0.996 for subcutaneous AT, 0.993 for visceral AT.

Ectopic fat infiltration evaluation

MR images were reviewed on a commercially available workstation (Leonardo, Siemens Medical Systems) by a single reader who was unaware of laboratory and clinical results.

The technique used to measure SI (signal intensity) values from regions of interest (ROIs) in the liver and spleen and to calculate the relative SI losses of the liver and the other organs was based on methods previously described for the liver.[18] The percentage of relative SI loss on opposed-phase MR images was considered to be a reasonable measurement of liver fat on the basis of the known effect of fat on SI values. The SI values of the liver and spleen were recorded on in- and opposed-phase T1-weighted MR images by positioning circular ROIs at anatomically matched locations on paired sequences, avoiding visible vessels, abnormalities and artefacts. Three circular ROIs were positioned in the liver (left lobe, upper right lobe and lower right lobe), three in the pancreas (head, body and tail, avoiding the main pancreatic duct), one each in the psoas muscles. The s.d. of the SI measurements within each ROI was kept to less than 10%. The SI of the spleen was similarly measured by positioning two circular ROIs in the splenic parenchyma. Size of the ROI was variable in the different organs: 1–2 cm diameter for the liver and spleen, equal or smaller for the pancreas. When more than one ROI was positioned in an organ, a mean SI was calculated to account for signal heterogeneity.

Liver fat was estimated on opposed-phase MR images as the percentage of relative SI loss of the liver on opposed-phase images with a previously used formula[18]

$$RSID = 100 \cdot (L_{in}/S_{in} - L_{op}/S_{op})/(L_{in}/S_{in})$$

where L_{in} is in-phase mean liver SI, S_{in} is mean in-phase spleen SI, L_{op} is mean opposed-phase liver SI and S_{op} is mean opposed-phase spleen SI.

Similar formulas were used to calculate the amount of fat in the pancreas according to previous reports.[17,19,20] The grading system for liver steatosis was based on that used in earlier studies:[21,22] grade 0 corresponding to less than 5% steatosis; grade 1 to 6–33% steatosis; grade 2 to 34–66% steatosis; and grade 3 to greater than 66% steatosis. The grading system incorporates the accepted normal value of histo-pathological liver fat, which is less than 5%, and is the standard applied in the clinical assessment of severity of liver steatosis. We calculated the intra- and inter-observer variability of our MRI measurements for pancreatic fat content and liver fat content, which were 8.2% and 9.7% and 7.8% and 8.2%, respectively (as determined in a subset of 20 obese patients).[17]

Biochemical analyses

Venous blood samples for all metabolic assessments were obtained after overnight fast. Plasma glucose was measured using a glucose analyzer (Beckman Instruments Inc., Palo Alto, CA, USA). The intra-assay coefficient of variation was 1.5%.

Plasma immune-reactive insulin underwent duplicate measurements by double-antibody radioimmunoassay using a commercial kit (Diagnostic Products Corp., Los Angeles, CA, USA). Sensitivity was 6 pvl $^{-1}$ and the intra-assay coefficient of variation was 4.9%.

Insulin resistance was estimated with the HOMA (homeostasis model assessment of insulin resistance) method.[23]

Cholesterol and triglyceride levels were determined using a Technicon Auto analyzer (Technicon Inc., Co, Tarrytown, NY, USA) and dextran-magnesium precipitation was used to separate high-density lipoprotein.

Serum leptin was measured using a specific ELISA kit (DBC-Diagnostic Biochem Canada Inc., London, Canada). Sensitivity was 0.5 ng ml $^{-1}$ and the intra-assay and interassay coefficient of variations were 7.4% and 9.6%, respectively.

Serum adiponectin was measured with a commercially available ELISA kit (B-Bridge International, Inc., Sunnyvale, CA, USA). Sensitivity was 20 pg ml $^{-1}$ and the intra-assay and interassay coefficient of variations were 3.5% and 5.2%, respectively.

Dietary intake

A trained dietician performed a 7-day dietary recall interview in order to assess the dietary habits of each subject enrolled in the study. A recall grid representing 7 days of the prior week and all possible food-encounter times was used by the dietician. Portion sizes were estimated for foods and fluids by comparing with reference foods and fluids in a booklet of photographs. The interview takes approximately 40 min, on average, for the study subject to complete. The record data were then processed by the dietician using special software to calculate daily intake of energy, protein, fat, carbohydrate and alcohol based on the tables furnished by the Italian National Institute of Nutrition.[24]

Hypoenergetic diet

All the subjects completed a weight-loss program designed to achieve a loss of 7–10% of the initial weight. The caloric restriction was 500 kcal

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below the resting energy expenditure, as evaluated by indirect calorimetry and multiplied by a physical activity level of 1.4. Each subject received a diet providing 62% carbohydrates, 24% fat, 14% protein and 20 g fiber. The only beverage allowed was water. The subjects underwent monthly clinical and nutritional follow-ups. Dietary compliance was checked by a 24-h recall every 4 weeks during an outpatient visit.

Statistical analyses

Results are shown as means ± s.d. Log transformations were performed for non-normal variables. Comparisons of anthropometric, metabolic and body-composition variables before and after weight loss were made by using paired *t*-test. Mcnemar test was used to test changes in the prevalence of liver steatosis before and after weight loss. Correlation analyses were used to test associations between variables. Linear regression analyses were used to test the joint effects of changes in liver and pancreas lipid content, as well as changes in leptin, high-sensitivity C-reactive protein (hs-CRP), visceral AT and waist circumference after weight loss on HOMA changes. The level of statistical significance was $P<0.05$ for all the variables. All statistical analyses were performed using the SPSS statistical package.[25]

RESULTS

The flowchart of study participants is shown in Figure 1. Anthropometric, body composition, fat distribution, hs-CRP, adipokines and metabolic variables before and after weight loss are shown in Table 1. The mean decrease in body weight was 8.9%. BMI and waist circumference significantly decreased after weight loss (both $P<0.001$) as well as fat mass and fat mass % (both $P<0.001$). Visceral, subcutaneous and total abdominal AT significantly decreased (all $P<0.001$). Among the metabolic variables, glucose, insulin, HOMA, total cholesterol, triglycerides and leptin significantly decreased (all $P<0.01$), whereas adiponectin did not change. A significant decline was also observed in gamma glutamyl transpeptidase and alanine amino transferase (both $P<0.001$).

Changes in liver and pancreas lipid content in each of the subjects enrolled in the study expressed as mean ± s.d. values are shown in Figures 2a and b. Liver lipid content significantly

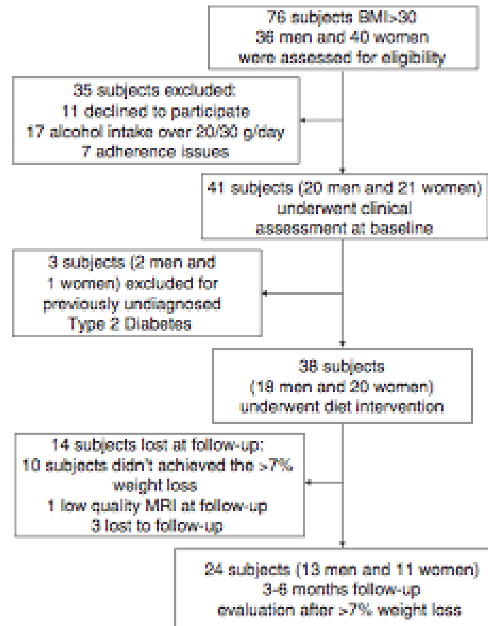

Figure 1. Flowchart of participant recruitment, screening and assessment.

Table 1. Anthropometric, body composition, fat distribution, metabolic variables, Hs-CRP and adipokines variables before and after weight loss

Variables	*Baseline* *mean ± s.d.* N = 24 *(M/F:13/11)*	*After weight loss* *mean ± s.d.* N = 24 *(M/F:13/11)*	*Percentage change (%)*	P
Weight (kg)	98.4 ± 15.9	89.7 ± 14.8	−8.88	<0.001
BMI (kg m^{-2})	35.4 ± 4.5	32.2 ± 4.0	−9.04	<0.001
Waist circumference (cm)	105.4 ± 12.3	98.6 ± 10.6	−6.04	<0.001
Fat mass (kg)	35.3 ± 6.9	29.6 ± 6.1	−16.1	<0.001
Fat mass percent (%)	36.4 ± 6.2	33.5 ± 6.4	−7.96	<0.001
Fat-free mass (kg)	63.1 ± 7.9	60.1 ± 7.2	−4.71	<0.001
Total abdominal AT (cm^2)	574.3 ± 154.1	464.5 ± 135.7	−19.1	<0.001
Subcutaneous abdominal AT (cm^2)	399.9 ± 124.4	345.5 ± 114.1	−13.6	<0.001
Visceral abdominal AT (cm^2)	174.8 ± 94.7	118.9 ± 76.3	−31.9	<0.001
Liver lipid content (%)	20.9 ± 25.4	3.3 ± 7.5	−84.1	<0.001
Pancreas lipid content (%)	19.7 ± 23.3	11.3 ± 14.3	−42.3	=0.001
Glucose (mM l^{-1})	5.1 ± 0.6	4.9 ± 0.6	−0.37	=0.01
Insulin (μUI ml^{-1})	14.9 ± 7.8	9.1 ± 4.3	−39.1	<0.001
HOMA	3.35 ± 1.6	1.98 ± 0.9	−40.9	<0.001
Total cholesterol (mM l^{-1})	5.43 ± 1.1	4.93 ± 0.8	−9.2	=0.002
HDL cholesterol (mM l^{-1})	1.23 ± 0.4	1.29 ± 0.4	4.8	=0.06
Triglycerides (mM l^{-1})	1.63 ± 0.86	1.19 ± 0.52	−26.99	=0.01
Gamma GT (UI l^{-1})	27.79 ± 19.8	19.33 ± 17.00	−30.4	<0.001
ALT (UI l^{-1})	37.25 ± 23.09	28.46 ± 17.48	−23.6	<0.001
Leptin (ng ml^{-1})	32.95 ± 20.24	20.86 ± 16.04	−36.6	<0.001
Adiponectin (μg dl^{-1})	15.12 ± 8.58	15.70 ± 8.90	3.8	=0.316
Hs-CRP (mg l^{-1})	5.55 ± 6.28	3.48 ± 3.30	37.3	=0.005

Abbreviations: ALT, alanine amino transferase; AT, adipose tissue; BMI, body mass index; Gamma GT, gamma glutamyl transpeptidase; HDL, high-density lipoprotein; HOMA, Homeostasis Model Assessment of insulin resistance; Hs-CRP, high-sensitivity C-reactive protein.

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decreased, as well as pancreas lipid content ($P<0.001$ and $P=0.001$, respectively). Interestingly, percent changes in liver lipid content were greater (84.1 ± 3%) than those in pancreas lipid content (42.3 ± 29%), visceral abdominal AT (31.9 ± 15.6%) and subcutaneous abdominal AT (13.6 ± 25.9%).

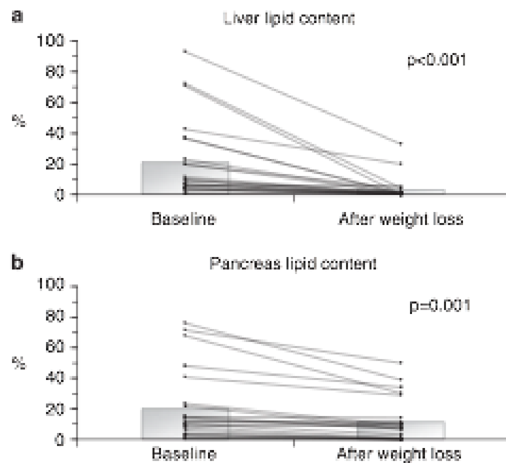

Figure 2. Liver lipid (**a**) and pancreas lipid (**b**) content at baseline and after weight loss.

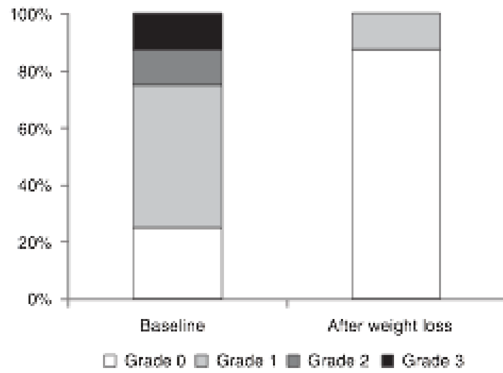

Figure 3. Prevalence of hepatic steatosis before and after weight loss.

As far as hepatic steatosis, as assessed by MRI, 75% (18/24) patients showed steatosis at baseline: respectively 50% (12/24) grade 1 steatosis, 12.5% (3/24) grade 2 steatosis and 12.5% (3/24) grade 3 steatosis. After weight loss, the percentage of subjects with steatosis significantly decreased to 12.5% ($P=0.008$) and none of these subjects had grade 2 or 3 steatosis (Figure 3).

Table 2 shows the correlations between changes in anthropometric variables, body composition, biochemical variables and changes in liver and pancreas lipid content and HOMA index after weight loss.

The loss of liver lipid content was related with the decrease in visceral AT ($r=0.664$, $P<0.001$), subcutaneous AT ($r=0.505$, $P=0.012$) and hs-CRP ($r=0.554$, $P=0.005$). Pancreas lipid infiltration decrease was related to visceral AT decrement after weight loss ($P<0.001$). An association between the respective changes in the liver and pancreas lipid content was also observed ($P=0.033$).

HOMA improvement was associated with reduction in waist circumference, visceral and subcutaneous abdominal AT and change in hs-CRP and insulin.

To evaluate independent determinants of insulin resistance improvement, a linear regression analysis was performed considering changes in HOMA index as dependent variable and the variables most closely correlated with it as independent variables. Table 3 shows different models considering gender, changes in liver and pancreas lipid content, visceral AT (or alternatively waist), leptin and hs-CRP as determinants of HOMA improvement.

The proportion of variance explained by gender and change in liver lipid content was 55%. When change in waist was added, 65.3% of variance was explained. When change in hs-CRP was also included in the model, a further increase in the proportion of variance was observed (71.9%) and F-test value decreased to 12.138. Building a model considering gender, changes in liver lipid content, visceral AT and hs-CRP as independent variables, 62.9% of variance in liver lipid content was explained.

A 49.7% and 49.1% of variance was explained, respectively, building models using gender and waist or alternatively gender and visceral AT.

DISCUSSION

Our study shows that a moderate weight loss reduces visceral AT as well as hepatic and pancreatic lipid content. After weight loss, percent changes are greater in liver lipid content than in pancreas lipid content and visceral abdominal AT. Furthermore, insulin resistance, hs-CRP and leptin were significantly reduced after

Table 2. Correlations between anthropometric, fat distribution, liver and pancreas lipid content measured with magnetic resonance and biochemical variables changes after weight loss

	Δ *body Weight*	Δ *body mass index*	Δ *waist circumference*	Δ *visceral abdominal AT*	Δ *subcutaneous abdominal AT*	Δ *liver lipid content*	Δ *pancreas lipid content*
Δ Insulin	0.56**	0.41	0.53**	0.37	0.24	0.32	0.09
Δ Glucose	0.01	−0.11	−0.14	0.22	0.37	0.09	0.26
Δ HOMA	0.50***	0.32	0.63**	0.67*	0.43***	0.68*	0.32
Δ Total cholesterol	0.29	0.17	−0.21	0.06	−0.02	−0.22	−0.07
Δ HDL cholesterol	−0.04	−0.00	−0.11	−0.18	−0.11	−0.17	0.17
Δ Triglycerides	0.12	−0.06	0.04	0.32	0.14	0.10	0.03
Δ hs-CRP	0.33	0.16	0.23	0.54**	0.38	0.55**	0.22
Δ Adiponectin	−0.10	−0.34	0.03	0.33	−0.03	0.08	0.19
Δ Leptin	0.11	−0.04	0.02	−0.13	0.44***	0.09	0.00

Abbreviations: AT, adipose tissue; HOMA, homeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; Sample size, $n=24$. *$P<0.001$, **$P<0.01$, ***$P<0.05$.

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Table 3. Different linear regression analysis enter method considering Δ HOMA as the dependent variable and gender, liver lipid content, pancreas lipid content, VAT, or alternatively waist circumference, leptin and hs-CRP as independent variables

Variables entered in the models	*Total R^2*	*β coefficient*	t	*Significance*	F
1- *Gender*	0.554	0.332	2.090	0.049	13.069
Δ liver lipid content		0.548	3.455	0.002	
2- *Gender*	0.653	0.219	1.453	0.162	12.535
Δ liver lipid content		0.448	2.992	0.007	
Δ waist circumference		0.360	2.380	0.027	
3- *Gender*	0.719	0.168	1.189	0.249	12.138
Δ liver lipid content		0.290	1.843	0.081	
Δ waist circumference		0.373	2.662	0.015	
Δ Hs-CRP		0.313	2.110	0.048	
1- *Gender*	0.554	0.332	2.090	0.049	13.069
Δ liver lipid content		0.548	3.455	0.002	
2- *Gender*	0.585	0.242	1.944	0.066	9.408
Δ liver lipid content		0.412	2.857	0.010	
Δ VAT		0.259	−0.162	0.873	
3- *Gender*	0.629	0.227	1.346	0.194	8.062
Δ liver lipid content		0.320	1.624	0.121	
Δ VAT		0.188	0.889	0.385	
Δ Hs-CRP		0.262	1.502	0.150	
1- *Gender*	0.497	0.343	2.011	0.057	10.390
Δ waist circumference		0.488	2.862	0.009	
1- *Gender*	0.491	0.258	1.380	0.182	10.114
Δ VAT		0.523	2.794	0.011	

Abbreviations: HOMA, homeostasis model assessment; hs-CRP, high-sensitivity C-reactive protein; VAT, visceral adipose tissue.

weight loss. Changes in liver lipid content were the best predictors of insulin resistance improvement after weight loss independently of changes in visceral fat distribution, pancreas lipid content, leptin, systemic inflammation as evaluated with hs-CRP and gender.

As expected, in agreement with previous studies,[4,26] after weight loss the amount of visceral abdominal AT loss was higher than that of subcutaneous abdominal AT. Preferential loss of visceral abdominal AT during initial modest weight loss, due to its higher lipolysis response, has been reported.[26]

After moderate weight loss, we also observed a significant loss in all ectopic fat compartments in our subjects. The decrease in liver lipid content after weight loss has been previously reported in patients with type 2 diabetes,[12,27] overweight[28] and obese.[29,30]

Interestingly, a decline in body fat % of about 3 units (from 36.4 to 33.5, Table 1), as in our study, has been shown to be associated with a much greater decline in liver lipid content and with a nearly total disappearance of liver steatosis. In fact, in our study sample, 75% of subjects had liver steatosis, as estimated according to Bahl *et al.*[22] by liver SI losses higher than 3%, at the beginning of the study and only 12.5% still had after weight loss, thus suggesting that a moderate weight loss is a landmark treatment for this condition.

To the best of our knowledge, no studies have evaluated changes in pancreas lipid content after weight loss. Our study shows a more than 40% reduction of pancreas lipid content after a moderate weight loss. Pancreas lipid content raises great interest because it has been suggested that fat deposition in and around pancreatic islets could be associated with impaired beta cells function in humans.[31,32] However, its decrease after moderate weight loss, as reported in our subjects, could contribute to the improvement of insulin resistance following weight loss.

Our data support that even moderate weight loss may decrease ectopic fat deposition in both, liver and pancreas, and that the amount of the decline in each compartment may be different. In fact, in our study group, mean changes in liver lipid content were greater than those in pancreas lipid content as well as than in visceral abdominal AT. Our findings of greatest decline in liver lipid content are at least partially in line with those of Colles *et al.*,[11] who demonstrated that intra-hepatic fat was mobilized faster than visceral and subcutaneous fat and with those of others.[8,12,27–29] Actually, our results seem to complement and expand these previous findings by also giving information on the effects of weight loss on pancreas lipid content.

Excess liver and pancreas lipid content have been shown to be frequently present in the same patients: Lee *et al.*[33] showed that concurrence of fatty pancreas and fatty liver, as estimated by sonography, was found in 70% of subjects and fatty liver without fatty pancreas just in 2%. However, our findings seem to suggest that dietary restriction may mobilize more ectopic fat stored in the liver than in the pancreas.

It has been recently observed, by using immuno-histochemistry, that fat in human pancreas could be stored in adipocytes between pancreatic cells in addition to vacuoles in pancreatic cells.[34]

No similar evidence has been shown in liver where fat is located inside hepatic cells; thus our findings of a higher decline of lipid content in liver than in pancreas could be at least partially explained by the fact that weight loss may easily mobilize triglycerides located inside pancreatic or hepatic cells than those located inside adipocytes between pancreatic cells.

As expected, in line with previous studies, a moderate weight loss improved all metabolic variables.[2–4,6] Insulin resistance, as estimated by HOMA index, was significantly improved after weight loss in our subjects.

Insulin resistance could result from the contribution of visceral fat through increased flux of free fatty acids to the liver[35] or by the activity of some adipokines, such as leptin and adiponectin[36] or by intracellular fatty acid metabolism through activation of serine kinase cascade.[37] In agreement with some[6] but not all studies,[18,19] adiponectin levels were not modified by moderate weight loss in our subjects, thus adiponectin changes did not seems to have a major role at least in our study in causing the observed improvement of insulin resistance.

On the contrary, it is possible to hypothesize that the significant changes in liver, pancreatic fat content, visceral AT and leptin, observed after weight loss could be all associated with the improvement in insulin resistance.

To test the joint effects of changes in liver and pancreas lipid content, fat distribution, leptin and hs-CRP after weight loss on HOMA improvement we performed a step-down multiple

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regression analyses: changes in liver lipid content were independently associated with HOMA improvement.

Changes in liver lipid content together with gender explained in our study 55.4% of the variance of insulin resistance improvement after weight loss, independently of visceral fat and pancreas lipid content decrease. This finding seems to be in line with recent observations that increased fat accumulation in liver is the main determinant of peripheral and hepatic insulin resistance[40] as well as with the findings that early and major decrease in liver fat after weight loss occurs at the same time of normalization of hepatic insulin sensitivity and fall of glucose levels.[41]

Some limitations of our study should be recognized. First, the relatively small study sample size. Further, although MR spectroscopy has been used in the majority of studies that evaluated liver lipid content before and after weight loss, we used the MR chemical shift technique. However, high accuracy for the quantification of liver lipid content, validated also against histological determined percentage of fat,[22] and pancreas lipid content[20] has been recently reported for this technique. Moreover, it has been recently shown that in/opposed phase technique is a valid and reliable tool for both, hepatic and pancreatic fat quantification, showing good agreement with fat-selective spectral-spatial gradient-echo imaging[20,42,43] and also with magnetic resonance spectroscopy.[44] MR spectroscopy still lacks general availability in current clinical practice, and the analysis can be performed only on one voxel at a time. On the contrary, MRI scan using T1-weighted gradient-echo in-phase and opposed-phase sequence is a rapid and available technique, already routinely included in MR-imaging protocols for the upper abdomen. Moreover, the analysis can be performed on whole organs, and not on single voxels.

Finally, we only evaluated a surrogate marker of insulin resistance such as HOMA index and then we were not able to evaluate hepatic insulin resistance. Thus, it is possible to speculate that our results could be even more relevant if more sophisticated methods tailored to test hepatic insulin resistance were used.

In conclusion, our data show that a moderate weight loss determines a significant decline in visceral abdominal fat, together with a decline in lipid fat content inside liver and pancreas. After weight loss percent changes are greater in liver than in pancreas lipid content and in visceral abdominal AT. Finally, changes in liver lipid content seem to be the most important predictor of insulin resistance improvement after weight loss. These results have clinical implications showing that even a small weight loss is able to reduce ectopic fat deposition in different splanchnic districts (liver and pancreas) and this reduction is significantly related to relevant metabolic improvements and showed that moderate weight loss is a cornerstone treatment of liver steatosis in obese people.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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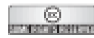

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