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ORIGINAL ARTICLE

Body mass index and the visceral adipose tissue expression of IL-6 and TNF-alpha are associated with the morphological severity of non-alcoholic fatty liver disease in individuals with class III obesity

Antônio Sérgio Barcala Jorge[a,b], João Marcus Oliveira Andrade[a], Alanna Fernandes Paraíso[a], Gislaine Candida Batista Jorge[a], Christine Mendes Silveira[a,b], Ludmilla Regina de Souza[a], Erivelton Pereira Santos[a], André Luiz Sena Guimaraes[a,c], Sérgio Henrique Sousa Santos[a,c], Alfredo Maurício Batista De-Paula[a,d,*]

[a] *Nucleus of Epidemiological and Molecular Research Catrumano. Health Research Laboratory. Health Science Post-graduate Programme, Universidade Estadual de Montes Claros, 39401-001 Montes Claros, MG, Brazil*
[b] *Department of Physiopathology, Universidade Estadual de Montes Claros, 39401-001 Montes Claros, MG, Brazil*
[c] *Department of Food Engineering, Universidade Federal de Minas Gerais, 39404-547 Montes Claros, MG, Brazil*
[d] *Department of Dentistry, Universidade Estadual de Montes Claros, 39401-001 Montes Claros, MG, Brazil*

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KEYWORDS
Class III obesity;
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Summary
Objectives: To analyze the mRNA expression of interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) in the liver and white adipose tissue samples of individuals

* Corresponding author at: Programa de Pós-graduação em Ciências da Saúde, Hospital Universitário Clemente de Faria, Universidade Estadual de Montes Claros, Avenida Cula Mangabeira, 562 Bairro Santo Expedito, 39401-001 Montes Claros, MG, Brazil. Tel.: +55 38 32248380.
E-mail address: ambpatologi@gmail.com (A.M.B. De-Paula).

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Anthropometric factors;
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Real-time-PCR

with class III obesity (body mass index $\geq$40.0 kg/m^2) with non-alcoholic fatty liver disease (NAFLD).
Methods: This cross-sectional study included patients with class III obesity exhibiting early or late morphological presentation of NAFLD (non-alcoholic hepatic steatosis [NAFL], $n = 8$ and non-alcoholic steatohepatitis [NASH], $n = 13$, respectively). All patients underwent bariatric surgery and peripheral blood, liver, and visceral white adipose tissue (WAT) samples were collected. Socio-demographic, anthropometric, clinical, plasma biochemical, and nutritional characteristics of each study subject were assessed and compared between patients presenting with NAFL and NASH. *IL-6* and *TNF-α* mRNA expression in the liver and WAT samples were measured by using quantitative real time-polymerase chain reaction (qRT-PCR).
Results: Individuals with class III obesity and NASH showed higher body mass index (BMI) and higher *IL-6* and *TNF-α* mRNA expression in the WAT compared to that of patients with NAFL ($p = 0.01$, for all associations).
Conclusions: Individuals with class III obesity with higher morphological severity of NAFLD exhibited higher BMI and higher IL-6 and TNF-α expression in the WAT. Future prospective studies are warranted to determine how BMI, IL-6, and TNF-α affect the progression of NAFLD in individuals with class III obesity.

Introduction

Obesity is one of the most common and injurious inflammatory and metabolic human disease [1]. This multifactorial inflammatory and metabolic disease is caused by the interaction of a series of genetic/epigenetic and environmental factors [1—3]. Obesity has high prevalence in many populations worldwide, affecting over 50% of the adult population and exhibiting high morbidity and premature mortality rates. Individuals with class III obesity (body mass index $\geq$40.0 kg/m^2) are associated with increased susceptibility to a wide range of inflammatory/metabolic diseases and high mortality rates [4].

The non-alcoholic fatty liver disease (NAFLD) is a prevalent inflammatory hepatic disease that represents the liver manifestation of obesity and metabolic syndrome. Individuals with NAFLD do not usually drink alcoholic beverage, have no history of viral hepatitis infection, and do not exhibit autoimmune or drug-induced liver diseases. Both environmental factors (sedentary lifestyle, stress, and hypercaloric diet) and a background of genetic/epigenetic host susceptibilities play a role in the pathogenesis and natural history of this disease [5—9]. NAFLD presents a morphological spectrum that varies from an early stage known as non-alcoholic hepatic steatosis (NAFL) to the progressive form known as non-alcoholic steatohepatitis (NASH). With progression to NASH, cirrhosis, hepatocellular carcinoma, or liver failure may develop [10—13].

Cytokines play pivotal roles in the pathogenesis of some inflammatory and metabolic human diseases, including obesity [14—16]. During inflammation, the tumour necrosis factor-alpha (TNF-α) is one of the main proinflammatory cytokines that regulate both innate and adaptive immune responses. This cytokine is primarily produced by endothelial cells and infiltrating immune cells in response to exogenous or endogenous etiologic factors in vascularised tissues [17,18]. The interleukin-6 (IL-6) is a proinflammatory cytokine that plays pivotal roles in local and systemic proinflammatory responses [19]. IL-6 is mainly produced from various cell types, including bone marrow cells and hepatocytes. IL-6 plays numerous proinflammatory roles such as B lymphocytes and T-cell activation, growth, and differentiation [20,21].

In this study, we analyzed the association between anthropometric, clinical, nutritional, and biochemical factors and the expression of IL-6 and TNF-α in the liver and white adipose tissue (WAT) in patients with class III obesity presenting with NAFL and NASH.

Patients and methods

Ethical statement

Ethical approval was obtained from the relevant ethic committee (CONEP — 85742/2012). All subjects agreed to participate to this study and signed

the informed consent form prior to the beginning of the study.

Study design

In this cross-sectional and analytical study, individuals with class III obesity ($n = 21$; male:female ratio: 1:6, mean age: 38.57 ± 11.52 years, ranging from 22 to 63 years) who were examined in a public health service for bariatric surgery in the Montes Claros city, Minas Gerais state, Brazil, were enrolled from 2013 to 2014.

Examinations

The NAFLD diagnosis was microscopically established in all patients with class III obesity. Preoperative evaluation of patients was conducted by a multidisciplinary health team that carried out a detailed anamnesis and a series of anthropometrical, nutritional, metabolic, cardiopulmonary, and psychological evaluations. All patients enrolled in this study underwent surgical treatment based on national and international guidelines [22,23].

Inclusion and exclusion criteria

Patients were included in this study if they were diagnosed with class III obesity, presenting or not any types of comorbidities that arisen after two years of treatment and during the follow-up. Major exclusion criteria for the selection of individuals were users of alcoholic beverages, presence of psychiatric or cognitive impairments, severe cardiopulmonary disease, portal hypertension and esophagogastric varices, acute inflammatory disease of upper aerodigestive tract, and Cushing's syndrome.

Sample collection

Peripheral blood samples from all individuals were collected after an overnight fasting period. Biopsies of the hepatic tissue and omental white adipose tissue (WAT) were performed surgically and a representative portion of the sample was rinsed in RNAse-free buffer and cryopreserved at -80 °C. Another representative portion of each tissue was formalin-fixed, paraffin-embedded, cut into serial sections, and stained with haematoxylin & eosin (H&E) and Gomori's trichrome. Posteriorly, these samples were submitted for molecular and morphological analysis.

Nutritional analysis

The 24 h dietary recall interview (24-HDR) dietary assessment was performed for all patients with class III obesity. The 24-HDR questionnaire is literacy and financially accessible, quickly applied, and provides a high response rate from subjects with different educational and socio-economic levels [24,25]. Dietary intake records for value caloric (VC), total lipid (TL), saturated fat (SAT), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were collected and the results were expressed as the percentage mean value $\pm$ standard deviation.

Plasma biochemical analysis

Clinical/biochemical blood analyses were performed to evaluate hepatic function and integrity. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by using an optimised UV method (IFCC). The plasma levels of alkaline phosphatase (ALP), glucose, albumin: A, ferritin, and glycosylated haemoglobin were measured by using enzymatic specific methods. The levels of C-reactive protein (CRP) were assessed using the Bio-Látex method. An enzymatic colorimetric test (AA Plus) method was used to measure the levels of low-density lipoprotein-cholesterol (LDL-C), very-low-density lipoprotein (VLDL), triglycerides (TG), high-density lipoprotein-cholesterol (HDLc), and total cholesterol (TC). The glucose levels were measured by the hexokinase method and serum insulin concentration was measured using an immunoradiometric assay. All biochemical analyses were assessed using Wiener Lab. BT 3000 Plus/CB 350i (Rosário, Argentina) on an automated chemistry analyzer.

Morphological and immunohistochemical analyses

All liver biopsies were reviewed by two trained pathologists (De-Paula, AMB and Mendes, CF). Morphological analyses were performed by using histopathological 5-μm-thick sections of archived formalin fixed-paraffin embedded-NAFLD ($n = 21$) tissues, which were deparaffinised, stained with H&E, and evaluated under a conventional light microscope. Typical morphological findings (hepatic steatosis, ballooning hepatocytes, lobular inflammatory reaction, and staging for hepatic fibrosis) were identified and graded in the liver samples. Additionally, the detection of ballooning hepatocytes was assessed immunohistochemically using an anti-human cytokeratin-18 (CK-18) mouse

monoclonal (clone DC-10, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody, with a streptavidin—biotin—peroxidase detection system (LSAB^{TM}-Kit Plus Peroxidase®, DakoCytomation, Glostrup, Denmark). The samples were then stained with a chromogen (3,3′-diaminobenzidine tetrahydrochloride, DAB), counterstained with Mayer's haematoxylin, cover slipped, and visualised under an optical microscope. Morphological and immunohistochemical analyses of NAFLD samples were performed by the pathologists who were blinded to the clinical and biochemical data. For each sample, the diagnosis for NAFL ($n = 8$) and NASH ($n = 13$) was reached by using previous morphological criteria. Morphologically, NAFL was diagnosed when the NAFLD activity score was <5 and NASH when the NAFLD activity score was ≥5 [26—28].

RNA isolation and real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis

Briefly, total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNAs were synthesised from 1 μg of total RNA by using the Moloney murine leukaemia virus (M-MLV) reverse transcriptase system (ThermoFisher Scientific, Waltham, MA, USA). The cDNA was used for qRT-PCR (ThermoFisher Scientific). All RT-PCR analyses were performed by using the Mx3005P Real-Time PCR System (ThermoFisher Scientific). For each condition, mRNA expression of biomarkers was quantified in duplicate. 18S rRNA was used as the endogenous control in the comparative cycle threshold (C_T) method. Gene expression was normalised to the expression of β-actin, which was considered as the reference. Additional details about primer sequences, GenBank and Entrez gene accession, and amplification conditions are provided in supplementary material (Table 1).

Statistical analysis

All data were statistically analyzed by using the SPSS® 18.0 software (SPSS Inc., Chicago, IL, USA). The association between NAFL and NASH groups based on clinical data was determined by using Pearson's, Chi-squared, and Fisher's exact tests. The comparison of anthropometric, nutritional, biochemical, and molecular data between NAFL and NASH groups was performed by using Student's *t* test and Mann—Whitney *U* test. The level of significance was set at 5% and associations with $p < 0.05$ were considered statistically significant.

Results

Descriptive analysis

The descriptive results of socio-demographic, anthropometric, clinical, nutritional, and plasma biochemical findings from patients enrolled in this study are summarised in Table 1. According to our clinical findings, the majority of patients with class III obesity and NAFLD exhibited hypertension (76.2%) and metabolic syndrome (61.9%), while dyslipidemia and diabetes mellitus were noted in 28.6% of the patients.

Comparison of sociodemographic, clinical, anthropometric, nutritional, and plasma biochemical data in patients with NAFL and NASH

The prevalence of diabetes *mellitus*, hypertension, dyslipidemia, and metabolic syndrome tended to be higher in patients with class III obesity and NASH than in patients with NAFL, but did not reach significance ($p > 0.05$). Notably, there was a significant association between NASH and higher BMI ($p = 0.01$). Nevertheless, our findings did not show associations between the others sociodemographic, clinical, anthropometric, nutritional, and plasma biochemical factors investigated in this study and the two groups ($p > 0.05$) (Table 1).

Comparison of molecular findings between NAFL and NASH groups

Our molecular findings showed that all patients with class III obesity and NAFLD expressed both *IL-6* and *TNF-α* mRNA in the liver (0.74 ± 0.31 and 0.68 ± 0.31, respectively) and WAT (1.05 ± 0.50 and 0.92 ± 0.33, respectively). In obese patients with NAFL, *IL-6* expression in the liver and WAT samples was 0.70 ± 0.31 and 0.90 ± 0.22, respectively. *TNF-α* expression in the liver and WAT samples was 0.65 ± 0.26 and 0.81 ± 0.19, respectively. In obese patient with NASH, *IL-6* expression in the liver and WAT was 0.77 ± 0.32 and 1.15 ± 0.60, respectively. *TNF-α* expression in the liver and WAT was 0.70 ± 0.35 and 0.99 ± 0.38, respectively. No significant association was observed between *IL-6* and *TNF-α* mRNA expression in the liver and NAFLD groups ($p > 0.05$). However, in WAT samples, a higher expression of *IL-6* and *TNF-α* mRNA was observed in patients with class III obesity and NASH when compared to obese patients with NAFL ($p = 0.01$, for both associations) (Fig. 1).

Table 1 Distribution and analysis of demographic, anthropometric, clinical, and plasma biochemical findings in a sample of individuals with class III obesity and non-alcoholic fatty liver disease (NAFLD).

Variables	All	NAFL	NASH	*p*
Age (years)	38.57 ± 11.52	36.25 ± 10.63	40.00 ± 12.27	0.50
Height (cm)	162.71 ± 6.73	159.50 ± 7.17	164.69 ± 5.87	0.39
Weight (kg)	112.63 ± 15.90	101.72 ± 8.39	119.34 ± 15.85	0.24
Waist circumference (cm)	118.52 ± 12.92	113.56 ± 8.92	121.58 ± 14.32	0.20
BMI (kg/m^2)	42.61 ± 4.08	40.13 ± 1.49	44.14 ± 4.46	0.01*
Systolic pressure (mm/Hg)	13.30 ± 1.44	13.08 ± 0.79	13.48 ± 1.88	0.85
Diastolic pressure (mm/Hg)	7.95 ± 0.54	7.84 ± 0.86	8.05 ± 0.33	0.55
VC	188.2 ± 77.54%	182.5 ± 68.62%	192.4 ± 86.38%	0.95
TL	32.2 ± 6.09%	31.3 ± 4.67%	32.8 ± 7.11%	0.08
SAT	13.1 ± 6.32%	11.9 ± 5.14%	13.9 ± 7.18%	0.28
MUFA	10 ± 4.54%	9.4 ± 4.05%	10.5 ± 5.01%	0.63
PUFA	4.5 ± 3.2%	4.2 ± 3.12%	4.7 ± 3.27%	0.91
Glucose (μmol/L)	97.33 ± 15.68	95.88 ± 21.35	98.23 ± 11.40	0.29
Glycohemoglobin (%)	5.75 ± 1.18	5.66 ± 1.21	5.8 ± 1.22	0.58
Albumin (g/dL)	3.94 ± 0.54	3.78 ± 0.60	4.03 ± 0.50	0.27
Triglycerides (μmol/L)	173.62 ± 24.63	191.25 ± 98.80	162.77 ± 96.81	0.49
Ferritin (ng/mL)	118.18 ± 71.32	91.66 ± 82.19	135.06 ± 61.54	0.18
Total cholesterol (μmol/L)	194.67 ± 37.12	204.63 ± 34.48	188.54 ± 38.68	0.27
HDL-c (μmol/L)	47.90 ± 17.33	48.88 ± 15.42	47.31 ± 19.00	0.74
VLDL-c (μmol/L)	33.24 ± 17.16	37.50 ± 20.66	30.62 ± 14.91	0.49
LDL (μmol/L)	115.05 ± 39.01	121.13 ± 41.26	111.31 ± 38.78	0.53
ALT (IU/L)	18.33 ± 8.00	17.75 ± 7.22	18.69 ± 8.70	0.88
AST (IU/L)	26.76 ± 9.31	31.62 ± 8.10	23.77 ± 8.99	0.07

Analyses were performed using Student's *t*-test and Mann—Whitney *U* test.
Results were expressed as mean ± standard deviation (S.D.). For nutritional variables (VC, TL, SAT, MUFA/MONSAT, and PUFA/UNSAT) the results are expressed as mean percentage value ± standard deviation.
NAFL: non-alcoholic fatty liver; NASH: non-alcoholic steatohepatitis; BMI: body mass index; HDL-c: cholesterol associated with ApoA-1/high-density lipoprotein particles; VLDL-c: cholesterol associated with ApoA-1/very low-density lipoprotein particles; LDL: low-density lipoprotein; ALT: alanine transaminase; AST: aspartate transaminase; VC: value caloric intake; TL: total lipid intake; SAT: saturated fat intake; MUFA: monounsaturated fatty acids intake; PUFA: polyunsaturated fatty acids intake.
* Significant *p* value ($p < 0.05$).

Discussion

A chronic, low-grade inflammation is the central feature of obesity [29], which is an important predictive factor for NAFLD [30—33]. Proinflammatory cytokines expressed in the liver and WAT participate to molecular signalling pathways that connect obesity and NAFLD [34,35]. Similar to other studies, our findings showed that both TNF-α and IL-6 were highly expressed in the visceral WAT and liver of obese individuals [36—40]. Obesity primarily affects the adipose tissue, but it also affects other metabolically critical organs such as the liver. WAT cells actively express genes related to proinflammatory signalling pathways [41—43]. In parallel, mutual damage mechanisms between resident hepatic cells and inflammatory cells infiltrating the liver are crucial to gradually trigger the inflammatory responses that promote hepatic injuries during the early stages of NAFLD [44,45].

Some studies reported that TNF-α and IL-6 may contribute to NAFLD progression in obese individuals [46—48]. In the current study, no significant difference was observed between the NASH and NAFL groups in term of *TNF-α* and *IL-6* mRNA expression in liver. TNF-α is produced by resident liver cells and infiltrative immune cells and contributes to NAFLD progression through different damage mechanisms. Initially, TNF-α stimulates hepatic steatosis, which, in turn, increases serum TG levels and stimulates fatty acid synthesis in hepatocytes [49,50]. TNF-α can also trigger cytotoxicity mechanisms, resulting in cell death of steatotic and normal hepatocytes [51]. Additionally, TNF-α might activate Kupffer cells which, in turn, stimulate the occurrence of hepatic fibrosis during NAFLD progression [52]. TNF-α expression has been predominantly investigated in peripheral blood and adipose tissues. In these studies, high TNF-α level is associated with the susceptibility and clinicopathological severity of NAFLD [53,54]. On the other hand, in the liver of individuals with NAFLD, IL-6 activates some resident and infiltrative immune cells and, once activated, these cells

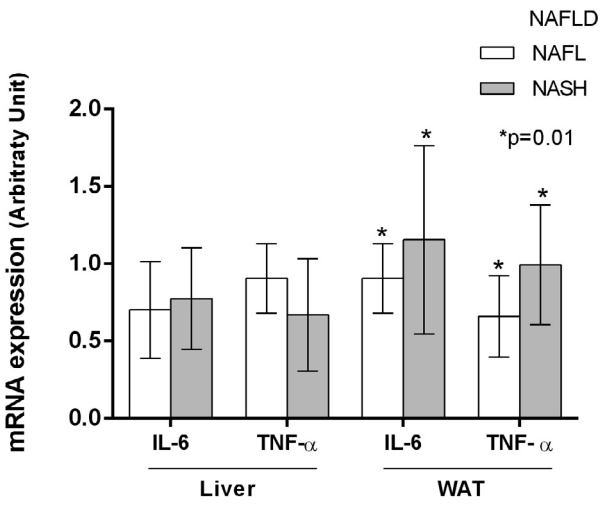

Figure 1 Expression of IL-6 and TNF-α in liver and visceral shite adipose tissue (WAT) samples of individual with class III obesity and non-alcoholic fatty liver disease (NAFLD). Our findings show a significant association between a higher WAT expression of TNF-α and IL-6 in individuals with class III obesity and NASH (non-alcoholic steatohepatitis; late stage) compared to individuals with class III obesity and NAFL (non-alcoholic hepatic steatosis; early stage) ($p = 0.01$, for both associations). The statistical analyses were performed using Student's *t*-test or Mann—Whitney *U* test. The level of significance was set at $\alpha = 5\%$ ($p < 0.05$).

promote, directly and indirectly, damage mechanisms on the hepatic tissue. Although the role of IL-6 in NAFLD progression is not established yet, the positive correlation between higher morphological aggressiveness of NAFLD and hepatic IL-6 expression has been observed [55—57]. Our finding suggests that the expression of IL-6 and TNF-α in the liver does not allow the differentiation between the NAFL and NASH stages in individuals with class III obesity.

Persistent systemic chronic inflammation in obese individuals affects normal storage of fat and WAT endocrine functions. WAT is a biologically active organ, in which endocrine disturbances promote the secretion of proinflammatory factors from adipocytes, which play an important role in NAFLD progression [58—60]. As previously demonstrated [60], we noted that individuals with class III obesity and NASH presented higher *TNF-α* and *IL-6* mRNA expression in the visceral WAT. In the visceral WAT of individuals with class III obesity and NAFLD, resident and immune cells release numerous chemical mediators that have target receptors in the hepatic parenchyma [39]. TNF-α produced by these WAT cells is known to promote lipolysis and secretion of free fatty acids in the plasma, which contribute to hepatic steatosis in obese individual [61]. In parallel, IL-6 expression in WAT is associated with high BMI, higher plasma levels of free fatty acids, and an increase of insulin resistance. These metabolic disturbances might, directly or indirectly, contribute to hepatic injuries from the early stages of NAFLD progression in obese patients [62,63]. Interestingly, in this study, a significant and positive correlation was observed between high *IL-6* expression in WAT and higher BMI and waist circumference values ($p = 0.02$ and $p = 0.04$, respectively) (data not shown). Thus, our findings provide additional evidence supporting the hypothesis of the ongoing negative feedback loop occurring at the interface of inflammatory and metabolic molecular networks between the WAT and liver of obese individuals. We hypothesize that the expression of TNF-α and IL-6 in WAT might trigger the hepatic damage mechanisms that occur during the early stages of NAFLD in individuals with class III obesity. Further experimental, prospective studies are warranted to confirm that hypothesis.

In this study, we showed that individuals with class III obesity and NASH presented higher BMI compared to individuals with class III obesity and NAFL. Although some studies reported controversial findings regarding this association [64], the BMI parameter has been considered as a significant predictor of NAFLD severity [65,66]. Patients with obesity often present with a significantly higher dyslipidemia and insulin resistance, which might contribute to NAFLD [67,68], which has been associated with NAFLD.

This study presents some limitations, which should be highlighted. The number of enrolled subjects was small. Moreover, this cross-sectional study does not allow us to establish the cause-effect relationship between cytokine expression in WAT and liver tissues and NAFLD progression. Due to technical reasons, we could not assess the WAT samples morphologically. Moreover, we only analyzed the mRNA expression, but not the protein expression or the function of TNF-α and IL-6 in the liver and WAT.

In conclusion, our findings showed that individuals with class III obesity and NASH exhibited a higher BMI and a higher IL-6 and TNF-α expression in the WAT. Future prospective studies are needed to determine how these factors affect NAFLD progression in individuals with class III obesity.

Conflict of interest statement

The authors have no conflict of interest to declare.

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