Interferon-alpha 2 but not Interferon-gamma serum levels are associated with intramuscular fat in obese patients with nonalcoholic fatty liver disease

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Abstract

Background: Intramuscular triglycerides (IMTGs) represent an important energy supply and a dynamic fat-storage depot that can expand during periods of elevated lipid availability and a fatty acid source. Ultrasonography (US) of human skeletal muscles is a practical and reproducible method to assess both IMTG presence and entity. Although a crosstalk between cytokines in skeletal muscle and adipose tissue has been suggested in obesity, condition leading to hepatic steatosis (HS) or better defined as nonalcoholic fatty liver disease and cancer, there are still questions to be answered about the role of interferons (IFNs), alpha as well as gamma, and IMTG in obesity. We aimed at discovering any correlation between IFNs and IMTG.

Methods: We analysed anthropometric data, metabolic parameters and imaging features of a population of 80 obese subjects with low-prevalence of co-morbidities but HS in relation to IFNs serum levels. A population of 38 healthy subjects (21 males) served as controls. The levels of serum IFNs were detected by a magnetic bead-based multiplex immunoassays.

Results: Serum concentrations of IFN-alpha 2 were increased, while serum levels of IFN-gamma were decreased confronted with those of controls; the severity of IMTG, revealed at US as Heckmatt scores, was inversely predicted by IFN-alpha 2 serum concentrations; IMTG scores were not predicted by serum levels of IFN-gamma; IMTG scores were predicted by HS severity, ascertained at US; HS severity was predicted by visceral adipose tissue, assessed by US, but the latter was not instrumental to IMTG.

Discussion and conclusion: This study has added some pieces of observation about the cytokine network regulating the interplay between IMTG and obesity in obese patients with HS.

Keywords: IFN-alpha, IFN-gamma, IMTG, obesity, NAFLD

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Background

Intramuscular fat, also known as intramuscular triglycerides, intramuscular triacylglycerol or intramyocellular triacylglycerol (IMTG), but also intramuscular adipose tissue (IntraMAT) and intramyocellular lipid (IMCL), when increased is thought to be linked to increased lipolytic activity in skeletal muscle, contributing to inducing insulin resistance (IR) [1]. Increased muscle TG stores are characterised by cytosolic accumulation of diacylglycerol and acyl-CoA-triglycerides, being lipase regulation central to skeletal muscle lipolysis [2]. Disturbances in pathways of lipolysis may play a role in the development and maintenance of these increased fat stores. Not only is IMTG an important energy supply for skeletal muscle, but represents a dynamic fat-storage depot that can expand during periods of elevated lipid availability [3]. Structural characteristics of IMCL seem to be similar between highly trained endurance athletes, type 2 diabetes patients, and overweight, sedentary men after an overnight fast. This observation is not in agreement with the hypothesis that elevated IMCL deposits are directly responsible for inducing IR [4].

A recent study, carried out to evaluate the exact localisation of IntraMAT, using $^3$H magnetic resonance spectroscopy or echo intensity (EI) determined by B-mode ultrasonography (US) of human skeletal muscles, has surprisingly suggested that IntraMAT primarily reflects extra-myocellular lipids, not IMCL [5].

Focusing on techniques unravelling IMTG, i.e., EI at US and high-resolution T1-weighted MRI, strong correlations were found between MRI percent fat and muscle EI after correcting for subcutaneous fat thickness [6].

But, apart evidence for being muscle US a practical and reproducible method, another point to be cleared consists in the choice of muscular district to be explored, i.e., the location of the region of interest. Here again, research has confirmed that the EI of biceps brachii and tibialis anterior was higher than that of all other muscles [7].

Beyond considerable insight into the role of IMTG in acute and chronic exercise training [3] and apart the proposed crosstalk between myokines and adipokines in skeletal muscle and adipose tissue [8], at our best knowledge there are no sufficient data about the link of specific cytokines, e.g., interferons (IFNs), alpha as well as gamma, and IMTG in obesity.

Indeed, a piece of evidence shows that IFN-gamma, which is released from inflamed omental adipose tissue, may contribute to the metabolic abnormalities seen in human obesity [9]. What is more, investigation pointed out to increased levels of IFN-gamma in obese subjects that were associated with central adiposity [10]. On the contrary, little research has been conducted to date on the role of IFN-alpha on visceral fat excess [11] and none on IMTG.

The subtype 2 of IFN-alpha was chosen to be evaluated in this study due to its action on memory CD8 cells and cytotoxic CD8 cells, which are activated by adipose tissue, in turn promoting the recruitment and activation of macrophages in this tissue [12], leading to the so-called chronic low grade inflammation, characterized by the abnormal production and activation of certain proinflammatory signalling pathways.

It is known type I IFNs are key cytokines involved in the early immune response to viral infections and it is interesting to evidence that obese subjects tend to have a decreased response to these infections [13] due to a reduced ability to produce IFN-alpha in response to Toll-like-receptors ligands, but its role in the obese without viral infections and its relation to IMTG is not still clarified.

Aiming at finding any correlations between serum concentrations of IFN-alpha 2 as well as IFN-gamma and IMTG, we analysed a population of obese subjects with low-prevalence of co-morbidities but nonalcoholic fatty liver disease (NAFLD) or hepatic steatosis (HS), evaluated by US.

Finally, there is no fresh evidence corroborating the link between NAFLD and IMTG outside exercise intervention [14], taking into account that IMTG is linked to increased BMI and visceral obesity [15] and shares common mechanisms with NAFLD.

Methods

Patients

We carried out a cross sectional type of observational study where at a particular point of time we described characteristics of obese patients without follow-up, with main variables, i.e., IFNs levels and IMTG scores compared to controls.

Specifically, this sub-study used the same original patient sample contained in a previous research [16], but with completely different analytical approaches resulting to be equally valid, according to The International Committee of Medical Journal Editors (ICMJE) at http://www.icmje.org/recommendations/browse/publishing-and-editorial-issues/overlapping-publications.html.

In this study 80 patients, who fulfilled the inclusion criteria and had given previous oral or, when possible, written consent were selected, comparing them to 38 healthy subjects (control group).

Inclusion criteria

Obese patients of different grade of obesity, on calorie-reduced, low-fat diet and sedentary lifestyle, with low
prevalence of co-morbidities, such as type 2 diabetes mellitus and hypertension but NALFD, US-documented.

Exclusion criteria
Patients were excluded if, at the time of blood specimen collection, they self-reported present or antecedent (past month) influenza, cold status, and gastroenteritis or there had been a history of unexplained weight loss in the past months (i.e., ±10% initial body weight) or recent illness/chronic disease, and the use of supplements or medications that might have affected body composition or muscle metabolism (e.g., steroids). Sarcopenic obese were ruled out from this selection.

Furthermore, any viral, autoimmune, metabolic liver disease (Wilson disease, hemochromatosis or antitrypsin deficiency) was ruled out by using appropriate testing, according to well-accepted diagnostic guidelines. Celiac disease was excluded by evaluating IgA anti-tissue transglutaminase antibodies. Alcohol abuse was disallowed, following the DSM-IV diagnostic criteria, by means of screening tests such as MAST (Michigan Alcohol Screening Test) and CAGE (Cut down, Annoyed, Guilty, and Eye opener), as well as random tests for blood alcohol concentration and the use of a surrogate marker, e.g., Mean Corpuscular Volume. Patients on antihypertensive drugs, and those treated with metformin or insulin, maintained a balanced therapeutic regimen throughout the study.

Anthropometric evaluation
The three degrees of obesity (light, moderate, and severe or 1–2–3) were established on the basis of BMI cut-off points of 30–34.9 and 35–39.9 and >40 kg/m², respectively.

Visceral obesity was identified by measuring WC at the midpoint between the lower border of the rib cage and the iliac crest. Hip circumference was measured around the widest part of the buttocks, with the tape parallel to the floor, and the waist-to-hip ratio (WHR) was calculated according to the National Institute of Diabetes, Digestive and Kidney Diseases stating that women with WHR of more than 0.8, and men with more than 1.0 are at increased health risk because of their fat distribution.

Metabolic profile
The canonical Adults Treatment Panel III was originally chosen to define the metabolic syndrome, considering at least three criteria: plasma glucose concentrations ≥100 mg dL⁻¹, WC>102/88 cm (male/female), serum HDL concentration<50 mg dL⁻¹ for women and <40 mg dL⁻¹ for men, blood pressure ≥130/85 mm Hg, and serum triglyceride concentration ≥150 mg dL⁻¹. But, to adhere to ethnic specific values, we added the metabolic syndrome criteria for Europids following the International Diabetes Classification (IDF), i.e., according to the IDF definition for a patient to be defined as having the metabolic syndrome they must have central obesity defined as WC with ethnicity specific values, e.g., for Europe’s males and females equal or superior to 94 and 80 cm, respectively, plus any two of the following four factors: Triglycerides >150 mg/dL or specific treatment for this lipid abnormality; cholesterol HDL<50 mg/dL for females and 40 mg/dL for males or specific treatment for this dyslipidemia; systolic and diastolic blood pressure equal or superior to 130 and 85 mm Hg, respectively; fasting plasma glucose >100 mg/mL or previously diagnosed type 2 diabetes mellitus. International Diabetes Federation, 2007; http://www.idf.org.

Triglyceride values of subjects who had fasted at least 12/14 h before the blood draw were evaluated, averaging the results of at least two determinations, made on different days.

Laboratory assessment
IFNs levels of 78 patients derived by a previously studied 48-cytokine/chemokine panel [16], which was performed on serum samples using a magnetic bead-based multiplex immunoassays (Bio-Plex) (BIO-RAD Laboratories, Milano, Italy) following manufactures' instructions. Data from the reactions were acquired using the Bio-Plex 200 reader, while a digital processor managed data output and the Bio-Plex Manager software returned data as Median Fluorescence Intensity (MFI) and concentration (pg/mL). Insulin resistance was studied by the Homeostatic Metabolic Assessment (HOMA) method with the formula: fasting insulin (μU/mL) × fasting glucose (mg/dL)/405 [17]. More than five determinations of HOMA in different situations were taken into account. HOMA-derived β-cell function (HOMA-B%) was also calculated, using the following formula: 20×fasting insulin (μU/mL)/fasting glucose (mmol/L)−3.5 [17]. A stringent value of HOMA>B was introduced as limit of the presence of insulin resistance [18]. We calculated a quantitative insulin sensitivity check index (QUICKI) as 1/[log(fasting insulin μU/mL)+log (fasting glucose mg/dL)], with range between 0.45 in healthy individuals and 0.30 in diabetics [19].

Ultrasoundography features
US measurements were obtained by an Esaote (Genoa, Italy) system. The classification of “bright liver” or hepatic steatosis (HS) was based on the following scale of echogenity: 0=absent, 1=light, 2=moderate, 3=severe, pointing out the difference between the densities of the liver and the right kidney [20], using a Convex Probe, with access to the liver through intercostal spaces along
the mid-axillary line. Transverse scanning was performed to measure the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) using an eleven and 3.5 MHz linear probe convex probe, respectively. The SAT was defined as the thickness between the skin-fat interface and the linea alba, avoiding compression, evaluated at the superior tertile of xifo-umbilical line. The VAT was defined as the distance between the anterior wall of the aorta and the internal face of the rectoabdominal muscle perpendicular to the aorta, measured one cm above the umbilicus. When the aortic walls were not visualized as they were obscured by bowel gas, the Doppler scan was used [21].

Muscle US, performed at the level of the biceps brachii of the left superior arm, is a convenient technique to visualise pathological muscle tissue, as it provides results in real time. Both infiltration of fat and fibrous tissue increase muscle echo intensity; that is, the muscles become whiter on the ultrasound image [22]. To describe muscle echo intensity, Heckmatt and coworkers developed a visual grading scale in which grade I represented normal muscle and grade IV represented a severely increased muscle echo intensity with total loss of bone echo (we chose biceps brachii versus humerus [23]. The levels of brightness of the liver and the biceps brachii, obtained by a single traverse image, were calculated three times directly from the frozen images. The choice of evaluating single traverse image findings was made according to Jenkins et al., who demonstrated that a single traverse imaging and panoramic US imaging are comparable [24].

Indirect calorimetry
RMR was measured by indirect calorimetry using a canopy system (V max 29 N, Sensor Medics, Anaheim, USA) in a quiet environment and with patients in the supine position for 30 min before measurement. After a 15–20 min adaptation to the instrument, oxygen consumption and carbon dioxide production were determined for 45 min. Energy expenditure was derived from CO₂ production and O₂ consumption with the appropriate Weir formula neglecting protein oxidation [25]. BMR, expressed as kcal/24h, was adjusted for changes in fat-free mass (FFM), which was evaluated by single-frequency bioimpedance analysis (BIA) obtaining a RMR/FFM ratio, expressed as kcal/24h·kg of body. Fat mass and FFM percentage were estimated using the device’s standard built in prediction equations and were displayed on the mid-axillary line. Transverse scanning was performed to measure the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) using an eleven and 3.5 MHz linear probe convex probe, respectively. The SAT was defined as the thickness between the skin-fat interface and the linea alba, avoiding compression, evaluated at the superior tertile of xifo-umbilical line. The VAT was defined as the distance between the anterior wall of the aorta and the internal face of the rectoabdominal muscle perpendicular to the aorta, measured one cm above the umbilicus. When the aortic walls were not visualized as they were obscured by bowel gas, the Doppler scan was used [21].

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Control group
Though IFN-alpha 2 is one of cytokines/chemokines not detected in any group of age of healthy subjects, either because they are under the lower limit of detection or because they are not produced [28] we took into account values of a population of 38 young healthy subjects to reduce the type I error. The control arm provided information when analysing difference of IFN-gamma levels in groups, too.

Statistics
Data, derived from a normally distributed population, were given as mean plus SD. Variables not normally distributed or ordinals are expressed as median plus 25–75 interquartile range (IQR). The difference in medians was assessed by the Mann–Whitney test. The two-way cross-tabulation was set by the Pearson correlation coefficient (Chi square). The Kruskal–Wallis equality-of-populations rank test was as used to evaluate differences when dealing with more that two variables.

The Spearman’s coefficient of rank correlation (rho) was employed to analyse the basic correlation between some data.

At univariate analysis, the linear regression analysis (ordinary least squares or OLS) was used evaluating the coefficient with its standard error, 95% confidence intervals (CI), the t (t-value) and R². In suspicion of heteroscedasticity, i.e., when there were sub-populations that have different variabilities from others in the homoscedastic model, and having detected the presence of few outliers, we analysed the correlation by the robust regression, using Least Absolute Deviations (LAD) Regression.

Contextually was conducted a residual analysis, a “residuals versus fits plot”. It is a scatter plot of residuals on the y axis and fitted values (estimated responses) on the x axis. The plot was used to detect non-linearity, unequal error variances, and outliers.

A simultaneous quantile regression was applied as a way to discover more useful predictive relationships.
between variables (bootstrap method). Quantile regression is more robust to non-normal errors and outliers.

At multiple linear regression also the factor Beta (\(\beta\)) was added.

An ordered probit model was employed to estimate relationships between an ordinal dependent variable (IMTG) or HS at US and a set of independent variables. These ordinal variable are variables that are categorical and ordered, expressed as severity score (I-IV) for the former or severity grade for the latter [1–3]. The output showed the coefficients, their standard errors, the z-statistic (also called a Wald z-statistic), and the associated P-values.

In a specific circumstance a Bayesian inference computed the posterior probability, expressed as mean, SD, Montecarlo standard error or MCSE, median and credibility intervals.

To highlight light unobserved confounding variables two methods were adopted: (i) Testing for mediation was performed as a four step approach in which several regression analyses were performed; the significance of the coefficients were examined at each step to study the so-called indirect effect [29]. (ii) The method of Instrumental Variables (IV) was utilised to estimate causal relationships. A valid instrument induces changes in the explanatory variable (covariate) but has no independent effect on the dependent variable, allowing to uncover the causal effect of the explanatory variable on the dependent variable. An instrument is a variable that does not itself belong in the explanatory equation but is correlated to the endogenous explanatory variables, conditional on the value of other covariates. The type of model was random effects and the estimator was the Baltagi–Changone.

The Factor Analysis was applied to detect the structure in the relationships among variables, selecting a subset of variables having the highest correlations with the principal component factors. In order to select a subset of variables, firstly Cattell Screen plot, with relative eigenvalues, was performed to screen the real factors, which resulted to be three. Secondly, extraction of the main components amounted to a variance maximizing (varimax) the rotation of the original variable space. The critical value was calculated by doubling Pearson’s correlation coefficient for 1% level of significance (5.152)/square root of patients minus 2 (n 78), i.e., 0.583. In bold will be shown the main components for any single factor, with a value superior to the critical one.

A closed form estimator of the uniqueness (unique variance) is proposed. It has analytically desirable properties, i.e., consistency, asymptotic normality and scale invariance. The concordance correlation coefficient (\(r_c\)), which measures precision and accuracy, was adopted to evaluate the degree of pair observations at US.

The power of this study was calculated on the difference of means of IFN-alpha and IFN-gamma levels between the obese and control group. In order to deepen this aspect, a further power analysis was performed using a slope test in the linear regression between the IFN-alpha levels and the IMTG scores.

Stata 15.1, Copyright 1985–2017, was the program on which we run statistics.

**Results**

The controls consisted of 17/21 healthy females/males with a BMI of 21 (20–21) and 23 (22–23), respectively. Their WC was 78 (78–79) and 94 (92–93) cm, respectively (values expressed as median plus IQR). Characteristics of the obese are shown in Table 1.

The median plus IQR for IFN-alpha 2 of healthy subjects was 2 pg/mL (0–2), the age-related reference intervals are shown in Fig. 1.

An interesting finding was that IFN-alpha 2 levels of obese patients were found to be significantly increased when compared to those of controls, i.e., 121.9 pg/mL (103.5–135.8) versus 2 (0–2), median plus IQR, \(P = 0.0001\), the Mann–Whitney test.

The median plus IQR for IFN-gamma of healthy subjects was 547.5 pg/mL (479–670), the age-related reference intervals are shown in Fig. 2.

The obesity degrees (1, 2 and 3), to which belonged 8, 26 and 46 patients respectively, did not show different distribution compared to gender, Pearson’s Chi square, \(P = 0.74\). IFN-alpha 2 levels were not significantly different among the three obesity degrees, \(P = 0.24\), Kruskal–Wallis equality-of-populations rank test.

Noteworthy, IFN-gamma levels in obese were lower than those of healthy subjects, i.e., 158 pg/mL (56–390) versus 547.5 pg/mL (479–670), median plus IQR, \(P < 0.001\), the Mann–Whitney test.

IMTG presence in our population was characterised by a light-moderate score of severity, as reported in Table 1.

The score of IMTG was not different when controlled for gender (Table 2), while the severity of HS at US was related to the obesity degree (Table 3). Finally, the score of IMTG was not dependent from the obesity degree (Table 4).

**Relationships**

First of all, IFN-alpha 2 and IFN-gamma levels were not correlated, \(P = 0.67\), Spearman’s rank correlation.

IMTG score were negatively predicted by IFN-alpha 2 levels at robust regression and ordered probit regression, Coeff. = −0.0149424 Std. err. = 0.0033541, \(t = -4.29\), \(P = 0.0001\), Conf. interval = −0.0220211–0.0078638 and Coeff. = −0.0312922, Std. err. = 0.0090407, \(Z = -3.46\),
### Table 1 Data of the studied patients

| Measure                               | Value                                                                 |
|---------------------------------------|-----------------------------------------------------------------------|
| Age (years)                           | 46 (34–53)                                                            |
| Gender                                | Males/females (n)                                                     |
|                                       | 36/44                                                                 |
| BMI                                   | 42 (38–47)                                                            |
| WC (Males) cm                         | 126 (121–135)                                                         |
| WC (Females) cm                       | 119 (110–128)                                                         |
| WHR males                             | 0.98 (0.96–1.05)                                                      |
| WHR females                           | 0.95 (0.93–0.97)                                                      |
| VAT                                   | Viseral adipose tissue                                                |
| SAT                                   | Subcutaneous adipose tissue                                           |
| US                                     | Ultrasound                                                            |
| WHR females                           | Grade I/II/III (n)                                                    |
|                                       | 8/26/46                                                               |
| HOMA                                  | 2.78 (1.85–4.18)                                                      |
| HOMA-B %                              | 37.11 (22.4–49.8)                                                     |
| QUICKI                                | 0.32 (0.31–0.35)                                                      |
| Insulin (μU/mL)                       | 11 (7.1–15.8)                                                         |
| HDL (males) (mg/dL)                   | 42.7 ± 8.98                                                           |
| HDL (females) (mg/dL)                 | 49 ± 13                                                               |
| Triglycerides (mg/dl)                 | 123.5 (83.5–188)                                                      |
| ALT (U/L)                             | 28 (21.5–39)                                                          |
| Gamma-GT (U/L)                        | 25 (16.5–42.5)                                                        |
| hsCRP (mg/mL)                         | 0.56 (0.27–1.3)                                                       |
| Ferritin (males) (ng/mL)              | 167.5 (85–234.5)                                                      |
| Ferritin (females) (ng/mL)            | 41.5 (20–69)                                                          |
| Fibrinogen (g/L)                      | 306 ± 74.7                                                            |
| Cholesterol                           | 190 ± 36.1                                                            |
| Fat mass %                            | 52 ± 7.5                                                              |
| FFM%                                  | 56.2 (41–67)                                                          |
| RMR/FFM/kg                            | 38.7 (33.9–43)                                                        |
| HS at US                               | Grade 1/2/3 (n)                                                       |
|                                       | 22/50/8                                                               |
| VAT at US (cm)                        | 7.5 (6–9.4)                                                           |
| SAT at US (cm)                        | 2.6 (2.1–3.1)                                                         |
| IMTG score                            | 2.25 (2, 3)                                                           |
| MS (APT III) yes/not (n)              | 51/29                                                                 |
| MS (IDF) yes/not (n)                  | 51/29                                                                 |
| IFN-alpha 2 pg^a                      | 121.9 (103.5–135.8)                                                  |
| IFN-gamma (pg/mL)^a                   | 158 (56–390)                                                          |

*IMTG intramuscular triglycerides, VAT visceral adipose tissue, SAT subcutaneous adipose tissue, US ultrasound, WHR Waist-To-Hip Ratio, WC waist circumference, RMR resting metabolic rate, FFM fat-free mass, MS metabolic syndrome, HS hepatic steatosis, n number of patients

* 78 patients were examined for IFNs. The mean plus/minus SD of IFN-alpha 2a levels of the obese was 120.1 ± 24.6

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**Fig. 1** Age-related reference intervals of IFN-a2 in healthy subjects
The residual-versus-fitted plot, Fig. 4, shows that fitted values do not have an obvious trend of failure. Conclusively, there is no problems of heteroskedasticity as residuals appear to have the same variance everywhere.

When adjusting for gender and age the prediction of IFN-a2 on IMTG overlapped the previous ones obtained by two methods (robust regression and ordered profit regression), i.e., Coeff. = −0.0149346, Std. err. = 0.0037878, t = −3.94, P = 0.000, Conf. interval. = −0.0224818 to −0.0073873.

IMTG grades were not predicted by IFN-gamma levels, analysing them both by LAD regression and order profit regression, i.e., Coeff. = 0.0000239, Std. Err = 0.0003695, t = 0.06, P = 0.94995%, Conf. interval. = −0.0007118 to −0.0007596 and Coeff. = 0.0000152, Std. err. = 0.0004215, z = 0.04, P > |z| = 0.971, Conf. interval. = −0.0008109 to −0.0008412, respectively.

Table 5 shows results of the simultaneous quantile regression (bootstrap method), highlighting that IMTG scores are predicted exclusively by intermediate and upper quantiles of IFN-alpha 2 levels.

Although IFN-alpha 2 was playing per se a significant role in predicting fibrinogen in the mediation method, its role was completely excluded as evident in Table 6.

The ordered probit regression showed that IMTG was predicted by HS at US as well as HS was predicted by VAT, Table 7 and Fig. 5.

To clarify this important aspect, instrumental-variables regression excluded the role of the confounding variable (VAT) as mediator between IMTG and HS, as evident in Table 8.

### Table 2 Correlation between IMTG and gender

| Gender | IMTG scale (scores) | Total |
|--------|---------------------|-------|
|        | I  | II | III | II |    |
| Females | 7  | 14 | 16  | 7  | 44 |
| Males   | 3  | 10 | 14  | 9  | 36 |

Two-way cross-tabulation, Pearson Chi square, P = 0.6; total: number of patients

### Table 3 Correlation between obesity severity and hepatic steatosis grade

| HS at US grade | Obesity degrees | Total |
|----------------|-----------------|-------|
|                | 1   | 2   | 3   |    |
| 1              | 5   | 9   | 8   | 22 |
| 2              | 3   | 17  | 30  | 50 |
| 3              | 0   | 0   | 8   | 8  |

Two-way cross-tabulation, Pearson chi square = 12.5536, P = 0.014; total: number of patients

P > |z| = 0.001, Conf. interval = −0.0490116 to −0.0135729, respectively, Fig. 3.

The residual-versus-fitted plot, Fig. 4, shows that fitted values do not have an obvious trend of failure. Conclusively, there is no problems of heteroskedasticity as residuals appear to have the same variance everywhere.
Table 4  Correlation between obesity severity and IMTG

| IMTG score | Obesity degrees | Total |
|------------|-----------------|-------|
|            | 1               | 2     | 3      |     |
| I          | 0               | 4     | 6      | 10   |
| II         | 3               | 10    | 11     | 24   |
| III        | 4               | 9     | 17     | 30   |
| III        | 1               | 3     | 12     | 16   |

Prevalence of moderate/severe grade of IMTG, i.e. 54 out of 80 patients = 67.5%; Two-way cross-tabulation, Pearson square = 4.9252, P = 0.553
Total: number of patients. Hepatic steatosis (HS) at UltraSonography (US)

At multivariate analysis, among VAT, SAT, WC, WHR, BMI only VAT predicted IMTG, i.e., Coeff. = 0.1384446, Std. err. = 0.0582611, t = 2.38; P > |t| = 0.020, 95% [Conf. interval] 0.0223568–0.2545323.

No prediction of IMTG by HOMA, HOMA-B % and QUICKI (P = 0.56, 0.15 and 0.71, respectively).

The finding concerning no link between IR and IMTG was confirmed by a more powerful tool evaluating whether IMTG might be predicted by HOMA, i.e., Coeff. = 0.0219949; Std. err. = 0.0367399 z = 0.60; P > |z| > 0.549; Conf. interval = −0.050014 to 0.0940038; ordered profit regression, robust method.

There was no prediction of IMTG by fat mass, FFM and RMR/FFM ratio (P = 0.550, 0.232 and 0.069, respectively, evaluated as ordered profit regression).

By the same technique, HS at US was not predicted by IFN-alpha levels (P = 0.079).

As expected, at univariate analysis HOMA-B % was predicted by HOMA: Coeff. 8.200134, Std. err. = 1.072445, t = 7.65, P > |t| = 0.000, 95% Conf. interval = 6.06506–10.33521.

Studying the hidden relationships between various variables comprehending anthropometric measures, fat and free-fat masses, US features of central, peripheral fat and intramuscular distribution, it is confirmed the link between IFN-a2 and IMTG (as evident in factor 3) as well as other parameters, Table 9.

Bayesian inference computing the posterior probability to appreciate the good level of confidence of the main linear regression results, i.e., IMTG/IFN-a2, was shown in Table 10.

The intra/inter-observational variability of UltraSound estimations was not significant, the mean difference being 1.9, 2.9, 2.4 and 3.1%, and 2.3, 3.1, 3.9 and 3.1% for the HS, IMTG, SAT, and VAT, respectively, with a ρc of 0.91.

Finally, the study turned out to be sufficiently powered (alpha = 0.01, power = 0.99) considering the sample size.
Fig. 4 Regression diagnostics, i.e., residuals plotted against the fitted values

Table 5 Quantile regression for predicting IMTG by IFN-alpha

| IMTG  | Coeff.  | Std. err. | t     | P    | 95% CI             |
|-------|---------|-----------|-------|------|--------------------|
| q25   | IFN-alpha 2 | 0         | 0.0036305 | 0.00  | 1.000 | −0.0072308 to 0.0072308 |
| q50   | IFN-alpha 2 | −0.0174917 | 0.004081  | −2.58 | 0.08  | −0.0302554 to 0.0047389 |
| q75   | IFN-alpha 2 | −0.0181554 | 0.0045649 | −3.98 | 0.000 | −0.0272471 to 0.0090637 |

Simultaneous Quantile regression Bootstrap SE (200 replications). The prediction of IMTG by IFN-alpha 2 levels is confined to their intermediate and upper quantiles CI confidence interval

Table 6 Mediation methods for predicting variables

|                   | Coeff.  | Std. err. | t     | P    | [95% Conf. interval] |
|-------------------|---------|-----------|-------|------|---------------------|
| At Univariate analysis (Robust regression) |         |           |       |      |                     |
| IMTG/IFN-alpha 2  | −0.0151912 | 0.0043683 | −3.48 | 0.001| −0.0238932 to −0.0064892 |
| Fibrinogen/IFN-alpha 2 | −0.1085108 | 0.0262153 | −4.14 | 0.000| −0.1607232 to −0.0562985 |
| IMTG/Fibrinogen    | 0.001491  | 0.0014679 | 1.02  | 0.313| −0.0014314 to 0.0044134 |
| At MULTIPLE regression |         |           |       |      |                     |
| IMTG/Fibrinogen    | −0.0002527 | 0.0015718 | −0.16 | 0.873| −0.0033839 to −0.0028785 |
| IMTG/IFN-alpha 2   | −0.0151912 | 0.0038637 | −3.93 | 0.000| −0.022888 to −0.0074944 |
| Beta of IFN-alpha 2 | 0.39, beta of Fibrinogen = −0.019 |            |       |      |                     |

The mediation effect of fibrinogen was excluded
The first variable the two showed is the dependent one
of the two studied populations on the basis of different means of IFNs. Furthermore, the estimated sample size for a linear regression slope test between IFNA levels and IMTG scores was inferior to that of our obese group.

**Discussion**

This study was designed to investigate any correlation of IFN-alpha 2a and IFN-gamma to IMTG in obese patients with US-detected HS or NAFLD.

Stating the major findings of our study, we should drawn attention on: (i) serum concentrations of IFN-alpha 2 were increased, while serum levels of IFN-gamma were decreased confronted with those of controls; (ii) the severity of IMTG, revealed at US as Heckmatt scores (I–IV) was negatively predicted by IFN-alpha 2 serum concentrations; (iii) IMTG scores were not predicted by serum levels of IFN-gamma; (iv) IMTG scores were predicted by HS severity ascertained by a US scale (grades 1–3); (v) HS severity was predicted by VAT but the latter was not instrumental to IMTG.

To try to explain the possible mechanisms of the core finding, i.e., the inverse association between IFN-alpha 2 and IMTG we can focus on serum lipid profile creating sort of parallelism between other situations/diseases in which increased concentrations of this cytokine were found and our obese patients.

Previous experiments in vitro showed that IFN-alpha inhibits lipoprotein lipase (LPL) activity directly or indirectly by inducing specific cytokines [30, 31]. In addition, increase in lipogenesis and VLDL secretion in the liver by IFN-alpha may contribute to hypertriglyceridemic subjects, as evident in cultured hepatocytes [32–34] and in patients on IFN-alpha therapy [35].

In contrast with aforementioned studies, other reports indicate that the elevated IMTG content found in obese women is not due to an up-regulation of key lipogenic proteins or to the suppression of lipolytic proteins [36].

| d.v.: IMTG scores | Coeff.  | Std. err. | z     | P > |z| [95% Conf. interval] |
|------------------|---------|-----------|-------|-----|---------------------|
| i.v.: HS grade   | 0.6427975 | 0.215061  | 2.99  | 0.003 | 0.2212858 to 1.064309 |

| d.v.: HS grade   | Coeff.  | Std. err. | z     | P > |z| [95% Conf. interval] |
|------------------|---------|-----------|-------|-----|---------------------|
| i.v.: VAT        | 0.8153024 | 0.1584942 | 5.14  | 0.000 | 0.5046595 to 1.125945 |

In these two regressions there is a suspicion of a confounding variable or covariate that is VAT
d.v.: dependent variable, i.v. independent variable

![Fig. 5 Prediction of IMTG scores by HS at US grades](image-url)
Table 8 Instrumental-variables regression for panel data (Baltagi–Chang estimator)

xtivreg IMTG HS at US (HS at US = VAT), re nosa (robust)
G2SLS random-effects IV regression
Group variable: WC
R-sq
Within = 0.1970
Between = 0.03012
Overall = 0.1028

Wald χ² (1) = 11.09
Corr (u_i, X) = 0 (assumed)

| d. v. | Coef. | Rob. Std. err. | z    | P > |z| | [95% Conf. interval] |
|-------|-------|----------------|------|-----|---|----------------------|
| IMTG  |       |                |      |      |   |                      |
| i.v.  |       |                |      |      |   |                      |
| HS at US | 0.5199847 | 0.156167 | 3.33 | 0.001 | 0.2139031 to −0.8260663 |
| HS at US (omitted) | 0 | | | | |
| _cons | 1.719254 | 0.270595 | 6.35 | 0.000 | 1.188903 to 0.249616 |
| Sigma_u | 0.48060539 | | | | |
| Sigma_e | 0.74235381 | | | | |
| rho | 0.2953461 (fraction of variance due to u_i) | | | | |

The instrument (VAT) cannot be correlated with the dependent (IMTG) in the explanatory equation. In other words, the instrument cannot suffer from the same problem as the original predicting variable (IMTG predicted by HS at US). If this condition is met, then the instrument is said to satisfy the exclusion restriction. As grouping variable was chosen an index of visceral adiposity, i.e., WC

IMTG intramuscular triglycerides, HS at US hepatic steatosis at Ultrasonography, VAT visceral adipose tissue, d.v. dependent variable, i.v. independent variable

Table 9 Factor analysis

| Variable | Factor 1 | Factor 2 | Factor 3 | Uniqueness |
|----------|----------|----------|----------|------------|
| Rotated factor loadings (pattern matrix) and unique variances | | | | |
| BMI | 0.8803 | 0.2118 | −0.0337 | 0.1791 |
| Obesity grade | 0.7365 | 0.3327 | 0.0669 | 0.3424 |
| WC | 0.8641 | −0.1363 | 0.0146 | 0.2345 |
| WHR | −0.1507 | −0.3176 | 0.5373 | 0.5878 |
| Fat mass% | −0.1197 | −0.9089 | 0.0098 | 0.1594 |
| IMTG | 0.2645 | −0.1249 | 0.6626 | 0.4753 |
| SAT | 0.4182 | 0.4665 | 0.0325 | 0.6064 |
| VAT | 0.7446 | −0.2465 | 0.2437 | 0.3255 |
| IFN-alpha 2 | −0.0214 | −0.1072 | −0.8050 | 0.3400 |
| RMR/FFM/kg | −0.1561 | 0.7631 | −0.0293 | 0.3925 |
| IFN-gamma | 0.1082 | 0.1913 | 0.3257 | 0.8457 |

| Factor rotation matrix | Factor 1 | Factor 2 | Factor 3 |
|------------------------|----------|----------|----------|
| Factor 1 | 0.9414 | 0.2736 | 0.1971 |
| Factor 2 | 0.1674 | −0.8865 | 0.4313 |
| Factor 3 | 0.2928 | −0.3731 | −0.8804 |

The extracted components explain nearly 60 (59.19)% of variability

The critical value was calculated by doubling Pearson's correlation coefficient for 1% level of significance (5.152)/square root of patients minus 2 (78), i.e., 0.583. In italics it will be shown the main components for any single factor, with a value superior to the critical one. HS was excluded due to collinearity with IMTG an VAT. The link between IFN-alpha 2 and IMTG (factor 3) as well as significative parameters in factors one and two are shown in italics text
Although IMTG synthesis rates were previously related to insulin sensitivity [2], we authors did not find a link between IMTG and IR. Indeed, we did not evaluate IR by glucose clamp technique but surrogate markers, even though quite reliable [37].

Relating our findings to those of available studies, we emphasise that in a research on HIV-1-infected men, a significant positive correlation was found between accumulation of IFN-alpha and increased levels of cholesterol, TG, VLDL cholesterol, VLDL TG, ApoB and ApoB-ApoA1 ratio [11], raising suspicion of active lipogenesis. Nevertheless, we should stress that the study of Teran-Cabanillas et al. showed that obese subjects have a decreased ability to produce IFN-alpha in response to TLR ligands [13].

Concerning the levels of IFN-gamma in our population we recognise that a comparison with results by Schmidt et al. [10], who found that in obese subjects physical activity may lower basal high levels of IFN-gamma, is not possible due to the lack of recognition of physical activity in our population. On the other hand, looking at the levels of IFNs in our obese and control arms, they overlap with those expressed into literature [38].

The association between IMTG scores and HS at US grades could hypothetically explained on the ground of common mechanisms leading to ectopic fat storage and utilisation. Indeed, the reason for which there is a lack of link between IMTG and VAT remains a point to be further clarified: this no association is in agreement with the absence of correlation between IMTG scores and IR and in contrast with the apparent but not confirmed, when adjusted for VAT presence, association between IMTG and HS at US. Further study is needed, at the light that NAFLD is considered a CV risk factor [39] and could lead to hepatocarcinoma.

The importance of relation between IFNs and VAT is italics by a recent work stressing that type 1 IFN signature gene expression in VAT correlates with both adipose tissue and systemic IR in obese individuals [40].

Up-to-date results in mice provide genetic evidence that plasmacytoid dendritic cells via type I IFNs, regulate energy metabolism and promote the development of obesity [41]. A possible mechanism explaining the latter event could be the signalling pathway activated by type I IFNs [42].

Although fatty acids induced type I IFN responses in murine hepatocytes/macrophages and exposure to a high-fat diet elicited type I IFN-regulated gene expression in the liver of wild-type mice, modulating susceptibility to metabolic or hepatic disease [43], we were not able to confirm this important link of IFN-alpha 2 with HS in our population.

Among limitations to study, we firstly acknowledge that ours was an observational study in which a clear relation of cause and effect is not possible to find; secondly the hypothetical mechanisms are far to be elucidated, being mechanistic studies not carried out; thirdly not having evaluated the physical activity with appropriate tests, even though there no univocal concordance on which one should be performed, because lifestyle strategies differentially affect IMTG accumulation [44].

As final remark, considering that IFN-alpha alters the human intestinal mucosa immune homeostasis [45] and looking at the main role of gut microbiome in obesity, our observation deserve controlled research in order to confirm these preliminary data.

**Conclusion**

The complex interplay between cytokines and ectopic fat excess is hopefully enriched by the observation that in obese patients with NAFLD the serum levels of IFN-alpha 2 are inversely related to IMTG scores, differently
from IFN-gamma levels that are not associated with severity of this ectopic storage.

Authors’ contributions
GT planned the study, analysed data and drafted manuscript. SC, FC and AS performed the immunological laboratory data. VC and FC gathered clinical data. DC contributed to drafting the manuscript and revised it critically as well other authors. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
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Consent for publication
It is specified above.

Ethics approval and consent to participate
Ethics approval and consent has been waived to to the fact that was a sub-study, which used the same original patient sample contained in a previous research (Tarantino G, Costantini S, Finelli C, Capone F, Guarriero E, La Sala N, Gioia S, Castello G. Carotid intima-media thickness is predicted by combined eotaxin levels and severity of hepatic steatosis at Ultrasonography in Obese Patients with Nonalcoholic Fatty Liver Disease. PLoS ONE. 2014;9:e105610, but with completely different analytical approaches resulting to be equally valid, according to The International Committee of Medical Journal Editors (ICMJE) at http://www.icmje.org/recommendations/browse/publishing-and-edito
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