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Original Article

Can obesity-induced inflammation in skeletal muscle and intramuscular adipose tissue accurately detect liver fibrosis?

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Abstract

Objectives: Obesity is characterized by a chronic, low grade, systemic inflammation. However, little is known about the role of skeletal muscle, which represents an active metabolic organ whose activities need to be determined. The purpose of our study was to detect relationships between skeletal muscle and adipose tissue inflammation with nonalcoholic fatty liver disease (NAFLD) and diabetes, as well as to explore associations with clinicopathological parameters. **Methods:** Our study population consisted of 50 morbidly obese patients undergoing planned bariatric surgery. Biopsies were taken from visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), skeletal muscle (SM), extramyocellular adipose tissue (EMAT) and liver. The expression of CD68 and CD3 was assessed by immunohistochemistry. **Results:** Our findings suggest a complex inter- and intra-tissue co-expression network that links obesity-induced inflammation in adipose depots and skeletal muscle with NAFLD. A novel finding is the intricate cross-talk between SM, EMAT and the liver and the probable correlation between SM, EMAT inflammation and the presence of liver fibrosis. **Conclusions:** Although the mechanisms of obesity-induced inflammation and its association with NAFLD and liver fibrosis are incompletely understood, our findings indicate an extensive and complex tissue network that needs to be further investigated.

Keywords: NAFLD, Obesity, Inflammation, Liver Fibrosis, Skeletal Muscle

Introduction

Obesity is a global epidemic and a major contributor to some of the leading causes of death including type II diabetes mellitus, cardiovascular disease, Non-Alcoholic Fatty Liver Disease (NAFLD) and some types of cancer[1]. According to World Health Organization (WHO), the rates of obesity worldwide have nearly tripled the last 40 years, but obesity still remains a neglected health problem with serious physical, social and psychological dimensions[2,3].

Visceral obesity, rather than total body fat, appears to be associated with increased cardiometabolic risk and along with hypertension, impaired glucose tolerance and lipid disorders constitutes Metabolic Syndrome (MetS)[4]. NAFLD is nowadays considered the hepatic manifestation of MetS and represents a wide spectrum of liver pathologies encompassing steatosis to Non-Alcoholic Steatohepatitis (NASH) and in rare cases cirrhosis and Hepatocellular Carcinoma (HCC)[5,6]. Although 30-40% of people with simple steatosis progress to NASH, 74% of NASH patients progress to fibrosis[7]. Liver fibrosis represents the consequences of a sustained wound healing response to chronic liver injury from a variety of causes including metabolic diseases, such as insulin resistance and impaired glucose tolerance[8]. This healing process in the liver is orchestrated by the Hepatic Stellate Cells (HSC), matrix molecules and several mediators, such as Tumor Necrosis Factor alpha (TNFa)[9]. The activation of stellate

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cells reflects paracrine stimulation by all neighboring cell types, including sinusoidal endothelium, *Kupffer* cells, hepatocytes, platelets, leukocytes and endothelial cells, that produce cellular fibronectin and Reactive Oxygen Species (ROS), alter adipokine/cytokine production and convert Transforming Growth Factor beta (TGFβ) from a latent to an active, profibrogenic form[10-12]. Additional factors that promote progression of NASH to fibrosis include increased sympathetic neurotransmitters, as well as angiotensin II and endocannabinoids and there is evidence to suggest that blockade of angiotensin II can attenuate fibrosis in animal models[13]. The modulation of HSC activation and Extramyocellular Matrix (ECM) remodeling is an area of active investigation and may also lead to novel therapeutic interventions[14].

It has already been proven that human metabolism and immunity are closely related to each other[15] and that obesity may represent an impaired immune function which predisposes to systemic inflammation[16-19]. Obesity is characterized by a chronic, low grade inflammation not only in all adipose tissue depots[20-27], but also in skeletal muscle[28-31] and liver[32,33], as a result of the systemic immunoactivation. There seems to be an imbalance between produced and circulating pro- and anti-inflammatory biomarkers and immune cells, including macrophages, T and B lymphocytes, which has important effects on systemic insulin sensitivity and will eventually lead to insulin resistance and type II diabetes. Adipose tissue and skeletal muscle represent active metabolic organs that produce and secrete a great variety of chemokines, adipokines[34,35] and myokines[36]. Adipocytes are the unique source of secreted adipokines such as leptin and adiponectin[37], which can promote insulin sensitivity, as well as resistin and Retinol-Binding Protein 4 (RBP4), which have the opposite action[38]. Crown-Like Structures (CLSs), which are described as accumulations of pro-inflammatory macrophages and extracellular matrix material around dead adipocytes, are considered the hallmark of adipose tissue inflammation and fibrosis[39-45].

Normal glucose homeostasis requires a communication network among several organs, including adipose tissue[46], skeletal muscle and the liver[47]. This inter-tissue cross-talk can be impaired in obesity by increased plasma Free Fatty Acid (FFA) and can therefore cause insulin resistance that leads to the development of type 2 diabetes mellitus and NAFLD. The mechanism through which FFA induces insulin resistance involves intramuscular and intrahepatocellular accumulation of triglycerides and diacylglycerol, activation of several serine/threonine kinases, reduction in tyrosine phosphorylation of the Insulin Receptor Substrate (IRS)-1/2, and impairment of the IRS/phosphatidylinositol 3-kinase pathway of insulin signaling. FFA also produce low-grade inflammation in skeletal muscle and liver through activation of Nuclear Factor-kappaB (NF-kB), resulting in release of several pro-inflammatory and pro-atherogenic cytokines[48].

Intramuscular fat is of particular interest amongst researchers because of the important role of skeletal muscle in insulin-mediated glucose uptake. Due to skeletal muscle's high insulin sensitivity and large percentage of body mass, fat accumulation and concomitant loss of insulin sensitivity potentially plays an important role in insulin resistance, obesity, and metabolic syndrome[49]. In obese individuals, intramuscular fat depot becomes infiltrated with pro-inflammatory macrophages, which may cause paracrine-like insulin resistance in skeletal muscle. In parallel with these inflammation-related changes, alterations in fatty acid metabolism can lead to the accumulation of fatty acid intermediates within the liver and skeletal muscle, which can serve as ligands to broadly activate inflammatory pathways in *Kupffer* cells and adipose tissue macrophages, possibly via Toll-like Receptor-2 and 4 (TLR2/TLR4) signaling pathways[50]. There is plenty of available data showing that increased intramuscular fat is associated with decreased insulin sensitivity. The underlying pathophysiological mechanisms are not fully understood, although it has been suggested that it may be caused by altered action of mitochondrial proteins as a result of increased lipid peroxidation products[51]. Kato et al published that liver steatosis is associated with insulin resistance in skeletal muscle rather than in the liver in patients with NAFLD, suggesting a central role of fatty liver in the development of peripheral insulin resistance and the existence of a network between the liver and skeletal muscle[52]. There is also evidence that SAT fibrosis seen in obesity, is positively associated with liver fibrosis and diabetes, but all these traits may be at least partially reversed after bariatric surgery[53].

The present study is a comprehensive "*in situ*" morphological evaluation of the underlying inflammation in morbid obesity. We used the well validated method of immunohistochemistry in 5 different tissues; Visceral Adipose Tissue/omentum (VAT), abdominal Ssubcutaneous Adipose Tissue (SAT), Skeletal Muscle (SM), Extramyocellular Adipose Tissue (EMAT) and liver. Biopsies were obtained from severely obese individuals who underwent planned bariatric surgery and we assessed the subcellular localization of CD68 and CD3 biomarkers, that suggests the presence of macrophages and lymphocytes respectively. The purpose of our study was to identify possible interactions between the investigated biomarkers within and between the different tissues and unveil any associations with the presence of diabetes, NASH and liver fibrosis at the time of surgery, as well as with important demographic and clinical parameters.

Materials and methods

Tissue samples and patients

Power analysis was performed using alpha error probability, power (1-β) and effect size. In particular, alpha error probability was set at 0.05, effect size was calculated using group means and standard deviations thus it was calculated to be 0.18 and power was set to 0.8. Estimated sample size was estimated to be 47 for patients with obesity. Power analysis was conducted on the hypotheses that body

Table 1. Descriptive characteristics for the continuous variables of our study population (*N*=50) (Legend: SD: Standard Deviation, IQR: Interquartile Range).

Continuous Variable	Mean	Median	SD	IQR	Min-Max
Age (years)	38.62	37.5	10.48	19.75	22-58
BMI	58.6	57.05	8.94	7.7	41.1-84.5
Body Fat (%)	49.63	49.8	5.02	5.9	30.1-58.2
SGOT (mg/dl)	27.92	23	15.23	11	14-93
SGPT (mg/dl)	38.06	32	20.24	19.25	13-104
CHOL (mg/dl)	196.8	197.5	38.37	56.75	114-288
LDL (mg/dl)	123.4	122.5	30.72	44.25	74-202
HDL (mg/dl)	46.3	44.5	13.25	13.25	28-88
TGs (mg/dl)	156.3	147	74.92	96	36-391
Total NAS score	4.33	5	1.93	3	1-8

weight follows the normal distribution and obese individuals (BMI>30) consist of the 12.5% of the population in the ages between 20 and 59[62]. Mean BMI was estimated at a mean of 25.9 for ages 20 to 59 years old and a standard deviation of 9.76, in the Greek population, for ages 20 to 59 years old[63].

In the present study, we included 50 severely obese patients, undergoing planned gastric bypass surgery at the Department of Surgery of the University Hospital of Patras in Greece.

Inclusion criteria for participating in the study were: age >18 years, clinically significant obesity (BMI>40) with a clear indication for surgical intervention, absence of major underlying pathology (i.e. renal failure, heart failure, cancer, known chronic infectious or autoimmune disease) and willingness to participate. Exclusion criteria were: increased alcohol consumption (defined as >20 g/day), previously diagnosed viral hepatitis or any other known chronic liver disease and long-term treatment with medication found to cause liver damage and steatosis. The vast majority of the patients underwent *Roux-en-Y* gastric by-pass surgery, accompanied by appendicectomy and cholecystectomy. During the planned surgical procedure, biopsies were taken from abdominal visceral adipose tissue (omentum) (*tissue α, VAT*), abdominal subcutaneous adipose tissue (*tissue γ, SAT*), skeletal muscle from rectus abdominis (*tissue δ.m, SM*) with its extramyocellular fat (*tissue δ.ad, EMAT*) and liver (*tissue ε*). All patients had abdominal (central) obesity, defined as a waist-hip ratio above 0.90 for males and above 0.85 for females, or a body mass index (BMI) above 30[54].

Unfortunately, no consensus has been reached regarding the definition of skeletal muscle's adipose tissue depot and as result there may be misunderstandings. According to Addison et al (2014) and Khan et al (2015)[55,56] intermuscular fat is typically the broadest definition of fatty infiltration in the muscle referring to storage of lipids in adipocytes underneath the deep fascia of muscle. This includes the visible storage of lipids in adipocytes located between the muscle fibers (also termed intramuscular fat: IMAT) and between muscle groups (literally intermuscular or perimuscular: PMAT). The IMAT and PMAT depots constitute the extramyocellular adipose tissue in general. While not frequently isolated as a separate fat depot, there also exists a smaller group of lipids stored within the muscle cells themselves, known as lipid droplets or intramyocellular lipids (IMCL). To facilitate comparisons with previous studies, we used the same definitions.

All tissue samples were fixed at the Pathology Department of the University Hospital of Patras and then embedded in paraffin. Serial thin sections were taken (4 μm) and mounted on gelatin-coated glass slides. We used tissue samples that had been collected from August 2005 until December 2006. Blood samples for routine testing were also taken prior to the planned surgical intervention.

Useful collected anthropometric parameters include sex, age, Body-Mass Index (BMI), body fat percentage (which was measured by bioelectrical impedance analysis, BIA) and serum biomarkers include total cholesterol levels (TC), HDL, LDL, TGs, SGOT (serum glutamic-oxaloacetic transaminase) and SGPT (serum glutamic-pyruvic transaminase). The characteristics of the enrolled patients are presented in Table 1 and Table 2.

Immunohistochemistry

Serial 4μm-thick sections were cut from the formalin fixed, paraffin embedded (FFPE) blocks and subjected to immunohistochemical analysis. We used the following primary antibodies: prediluted monoclonal mouse antibody against macrophages (Flex monoclonal mouse anti-human CD68 ,Clone PG-M1, Ready to use, Dako) and prediluted polyclonal rabbit antibody against lymphocytes (FLEX Polyclonal Rabbit, Anti-Human CD3, Ready to use, Dako, Code: IS503).

Briefly, sections were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating with 0.3% hydrogen peroxide for 15 min at room temperature. For antigen retrieval, sections were heated in 1 mM ethylenediamine tetraacetic acid (EDTA)-NaOH, pH

Table 2. Absolute (N) and relative (%) frequencies for the nominal variables of our study population (*N*=50) *(Notes: *In liver biopsy, ¥According to Kleiner histological scoring system[57], there are 5 stages in liver fibrosis and stage 1 is subdivided in other 3 parts [0: none, 1a: mild zone 3 perisinusoidal fibrosis, 1b:moderate zone 3 perisinusoidal fibrosis, 1c: portal/periportal fibrosis only, 2: perisinusoidal and portal/periportal, 3:bridging fibrosis, 4: cirrhosis]. For statistical reasons and in order to eliminate sample fragmentation, we excluded stages 0 and 4 from further statistical analysis and we did not use the subdivision for stage 1 [The values in the right columns were finally used]).*

Nominal Variables		N	(%)
Sex	*Man*	14	28%
	Woman	36	72%
Hypertension	*Yes*	19	38
	No	31	62
Diabetes Mellitus	*No*	36	72
	IGT (Impaired Glucose Tolerance)	5	10
	Yes	9	18
Antidiabetic Treatment	*Nill*	45	90
	Tablets	5	10
	Insulin	0	0
Lobular inflammation*	*0*	1	2
	1	21	43
	2	23	47
	3	4	8
Ballooning*	*0*	10	20
	1	19	39
	2	20	41

Nominal Variables		N	(%)
Steatosis*	*0*	16	33
	1	8	16
	2	10	20
	3	15	31
NASH	*No*	12	25
	Borderline	11	22
	Yes	26	53
FIBROSIS*,¥	*0*	1	2
	1	13 13	26.5 27.7
	2	16 16	32.7 34
	3	18 18	36.7 38.3
	4	1	2
Intramuscular Adipose Tissue (IMAT)	*No*	9	18
	Yes	41	82
Lipid droplets (Intramyocellular lipids, IMCL)	*No*	27	54
	Yes	23	46

8 for 15 min in a microwave oven. After cooling to room temperature, sections were incubated with blocking serum (1% bovine serum albumin fraction V; Serva Electrophoresis, Germany) for 30 min and then with the primary antibody for 1 h at room temperature. Slides were next incubated with Dako EnVision labeled polymer (Dako, CA, USA). Diaminobenzidine (Dako, CA, USA) was used as the chromogen. Nuclei were counterstained with Harris hematoxylin. Sections from tonsil were used as positive control for CD68 and CD3, according to manufacturer's advice. Furthermore, consistent positive staining of liver *Kupffer* cells was also used as internal control for CD68 and of lymphocytes for CD3. For negative control slides, the same method was performed, but the primary antibody was substituted by 1% TBS.

Staining evaluation

Haematoxylin and Eosin (H&E) stained sections were initially reviewed to evaluate each patient's underlying histopathology and assess the presence and severity of nonalcoholic fatty liver disease (NAFLD) according to *Kleiner* histological scoring system[57]. The diagnosis of NASH was based on the NAFLD Activity Score (NAS), which has three components: steatosis amount (0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2), which divides the patients into three categories: i. definite (NAS ≥5), ii. borderline (NAS: 3-4) and iii. no NASH (NAS <3). The degree of liver fibrosis was assessed separately[58]. For some of the cases, we also used extra histochemical stains, such as Masson's Trichrome for better assessing liver fibrosis.

Each slide was individually evaluated and scored by two independent observers blinded to all clinical data. Discrepancies in scoring between the observers were resolved by additional review of the slides under a double headed microscope until a consensus was reached. For the evaluation we used Olympus light microscope. The whole section was initially reviewed and representative areas were selected at low magnification (×100). Cell count was performed at high magnification (×400). Cytoplasmic expression of CD3 and CD68 biomarkers was assessed. The number of positive stained cells along with the total number of adipocytes were counted in 10 different, non-overlapping fields per section. Then, the average of the cells was taken and the percentage of positive stained cells for each section was calculated (positive stained cells/adipocytes %). For the assessment of the inflammatory cells in the skeletal muscle and EMAT, we used a slightly different approach in order to be in line with previous studies and thus able to make comparisons[59-61]. We therefore evaluated the absolute number of CD68+ macrophages and CD3+ lymphocytes per mm^2 of skeletal muscle and EMAT, by using a special microscope eyepiece with grading scale (Olympus BX41, Infinity HD Lumenera/ WHN 10x-H-1-3). We did not count and therefore statistically analyze the inflammatory cells in the liver, as normal *Kupffer* cells were also CD68+ positive-stained and lymphocytes were present in all cases of NASH.

Table 3. Relative expression (Mean value ± SD and Median in brackets) of the investigated biomarkers (%) in each tissue (*Notes: *We did not detect any CLSs in skeletal muscle and EMAT. ≠In SM and EMAT the values represent cells/mm², whereas in SAT and VAT positively stained cells/adipocytes %*).

Antibody	Tissue			
	α (VAT)	*γ (SAT)*	*δ.m (Muscle)*	*δ.ad (EMAT)*
CD68≠	28.48±17.54 (26.4)	23.33±18.06 (17.5)	17.44± 14.72 (13)	45.8±25.5 (39.5)
CLS*	0.62±1.26 (0)	2.84±4.52 (1.5)	-	-
CD3≠	21.54±14.36 (18.67)	6.31±3.49 (5.78)	7.9±7.9 (6)	12.62±6.46 (12)

Table 4. Cut-off values used in our study for dichotomizing biomarkers' expression in low and high expression.

Antibody	Tissue			
	α	γ	δ.m	δ.ad
CD68	27%	20%	15cells/mm²	42cells/mm²
CLS	≥1CLS/HPF	≥1CLS/HPF	-	-
CD3	20%	6%	7cells/mm²	12cells/mm²

Statistical analysis

All data was analyzed by using the SPSS statistical package (SPSS release 17.0, Chicago, IL, USA) and the R Statistical Foundation (3.3.1. Edition, Austria). The level of statistical significance was set at *p*-value <0.05. We correlated the biomarkers' expression levels between and within the tissues, with the anthropometric and clinical parameters and histopathological findings of our population. Final endpoints of our study were considered: (i) the presence of diabetes, (ii) the presence of nonalcoholic steatohepatitis (NASH) and the (iii) presence of fibrosis in liver biopsy.

The nominal variables are described with absolute (N) and relative frequencies (%), whereas the continuous with mean (M) and standard deviation (SD) in case of normal distribution, or with median (MED) and interquartile range (IQR) in case of abnormal distribution. The normal distribution of each of the investigated parameters was assessed with *Shapiro-Wilk* test.

Levels of biomarkers' expression were initially analyzed as continuously scaled measures, but for statistical purposes they were finally dichotomized into low and high expression. The mean expression of the investigated biomarkers in each tissue can be seen in Table 3. Due to lack of evidence in the literature, we used as cut-off points, values between the mean and median expression of each antibody per tissue[64], in such a way so as the 2 categories would have almost equal number of patients. The cut-off values that we used are displayed in Table 4. CLS status was assessed after examination of all fields available per slide at high-power field (HPF) magnification using light microscopy. A CLS was defined as CD68+ macrophage aggregates comprising at least 50% of the circumference circularly surrounding necrotic adipocytes. Subcutaneous and visceral (omental) fat depots from each participant were dichotomously categorized based on the presence (CLS+) or absence (CLS-) of CLS in fat. In additional analyses, CLS+ depots were characterized as low density (1 CLS per HPF) or high density (≥2 CLS per HPF)[39]. The correlations between the continuous variables were examined by using the *Pearson's (r)* and *Spearman's (p)* rank correlation tests, depending on the symmetry of the distribution. The possible associations between a nominal and a continuous variable were assessed with t-test, Wilcoxon and the non-parametric Kruskal-Wallis test. Finally, comparisons between nominal variables were performed by using *Pearson's Chi square (x^2) or Fisher test*.

We assessed liver fibrosis, according to *Kleiner* histological scoring system. For statistical purposes and in order to eliminate sample fragmentation, we excluded stages 0 and 4 from further statistical analysis and we did not use the subdivision for stage 1.

The final endpoint of our study is to investigate any possible correlations between the biomarkers' expression levels in each one of the tissues, the clinical parameters and the development of diabetes, NASH and liver fibrosis.

Results

Descriptive statistics of demographic and clinical characteristics

In the present study we enrolled 50 morbidly obese individuals who underwent planned bariatric surgery. The majority of the participants were women (72%), the median age was 37.5 years and the median BMI 57.05. The demographic and clinical characteristics of our population are displayed in Table 1 and Table 2 for the continuous and nominal values respectively. According to *Shapiro-Wilk* test, only total cholesterol and LDL levels

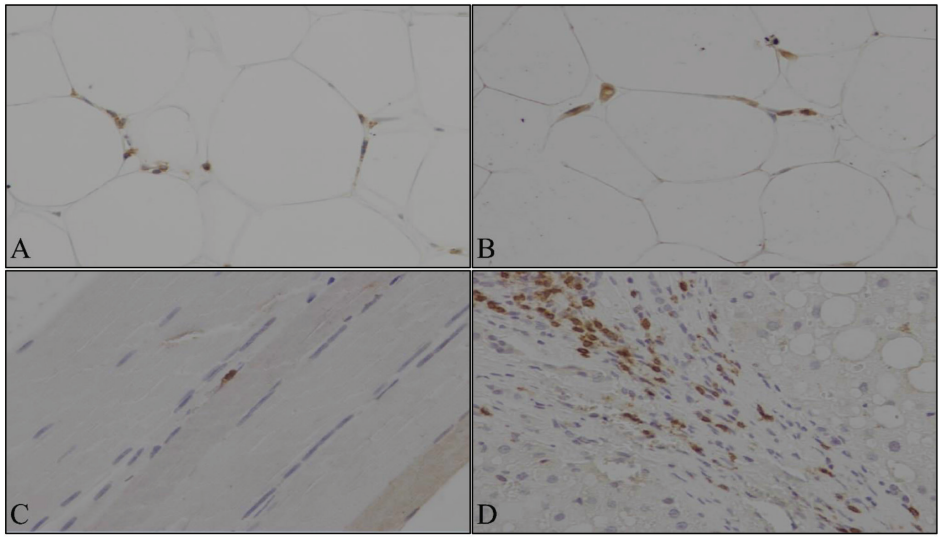

Figure 1. Representative images of CD3 immunostaining in Subcutaneous Adipose Tissue (SAT) (**A**), Visceral Adipose Tissue (VAT) (**B**), Skeletal Muscle (SM) (**C**), liver with fibrosis (**D**) (Legend: SAT: Subcutaneous Adipose Tissue, VAT: Visceral Adipose Tissue, SM: Skeletal Muscle) (original magnifications ×400).

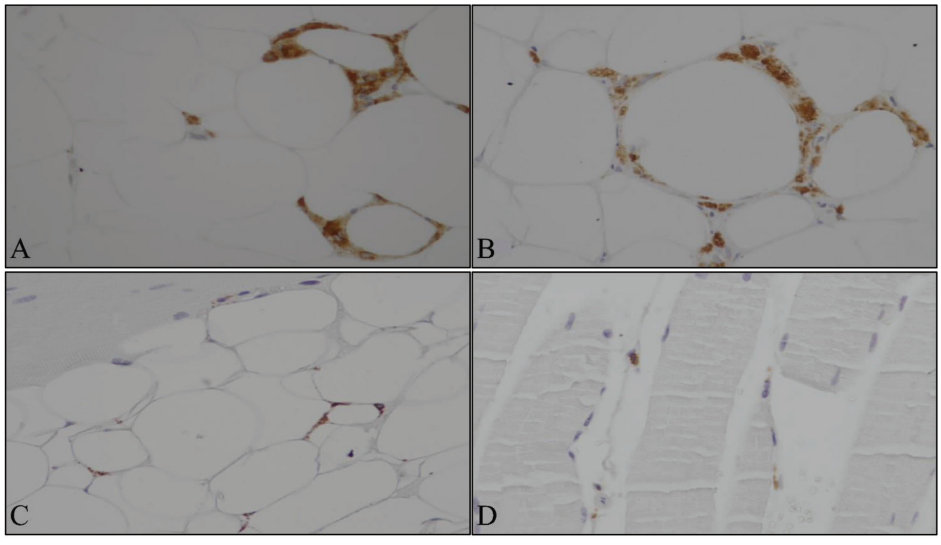

Figure 2. Representative images of CD68 immunostaining in Crown-Like Structure (CLS) seen in Visceral Adipose Tissue (VAT) (original magnification ×200) (**A**), CLS seen in Subcutaneous Adipose Tissue (SAT) (original magnification ×400) (**B**), Extra-myocellular Adipose Tissue (EMAT) (original magnification ×200) (**C**) and SM (original magnification x400) (**D**) (Legend: SAT: Subcutaneous Adipose Tissue, VAT: Visceral Adipose Tissue, SM: Skeletal Muscle, EMAT: Extramyocellular Adipose Tissue, CLS: Crown-Like Structure).

were normally distributed and thus median and IQR are more representative and accurate, than *mean*±*SD*, for describing the rest of the variables.

It is noticeable that 38% of the enrolled patients suffer from hypertension and 28% from either impaired glucose tolerance or full-blown diabetes mellitus. Moreover, 75% was found to have some degree of non-alcoholic fatty liver disease (NAFLD) and the vast majority (98%) some degree of fibrosis as assessed on liver biopsy, by *Kleiner* histological scoring system[57]. Finally, 82% of our population had intramuscular and 46% had intra-myocellular fat as well.

Further on, due to the predominance of the female subjects

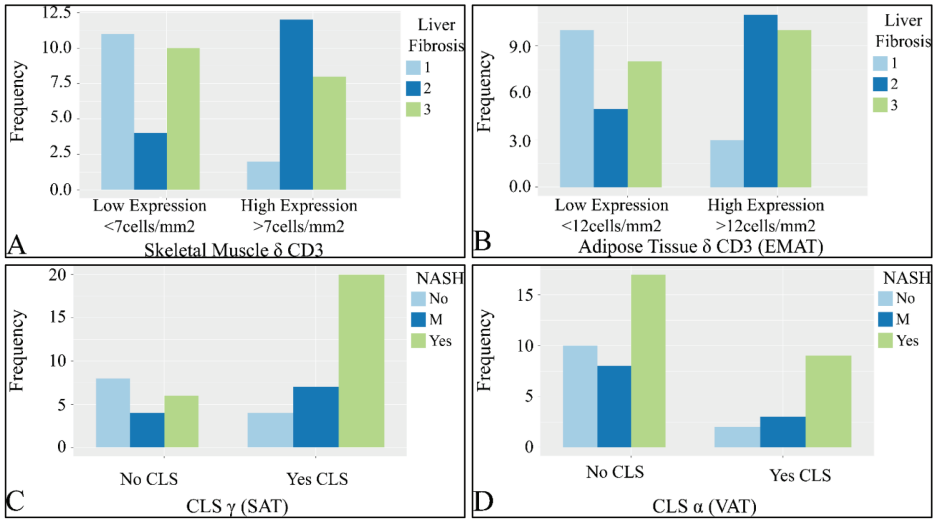

Figure 3. Bar plots showing the correlation between CD3 expression levels in Skeletal Muscles (SM) (**A**) and Extra-myocellular Adipose Tissue (EMAT) (**B**) with liver fibrosis and correlation between the presence of Crown-Like Structure (CLS) in Subcutaneous Adipose Tissue (SAT) (**C**) and Visceral Adipose Tissue (VAT) (**D**) with the development of Non Alcoholic Steatohepatitis (NASH) (Legend: SAT: Subcutaneous Adipose Tissue, VAT: Visceral Adipose Tissue, SM: Skeletal Muscle, EMAT: Extra-myocellular Adipose Tissue, CLS: Crown Like Structure, 1-3: stages of liver fibrosis, M: Marginal stage. Borderline for NASH).

we have investigated possible differences in our population due to gender. In particular, significant differences were found between males and females with respect to height ($p=3\times10^{-8}$), weight ($p=0.0023$), hematocrit ($p=0.0001$), hemoglobin ($p=0.0002$), Fe ($p=0.03$) and Ferritin ($p=0.009$). Body fat content (%) appeared also to differ significantly between men and women ($p=0.004$) with women showing a higher median value (*Median: 50.2%, IQR=5.0%*) as compared to men (*MED=46.1%, IQR=6.8%*). Despite the fact that body fat was significantly associated with BMI ($p=0.006$), BMI did not show major differences between the two sexes ($p=0.72$). Hypertension, diabetes and lipid levels did not differ significantly between men and women. Finally, no further differences were observed with respect to CD3 and CD68 expression levels, in all tissue biopsies.

Statistical analysis revealed no correlation between sex, hypertension, BMI, body fat percentage, total cholesterol, LDL and HDL levels with the presence of diabetes, NASH or liver fibrosis in our study population. The likelihood of developing diabetes appeared to increase with age (median age 34 years in non-diabetic patients, whereas median age was 43 in diabetic or prediabetic group), but this association was not found to be statistically significant. Moreover, there was an apparent but not statistically significant correlation (statistical trend) between both intramuscular ($p=0.059$) and intrahepatic fat ($p=0.063$) and the development of type II diabetes. Finally, we detected a statistically significant positive link between SGOT ($p=0.009$) and SGPT ($p=0.013$) levels with the presence of NASH, making liver transaminases useful diagnostic and prognostic biomarkers.

Descriptive statistics of biomarkers' expression

All 5 collected tissues (*a, γ, δ.m, δ.ad, ε*) from the 50 enrolled patients were assessed for the presence of inflammatory cells by immunohistochemistry. Specifically, we investigated the expression of CD68 and CD3 biomarkers, for identifying macrophages and T-lymphocytes respectively and we also assessed the presence of crown-like structures in all three distinct adipose tissues. Both biomarkers were expressed in all tissues, indicative of the underlying systemic inflammation. The mean, standard deviation and median values of the positively stained cells are displayed in Table 3. Representative images of CD63 and CD68 immunostaining are shown in Figure 1 and Figure 2 respectively.

As it is presented in Table 3, the median expression levels for both CD68 and CD3 are higher in VAT compared to SAT, with CD68 levels being higher than CD3 in all investigated tissues. Moreover, CD68 and CD3 expression levels in EMAT were found to be higher in EMAT than in skeletal muscle, but cannot be directly compared to SAT and VAT as we used slightly different methods. Interestingly, we detected more CLSs in SAT rather than in VAT, but we did not identify any in EMAT.

Correlations between biomarkers' expression and clinical characteristics of our population

We found that age has statistically significant positive correlation with CD68+ expression in both skeletal muscle ($p=0.012$) and EMAT ($p=0.014$). We also observed a strong positive association between body fat content and CD3 expression levels in VAT ($p=0.013$).

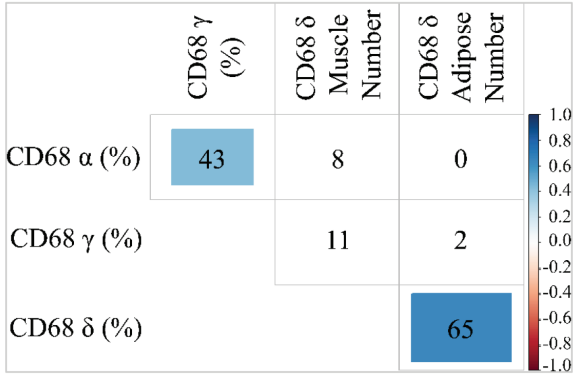

Figure 4. Correlation matrix (with Spearman values) that gives a comprehensive view of all possible inter-tissue correlations for CD68 expression (all parameters are shown as continuous variables). All strong positive correlations (>30%) are shown in bold blue color.

Moreover, there was a strong positive link between liver transaminases' levels and CD68+ expression in SAT (p=0.006 for SGPT), VAT (p=0.035 for SGPT) and EMAT (p=0.024 for SGOT), indicative of systemic inflammation in all adipose deposits and its association with NAFLD. Supporting finding is the prominent positive correlation with CD3 expression in VAT (p=0.019 for SGOT, p=0.009 for SGPT). Finally, we noted a strong positive link between NAS score and the presence of CLSs in SAT (p=0.018), but we did not detect any statistically significant links between lipid levels and inflammatory biomarkers' expression.

Correlations between biomarkers' expression and study endpoints

We found a significant positive link between CD3 expression levels in skeletal muscle (p=0.006) and EMAT (p=0.045) with the presence of liver fibrosis. This is a novel finding that in our knowledge has not been described so far.

Moreover, NASH was found to be strongly positively correlated with the presence of CLSs (p=0.034) in SAT, finding which is in line with the association between NAS score and the presence of CLSs in SAT (p=0.018).

We did not detect any statistically significant correlation between the presence of diabetes mellitus and the inflammatory biomarkers' expression. We noticed though, a marginally significant correlation (statistical trend) with liver steatosis (p=0.063) and IMAT (p=0.059). Representative bar-plots are shown in Figure 3.

Correlations between biomarkers' expression within the same tissue (Intra-tissue Co-expression Networks)

VAT

In VAT, there seems to be a strong positive link between CD3+ and CD68+ expression levels (p=0.01), which is expected as it confirms the local inflammation.

SAT

In SAT there is a remarkable positive association between the presence of CLSs and the expression levels of CD68+ (p~0) and CD3+ (p=0.019). However, we failed to demonstrate a statistically significant correlation between CD68+ and CD3+ expression levels , although there was a clear trend (positive, p=0.059).

Skeletal muscle

As in the case of SAT and VAT, considerable positive correlation was found between CD3+ and CD68+ in skeletal muscle (p=0.026). This result, was also expected as it confirms the tissue's local inflammation.

EMAT

There was a statistically significant positive correlation between CD3+ and CD68+ (positive, p~0), as in the rest of the tissues, which is indicative of systemic inflammation.

Correlations between biomarkers' expression between the tissues

CD68

We found an outstanding positive link between CD68+ expression levels in SAT and VAT (p=0.002), as well as between skeletal muscle and EMAT (p~0). Moreover, a significant correlation was noted between the presence of CLSs in SAT and VAT (p=0.027). All inter-tissue correlations regrading CD68 expression are shown in Figure 4.

CD3

Our findings regarding CD3 expression between tissues are in agreement with the ones for CD68. Thus, we noticed a statistical significant positive correlation between SAT and VAT (p=0.0004), as well as between skeletal muscle and EMAT (p=0.002).

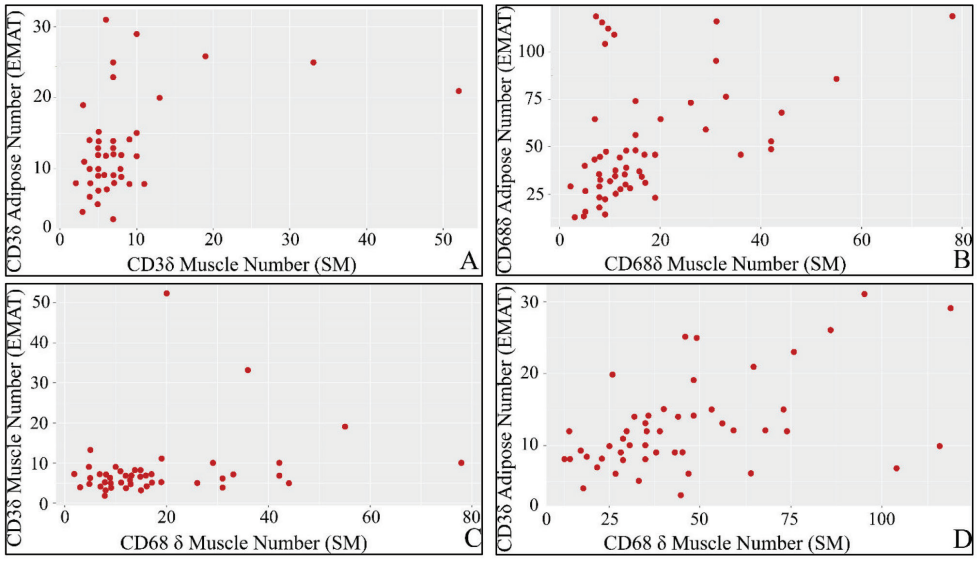

Figure 5. Scatterplots showing inter- and intratissue correlations between the investigated biomarkers (**A**) CD3 expression between SM and Extra-myocellular Adipose Tissue (EMAT) (**B**) CD68 expression between Skeletal Muscle (SM) and EMAT (**C**) CD3 and CD68 expression in SM (**D**) CD3 and CD68 in EMAT (Legend: SM: Skeletal Muscle, EMAT: Extra-myocellular Adipose Tissue).

Representative scatter plots regarding inter- and intratissue biomarkers' expression are shown in Figure 5.

Discussion

Intra- and inter-tissue co-expression networks also apply to inflammation and the mediated biomarkers

Obesity is characterized by a chronic, low grade systemic inflammation and the pathogenesis of the disease can be better understood in the context of "disease network analysis". Recent studies[65-69], including own data under submission, denote the significance of inter-tissue, cross-talk comprehension in unmasking the underlying pathologies in obesity, inflammation, diabetes and NAFLD. We detected a complex and extensive intra- and inter-tissue inflammatory co-expression network that may shed light on the understanding of chronic inflammatory diseases. Specifically, we revealed a strong positive link between CD3 and CD68 expression levels in VAT (p=0.01), SM (p=0.026), EMAT (p~0) and a weaker in SAT (p=0.059). Moreover, we showed a statistically significant association between the presence of CLSs with both CD3 (p=0.019) and CD68 (p~0) expression levels in SAT. Regarding inter-tissue communication, we detected significant positive links between two tissue-pairs: (i) SAT and VAT (p=0.002 for CD68, p=0.027 for CLSs, p=0.0004 for CD3) and (ii) SM and EMAT (p~0 for CD68, p=0.002 for CD3). Our findings are suggestive of obesity-induced, systemic inflammation which appears to be related with the presence of its associated comorbidities.

Obesity is related to systemic inflammation

Previous studies have demonstrated that the presence of macrophages and lymphocytes in adipose tissue and skeletal muscle in obese subjects, suggests a low grade chronic inflammation and is associated with insulin resistance, endothelial dysfunction and NAFLD[70]. Obesity alters the architecture and microenvironment of adipose tissue and leads to the infiltration of proinflammatory cells, which in some cases form the so-called Crown-Like Structures (CLSs) that surround dead adipocytes and are nowadays considered the hallmark of adipose tissue inflammation and fibrosis[40-45]. Bigornia et al (2012) correlated the presence of CLSs, mostly in VAT, with insulin resistance in obese individuals[39] and he suggested that subcutaneous adipose tissue biopsy may be considered for better assessing patients' metabolic profile. There is also strong evidence that adipose tissue macrophage infiltration, especially in visceral rather than subcutaneous fat, is associated with liver dysfunction and histopathological lesions possibly via "portal-hypothesis" mechanism[71]. Cancello et al (2006) published that macrophage concentration in VAT is double than in SAT, although adipocytes in VAT tend to be smaller in diameter and concluded that adiponectaemia, SGOT levels and VAT macrophages can accurately predict the severity of liver disease[72]. In our study population, macrophage and lymphocyte concentration in VAT was 1.5 and more than three times respectively, higher than in SAT. It is notable though that, in contrary with previous studies[39,73], we found that CLS were more abundant in SAT than in VAT. This finding may be explained by the fact that we used abdominal rather than gluteofemoral SAT and thus this depot

could represent the "metabolically active" deep instead of the dormant superficial sub-compartment of subcutaneous adipose tissue[74].

Apart from adipose tissue, skeletal muscle can also be characterized by a chronic low-grade inflammatory status, especially in obese, diabetic and elderly individuals[30]. Increased T cell and macrophage infiltration in obese subjects, may contribute to metabolic dysfunction at a later stage. It has been proposed that T cells and macrophages residing in EMAT, may exert effects on the neighboring myocytes via a paracrine mechanism and induce the expression of pro-inflammatory chemokines, such as Monocyte Chemoattractant Protein-1 (MCP-1)[75] and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)[76], further mediating blood monocyte and T lymphocyte migration into skeletal muscle, resulting in expansion of the inflammation in skeletal muscle and insulin resistance (via Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signaling pathway). In contrast to previous studies[77,78], Tam et al. (2013, 2014) found relatively few macrophages (2-3%) and low inflammation gene expression (CD68, CCL2, CD40, CD206, CD11c, Arginase 1) in skeletal muscle of obese subjects, that remained unchanged after exercise and concluded that greater macrophage accumulation seen in other studies (4-5%) may potentially be due to contamination with adipose tissue[79]. This result was in agreement with our results, where the median expression of macrophages (CD68 positively stained cells) in skeletal muscle was 16 cells/mm^2 and of T cells (CD3 positively stained) was 7 cells/mm^2, corresponding to 5% and 2% infiltration respectively[77]. Comparing results between different studies could be a challenging task. Different groups have used dissimilar methods for assessing the accumulation of inflammatory cells. However, the absolute number of cells/mm^2 is becoming the method of choice amongst researchers and it will probably be established as the gold standard for assessing the density of different cell types in the investigated tissues[59-61].

According to recent studies, extramyocellular fat expansion in obesity correlates with skeletal muscle T cell and macrophage infiltration, systemic inflammation and insulin resistance[28,56,80]. It appears that EMAT has altered phenotype ("adiposopathy") similar to that observed in VAT, and its metabolic actions are mediated through paracrine and endocrine mechanisms. EMAT increases with age (~9g/year) after adjustment for total fat, but there seems to be no significant difference between men and women[81]. The infiltration of macrophages occurs during an early stage of obesity and precedes T cell accumulation. It is notable that inflammatory cells in EMAT can cluster in CLSs, as seen in VAT[82] although we did not detect such structures in our EMAT specimens.

We detected a strong positive link between age and CD68 expression both in skeletal muscle (p=0.012) and EMAT (p=0.014), despite the fact that the median age of our population was only 37.5 years. There is evidence that aging is accompanied by chronic inflammation due to elevated circulatory inflammatory cytokine production[83-86]. Several inflammatory cytokines (CRP, IL-6, IL-10, IL-15, TNFa) have been shown to be responsible for a decrease in muscle mass and an increase in the infiltration of macrophages, which are primarily responsible for the shift toward a more fibrotic state of skeletal muscle ("sarcopenia"). One the other hand, Tam et al found that CD68+ macrophage number is independent of aging and sex[30]. Moreover, it has already been proven that inter- and intramuscular fat increases with age ("myosteatosis"), a process that may contribute further to sarcopenia, inflammation and thus the development of insulin resistance[87]. However, little is known about the possible relationship between inflammation and sarcopenia due to aging and more studies are needed in order to shed light on the underlying mechanisms.

Systemic inflammation is strongly associated to the presence of NASH and liver fibrosis

We demonstrated a strong positive link between total NAS score and the development of NASH, with the presence of CLSs in SAT (p=0.018 and p=0.034 respectively). CLSs are considered the hallmark characteristic of adipose tissue inflammation and fibrosis, which is related to the presence of non-alcoholic fatty liver disease and insulin resistance[33,39,70,88]. Although previous studies[71,89,90] correlate visceral adipose tissue inflammation and the presence of CLSs with liver damage, it seems that subcutaneous fat may play a role as well. In our study we used subcutaneous tissue from the abdominal wall, which could represent its deep component that has morphological and functional characteristics more similar to VAT[91].

Liver transaminases, alanine (ALT/SGPT) and aspartate aminotransferase (AST/SGOT) are assertive indicators of hepatocellular injury and can be used as diagnostic and prognostic markers of liver disease[92-94]. Several studies have demonstrated that SGPT appears to have a role in gluconeogenesis and seems to be more related to hepatic fat accumulation than SGOT. High levels of SGPT are correlated with a higher risk of NASH, however, patients with normal SGPT levels may also have abnormal histological features, suggestive of steatohepatitis[95,96]. Additionally, it has been introduced a new SGPT upper limit for healthy individuals which is ≤40 U/L for both genders[97,98]. In our study, both SGOT and SGPT were significantly correlated with the presence of NASH (p=0.009 and p=0.013 respectively). We also found a significant association between liver transaminases and CD68 expression in all adipose depots: SAT (p=0.006 for SGPT), VAT (p=0.035 for SGPT) and EMAT (p=0.024 for SGOT), as well as with CD3 expression in VAT (p=0.019 for SGOT and p=0.009 for SGPT). This finding is particularly important, as it links adipose tissue inflammation with non-alcoholic fatty liver disease, supporting the existing evidence for a potential key role of adipose tissue inflammation in the pathogenesis of NAFLD[90,99].

As liver biopsy is considered a painful and risky procedure, we could therefore use subcutaneous tissue instead, along with serum transaminases levels for detecting and following

liver disease. Moreover, the last few years FibroScan or Transient Elastography (TE) has become increasingly available in assessing non-invasively liver fibrosis[100]. Fibrotic livers have reduced elasticity due to the deposition of fibrous tissue in the hepatic parenchyma. TE gives a liver stiffness measurement (LSM) using pulsed-echo ultrasound as a surrogate marker of fibrosis and allows for the identification of disease severity[101]. A low LSM reliably excludes advanced fibrosis, but the optimum cut-offs for clinical use are yet to be determined. Although TE is a safe, simple and cost-effective technique, there are considerable limitations that have to be addressed. There is evidence that results may be inaccurate in older patients (>52 years), those with central obesity, ascites and type II diabetes[102]. The use of new imaging modalities as noninvasive measures of liver fibrosis is undoubtedly an important step forward in the clinical management of patients with chronic liver disease, but due to their current limitations, liver biopsy still remains the gold standard for the diagnosis, staging and prognosis of liver disease.

Our most remarkable finding though, is the strong positive link between liver fibrosis and CD3 expression levels in skeletal muscle (p=0.006) and EMAT (p=0.045) and to the best of our knowledge, we are the first to demonstrate such a correlation. Takata et al (2017) published very recently that liver fibrosis markers, such as Fibrosis 4 Index (FIB4), can be helpful for predicting skeletal muscle mass loss in patients with chronic hepatitis C, suggesting a link between liver fibrosis and skeletal muscle[103]. There is no doubt that further research is needed in order to identify the complex underlying mechanisms and the signaling pathways that associate skeletal muscle inflammation with liver fibrosis.

NAFLD: Is it finally related to the development of insulin resistance?

Nonalcoholic fatty liver disease (NAFLD) encompasses a wide histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and in rare cases cirrhosis and hepatocellular carcinoma. NAFLD is considered the hepatic manifestation of metabolic syndrome and is strongly associated with insulin resistance and diabetes[5,99,104,105]. While it is fairly clear that insulin resistance causes hepatic steatosis, it is not known if NAFLD causes insulin resistance[106]. In our study, we detected a marginally significant correlation (statistical trend) between liver steatosis and the presence of diabetes (p=0.063). Hepatic steatosis is caused by triacylglecerol (TAGs) accumulation in the liver due to an imbalance between lipid storage and lipid removal, that can be caused by a high dietary fat intake, increased de novo lipogenesis, and increased lipolysis in adipose tissue[6,107,108]. Moreover, macrophages and other immune cells are recruited to the liver and secrete pro-inflammatory cytokines that activate NFkB/JNK (c-Jun N-terminal Kinase) signaling pathway. This low-grade chronic inflammation and lipid accumulation in the liver and other organs, are believed to be the main drivers of hepatic insulin resistance in NAFLD[108-110]. There is evidence that bariatric surgery in morbidly obese subjects can ameliorate or even reverse liver steatosis and inflammation and thus improve patients' metabolic profile[111,112]. Weight loss is still the cornerstone in the treatment of NAFLD, but many novel compounds are being studied and new weight-loss inducing agents are eagerly awaited[112].

Study limitations

Our study is an *in situ* morphological evaluation of the underlying inflammation in morbid obesity through biopsies obtained from severely obese individuals, who underwent planned bariatric surgery. One of the limitations was the fact that we were not able to include a control group of lean individuals in order to examine the presence of inflammation due to the surgical procedure per se and allow for comparisons between the two groups. It was challenging to obtain simultaneously biopsies from 5 different healthy organs (including liver) of non-obese individuals undergoing various types of abdominal surgery. Further on, several studies have demonstrated that not only morbidly obese, but also healthy overweight subjects (BMI: 25-30 kg/m^2) have higher levels of inflammatory biomarkers, pro-inflammatory cells and adipokines than their lean counterparts[113,114]. Moreover, lean individuals with NAFLD represent a wide spectrum of diseases including genetic predisposition, toxins, fructose- and cholesterol-rich diet and inherited lipid disorders. Including such patients with different underlying pathophysiological conditions in our study, would most likely complicate rather than clarify our results[115]. Thus, we have moved towards the solution of increasing the sample size and at the same time we have searched the literature for similar studies that used control populations in their investigations. To the best of our knowledge, all previous reports did not use directly control samples yet, they assessed the inflammatory effects based on the changes within the studied populations. This was true for both the CD3[116-120] as well as the CD68 inflammatory biomarkers[121-124]. Further on, although our study did not entail control samples it is one of the largest in sample size (n=50). Previous ones have reported investigations with morbidly obese individuals of 87 samples in the study of Atef E et al. (2016)[118], 59 samples in the study of Linkov F et al. (2014)[119], 27 samples in the study of Adler M et al. (2011)[116], 20 samples in the study of Merhi ZO et al. (2009)[120], 40 samples in the study of Guglielmi V et al. (2015)[123], 27 samples in the study of Corbould A et al. (2014)[122], 110 samples in the study of Caballero T et al. (2012)[121] and 9 samples in the study of Tchoukalova YD et al. (2004)[124]. The sampling size of previous reports makes our study the fourth largest in population.

Conclusions

In summary, obesity should not be regarded as a "lone" entity but as part of a complex, highly interlinked disease network. In this context we should base our thinking and research in order to identify the common genetic origins and

address the key biomarkers that need to be targeted so as to provide novel and efficient treatments. Our findings support the "disease network theory", as we detected a complicated inter- and intra-tissue co-expression network that links obesity-induced inflammation in the investigated tissues with non-alcoholic fatty liver disease. Specifically, inflammatory biomarkers (CD68, CD3) in all adipose depots were found to be positively related with serum liver transaminases and the presence of NASH, whereas CD3 expression in skeletal muscle and EMAT is linked to liver fibrosis as assessed by biopsy. Therefore, adipose or skeletal muscle biopsy, along with liver transaminases' levels and novel imaging techniques, can accurately detect subtle hepatic disease and may substitute for liver biopsy in the near future. There is no doubt that further research is needed in order to advance our understanding of the molecular mechanisms that contribute to liver steatosis, NASH, and fibrosis in obese subjects and identify novel and promising therapeutic targets.

Ethics statement

Informed written consent was given by all patients prior to participation. The protocol of our study was approved by the Institutional Review Board of the University Hospital of Patras and the ethical considerations were fully consistent with the Declaration of Helsinki (1975, review 2000).

Authors' contributions

AC: collected samples, collected biopsies, processed samples, performed experiments, KB: collected samples, collected biopsies, processed samples, performed experiments, EK: provided clinical insight, FK: provided clinical insight, VL: provided clinical insight, GIL: proof-read the manuscript, reviewed the manuscript, performed and provided critical insight in data analysis, MM: provided clinical insight, GSB: provided critical insight, proof-read the manuscript, gave final permission for submission.

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