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FTO rs9939609 influence on adipose tissue localization in the Italian population

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Abstract. – OBJECTIVE: Among the genes involved in obesity, the Fat mass and obesity-associated gene (FTO) is certainly one of the most known and the relation between FTO rs9939609 and BMI is highly discussed; nevertheless, data about its influence on body composition are limited.

MATERIALS AND METHODS: We carried out a study on a sample of 1066 Italian subjects, whose body composition and FTO rs9939609 were analyzed.

RESULTS: We found significant relations between FTO with arm (*p*=0.01), abdomen (*p*=0.00), and trunk circumferences (*p*=0.00), BMI (*p*=0.01), FM% (p=0.00), and android FM% (*p*=0.01), whereas no relations were found between FTO and both gynoid fat and lean mass.

CONCLUSIONS: To conclude, the relation between FTO and BMI is confirmed and is related specifically with android FM%. These results indicated that FTO rs9939609 may be a genetic etiological factor for obesity. Indeed, the specificity for the android FM% would indicate FTO as an etiological factor in the development of cardiovascular diseases.

Key Words:
Obesity, Fat Mass, FTO, SNPs.

Abbreviations

Appendicular Skeletal Muscle Index (ASSMI), Bioelectrical Impedance Analysis (BIA), Body Cell Mass (BCM), Body Cell Mass Index (BCMI), Body Lean Mass (BLean), Body Mass Index (BMI), Bone Mineral Content (BMC), Bone Mineral Density (BMD), Cardiovascular Diseases (CDVs), Dual-energy X-ray absorptiometry (DXA), Extracellular Water (ECW), Fat Mass Percentage (FM%), Fat Mass and Obesity-associated gene (FTO), Generalized Linear Models (GLM), Hardy-Weinberg equilibrium (HWE), Hydration (HYDR), Intracellular Water (ICW), Intra-Muscular Adipose Tissue (IMAT), Logistic regression models (LRM), Normal-weight (NW), Normal-weight Obese (NWO), Obese (Ob), Odds ratios (OR), Phase Angle (PA), Pre obese (PreOb), Reactance (Xc), Resistance (R), Single Nucleotide Polymorphism (SNP), Sodium-Potassium exchange (NA/K), Toscans in Italy (TSI), Total Body Bone (TB-Bone), Total Body Lean Mass (TBLean), Total Body Water (TBW), Underweight (UW), Visceral Adipose Tissue (VAT), Waist Hip Ratio (WHR), World Health Organization (WHO).

Introduction

According to the epidemiological analysis of the World Health Organization (WHO), obesity is one of the most widespread healthcare problems in the world. In 2016, more than 1.9 billion adults were considered overweight and, of these, over 650 million were obese. Both overweight and obesity are defined as anomalous or undue fat accumulation that may lead to a worsening of the health condition[1], which can be diagnosed through the calculation of Body Mass Index (BMI). According to the WHO[2], a subject is considered obese if BMI≥30 kg/m^2, overweight with a BMI between 25 kg/m^2 and 29.99 kg/m^2, and normal weight if 18.5 kg/m^2≤BMI<25 kg/m^2. Nevertheless, it is also true that BMI has a limited diagnostic accuracy to diagnose obesity. In fact, normal weight subjects can be considered obese if their fat mass is higher than 30% for females

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and 25% for males[3]. As regards the main causes of the condition of obesity, in the Western world, incorrect dietetic habits are the most common cause of obesity, but also psychological problems, such as food addiction, the expanding market of junk foods, and a sedentary lifestyle, are increasing the incidence of this medical condition[4]. Moreover, the higher percentage of body fat present in obese patients is an important risk factor for other pathologies, such as cardiovascular diseases, type-2 diabetes, metabolic syndrome, cancer, depression, and pulmonary complications[5]. Moreover, obesity is a preventable medical condition and it is important to highlight that predictive and preventive medicine might be fundamental to reduce its incidence. Dietetic therapies, nutritional education, and psychological support are in the frontline for the fight against obesity; nevertheless, it is important to remember that genetics also play a fundamental role in the development of body composition[6]. Among the genes involved in obesity, Fat mass and obesity-associated gene (FTO) is certainly one of the most known. This gene, located on chromosome 16 (16q12.2), encodes for the enzyme alpha-ketoglutarate-dependent dioxygenase, and it is widely expressed in all the adult tissues[7]. This enzyme has several functions; indeed, it regulates both the control of adipocyte differentiation and their thermogenesis, contributing sensibly to the body fat accumulation[8]. Additionally, it seems to contribute to the regulation of both metabolic rate and energy homeostasis[9]. The FTO rs9939609 variant, located in the first intron of the gene, is associated with BMI in different ethnicities[10]. Specifically, the allele A of FTO rs9939609 is related both to a higher body mass index and higher risk of developing Type-2 diabetes compared to the allele T[11]. Nevertheless, BMI alone cannot define obesity[3]. So, the relations between Single Nucleotide Polymorphism (SNP) and fat mass have to be done. To date, only few studies are complete. The allele A seems to be associated both with a higher amount of both fat and lean mass[12], suggesting that the activities of this SNP are not only limited to the fat mass, but to the entirety of body mass. Finally, SNP seems to play a role in the development of metabolic syndrome[13], since it also seems to influence cholesterol and triglyceride levels in the blood[14] and it may influence food intake and food choice[15]. Unfortunately, Italian population is poorly studied for FTO rs9939609. In particular, a study[16] of subjects from the island of Sardinia has shown a significant relation between BMI and FTO rs9939609, and, even if the Sardinian population may have peculiar genetic features, different from the rest of Italians[17], the same results were demonstrated in another study[18] on the general Italian population. Moreover, there are significant data for the metabolic syndrome[19] but not for body composition in Italian population.

The main objective of this study was to investigate the possible relation between FTO rs9939609 variant and body composition, which was evaluated through a full range of methods like anthropometry, Dual-energy X-ray absorptiometry (DXA), and bioelectrical impedance analysis (BIA), in a large Italian population sample. The secondary aim of the study was to confirm the association of the SNP with obesity, using a more specific obesity classification based not only on BMI, but also on the fat mass percentage (FM%).

Trial Registration: ClinicalTrials.gov Id: NCT01890070. Registered 01 July 2013, https://clinicaltrials.gov/ct2/show/NCT01890070.

Materials and Methods

Study Design and Subjects

The study was conducted at the Section of Clinical Nutrition and Nutrigenomics, Department of Biomedicine and Prevention of the University of Rome Tor Vergata. The initial sample was composed of 1095 subjects, who were recruited within routine medical checkups. To be eligible, each individual had to belong to the Caucasian race, to be Italian, and be older than 16 years old. Exclusion criteria included pregnancy, breast-feeding, active smoking, arterial hypertension (≥140/90 mmHg), acute or chronic diseases, intestinal disorders, neoplastic disease, cardiovascular diseases, type 1 diabetes, hepatitis C and B virus, HIV.

Furthermore, for each subject, a medical assessment was evaluated before the application of any nutritional treatments. For statistical purposes, we have pooled the sample into several groups.

In the first instance, we classified our population regarding FM%, where male subjects with a FM%<25% and female subjects with FM%<30% were considered normal weight, otherwise they were considered pre-obese/obese. The same classification was used to define an excess of FM% for gynoid and android areas. Then, we classified our population on phenotypes according to BMI and FM% as follow: underweight (UW) (BMI<18.50 kg/m^2); normal-weight (NW) (18.50 kg/m^2≤BMI<25 kg/m^2 or BMI≥25 kg/m^2 but FM% lower

than 30% for women and 25% for men); normal weight obese (NWO) (18.50 kg/m^2≤BMI<25 kg/m^2 and FM% higher than 30% for women and 25% for men); PreObese (PreOb) (25 kg/m^2≤BMI<30 kg/m^2 and FM% higher than 30% for women and 25% for men); obesity I (30 kg/m^2 ≤BMI<35 kg/m^2); obesity II (35 kg/m^2 ≤BMI<40 kg/m^2); obesity III (BMI≥40 kg/m^2)[3].

Phase angle (PA) was used to classify malnutrition risk. Individuals under 30 years old with PA values of 6-8 for males and 6-7 for females, individuals between 30 and 50 years old with values of 5.5-6 for males and 5-6 for females, and individuals older than 50 years old with values of 5-5.6 for males and 4.8-5.3 for females, were considered healthy. In order to define muscle mass status in our population, we evaluated both the Appendicular Skeletal Muscle Mass Index (ASMMI) and Body Cell Mass Index (BCMI). Subjects with low muscle mass, who were then considered unhealthy, were distinct by ASMMI values <7.26 for men and <5.53 for women or BCMI lower than 10 for men and 7.5 for women. Thus, to estimate the Intramuscular Adipose Tissue (IMAT), considered as a potential contributor to declining strength and muscle quality[20], all the subjects with a value higher than 0.5 were considered unhealthy. Finally, to define bone health status, we used T-score total body for the following classification: osteoporotic (T-score≤-2.5), osteopenic (-2.5< T-score <1), and normal (T-score≥1). All participants enrolled in the study approved their participation studying and signing the informed consent, carried out in accordance with the Helsinki Declaration of 1975 as revised in 1983. The study protocol was approved by the Ethical Committee of the Calabria Region Center Area Section (Register Protocol No. 146 17/05/2018).

Trial registration: this protocol has been registered with ClinicalTrials.gov Id: NCT01890070.

Body Composition Analysis

A flexible steel metric tape to the nearest 0.5 cm was used to measure waist, hip, neck, and abdomen circumferences, according standard protocol, as reported in De Lorenzo et al[21,22]. Height was evaluated to the nearest 0.1 cm using a stadiometer (Invernizzi, Rome, Italy) whereas BMI was estimated using the formula BMI=body weight/height^2 (kg/m^2). Body composition analysis was carried out using both bioelectrical impedance analysis (BIA 101S, Akern/RJL Systems, Pontassieve, Florence, Italy) and dual-energy X-ray absorptiometry (DXA; I-DXA, GE Medical Systems, Milwaukee, WI, USA). Bioelectrical impedance analysis was carried out to evaluate resistance (R), reactance (Xc), phase angle (PA), hydration, exchange Na/K, total body water (TBW), extracellular water (ECW), intracellular water (ICW), body cell mass (BCM), body cell mass index (BCMI). DXA was performed to assess FM%, android FM%, gynoid FM%, arm, leg, trunk of body fat (percentage and kg), and lean mass (kg). Moreover, also bone mineral density (BMD) and bone mineral content (BMC) of head, arm, leg, trunk, ribs, pelvis, spine, total body were evaluated along with total Z-score and T-score. FM% was calculated as FM (kg) divided by the total mass of all tissues, including the total body lean (TBLean) total body bone (TBBone), as the following: FM%=[FM/(FM + TBLean + TBBone)] x 100[23]. Furthermore, the Appendicular Skeletal Muscle Mass Index (ASMMI) was evaluated using the following formula: Legs Muscle Mass (kg)+Arms Muscle Mass (kg)/Height (m^2) (Men<7.59 kg/m^2, Women<5.47 kg/m^2)[24], whereas the IMAT was calculated according to Bauer et al[25] with the following formulas: Log(IMAT)=[-2.21+(0.12×fat)+(-0.0013×fat^2)] for women and Log(IMAT)=[-2.05+(0.12×fat)+(-0.0013×fat^2)] for men.

DNA Isolation and qRT-PCR Analysis

Genomic DNA was extracted from saliva swabs using the phenol-chloroform extraction described by Shrey et al[26]. A master mix containing Taq DNA Polymerase and dNTPs (TaqPath ProAmp Master mix Life Technologies, Carlsbad, CA, USA) and a two allele-specific fluorescent probe containing a PCR primer pair (TaqMan SNP Genotyping Assays, Life Technologies, Carlsbad, CA, USA) were used to prepare the gDNA for the genotyping. The FTO rs9939609 variant allele (homozygous=AA and heterozygous=AT) context sequence was the following: GGTTCCTTGCGACTGCTGTGAATTT [A/T] GTGATGCACTTGGATAGTCTCTGTT. Then, SNP genotyping assessment was performed using a Real Time-PCR analysis (Applied Biosystems StepOnePLus Real-Time PCR, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. To normalize fluorescence fluctuations not related to the amplification reaction, an internal reference fluorophore (ROX), also called "passive", present in the reaction buffer is used. The following equation was calculated: ΔRn=(Rn +)-(Rn-) where: the first (Rn +) corresponds to the value of the reaction which

includes the template, while the second (Rn-) corresponds to the value of the sample that does not react, that is to the mixture without the template. The values of ΔRn reflect the quantity of degraded fluorescent probe and increase exponentially during the reaction.

Statistical Analysis

The Hardy-Weinberg equilibrium (HWE) was assessed for FTO rs9939609 using the SNP-HWE program and tested using the χ^2 analysis[27]. To analyze the sample, the subjects were divided into carrier/non-carrier (carrier for A allele *vs.* homozygous T). Power calculations for obesity for total FM% association were performed using QUANTO (USC Biostatics, Los Angeles, CA, USA) based on sample size[28]. A normality test was conducted in order to determine parametric and nonparametric data. Linear Generalized Linear Models (GLM) were performed on parametric values, whereas GLM gamma log distribution was used to analyze positive nonparametric values. These two analyses were carried out to analyze BMI, waist, hip, arm, and abdomen circumferences, FM%, android, gynoid, arm, leg, trunk of both fat and lean mass, and BMD of head, arm, leg, trunk, ribs, pelvis, spine, and total body. Logistic regression models (LRM), such as linear or multinomial, were performed on BMI, waist circumference, FM%, android and gynoid FM%, PA, BCMI, ASMMI, IMAT, and T-score as categorical variables. Odds ratios (OR) were reported with the related confidence interval of 95% (min-max). All values were adjusted by gender and age, significance was set as $p<0.05$ and the statistical analysis was performed using IBM Statistical Product and Service Solution (SPSS) 21.0 for Windows (IBM Corp., Armonk, NY, USA).

Results

Population Characteristics

Of the 1095 subjects enrolled, 29 were excluded because they did not respect the eligibility criteria. Finally, 1066 subjects were considered for this study. In our sample the HWE was respected ($p>0.05$) and the power of our study was 0.99, with fixed $\alpha=0.05$ and 2-sided. The overall description of the total sample population at baseline can be seen in Table I.

According to exclusion criteria, all subjects were healthy and had no evidence of chronic disease.

The description of the sample population divided for TT genotype and A carriers at baseline can be seen in Table II.

Genotype frequencies shown in TSI population (TT: 0.327; AA: 0.252; AT: 0.421) are similar to the ones of our subjects (TT: 0.337; AA: 0.200; AT: 0.463), as well as the allele frequencies for TSI (T: 0.537; A: 0.463) and for our sample (T: 0.568; A: 0.438) (Table I). The average age of subjects was 43.36 ± 15.63 years, 68.9% female, and 31.1% male (Table I). According to BMI, we obtained that A carriers frequency in the three grades of obesity were higher than the TT genotype (A carriers: obesity I=18.95%, obesity II=10.61%, obesity III=11.88%; TT genotype: obesity I=13.65%, obesity II= 7.52%, obesity III=10.03%), as well as for the obese population according to FM% (A carriers=86.44%; TT genotype=80.40%). Android FM% followed the same pattern (A carriers=85.92%; TT genotype=80.73%). Conversely, gynoid FM% of obese population was similar between FTO groups (A carriers=93.76%; TT genotype=92.66%). Unhealthy and healthy groups are divided according BCMI, ASMMI, IMAT, and T-score (both osteopenic and osteoporotic subjects) and had a similar distribution among A Carrier and TT genotype populations (Table III).

Relationship Between FTO rs9939609 and Bone Tissue

In our population, A carriers present higher BMD than TT genotype ($p=0.00$; $\beta=-0.98$; $\Delta\%=1.12$), in total body analysis and in particular BMD areas, like the head ($p=0.00$; $\beta=-0.52$; $\Delta\%=0.65\%$), leg ($p=0.00$; $\beta=-7.15$; $\Delta\%= 0.71\%$) and lower pelvis BMD ($p=0.00$; $\beta=-1.58$; $\Delta\%=-0.46\%$) (Table IV).

However, no differences were highlighted for T-score and Z-score among A carriers and TT genotype ($p\geq0.05$). Furthermore, compared to normal subjects, LBM analysis on total T-score did not show any statistical significance in osteopenic and osteoporotic categories among the FTO groups ($p\geq0.05$).

Relationship Among FTO rs9939609, BMI and Body Composition

In this study, the GLM analysis underlined the statistical significance between FTO rs9939609 carriers A and TT genotype. In our population, carriers A present higher BMI than TT genotype ($p=0.01$; $\beta=0.45$; $\Delta\%=5.29\%$). Furthermore, the LRM analysis, adjusted for age and gender, showed carriers of the A allele associated with pre-obese/obese subjects (BMI≥25) ($p=0.01$; OR=1.42 (1.07-1.89).

Table I. Descriptive characteristics of study population.

Gender	Male (No.=332; 31.1%)	Female (No.=734; 68.9%)
Allele frequencies	A allele 0.438	T allele 0.568
	Median	Minimum; Maximum
Age (years)	43.36	16.00; 86.00
Height (cm)	163.55	137.00; 196.50
Weight (kg)	75.80	33.60; 185.60
BMI (kg/m^2)	27.31	13.56; 63.70
Neck circumference (cm)	38.00	29.00; 100.00
Waist circumference (cm)	87.30	40.00; 161.00
Abdomen circumference (cm)	98.25	56.00; 156.00
Hip circumference (cm)	105.00	58.50; 160.00
WHR	0.83	0.50; 1.4
Ra	517.00	226.00; 851.00
Xc	55.00	23.00; 111.00
PA	6.10	3.00; 26.00
BCM (kg)	26.70	15.00; 67.00
HYDR	73.25	65.00; 87.00
Na/K	0.90	0.30; 2.00
TBW (L)	36.90	25.00; 89.00
ECW (L)	16.89	6.00; 40.00
ICW (L)	20.10	11.00; 49.00
BCMI	10.50	5.00; 55.00
BMD Head (g/cm^2)	2.25	1.00; 3.71
BMD Arm (g/cm^2)	0.80	0.48; 1.80
BMD Leg (g/cm^2)	1.20	0.78; 1.95
BMD Trunk (g/cm^2)	0.98	0.61; 1.43
BMD Ribs (g/cm^2)	0.79	0.32; 1.25
BMD Pelvis (g/cm^2)	1.08	0.59;1.84
BMD Spine (g/cm^2)	1.13	0.68; 1.70
BMD Tot (g/cm^2)	1.17	0.80; 1.65
Total T-score	0.40	-4.00; 6.00
Total Z-score	0.40	-3.00; 9.00
BMC Head (g)	505.00	84.00; 791.00
BMC Arm (g)	292	141.00; 931.00
BMC Leg (g)	903	151.00; 1623.00
BMC Trunk (g)[a]	736.00	82.00; 1360.00
BMC Ribs (g)	249.00	39.00; 576.00
BMC Pelvis(g)	297.00	87.00; 732.00
BMC Spine (g)	197.50	60.00; 389.00
BMC Android (g)	44.06	5.01; 112.47
BMC Gynoid (g)	244.00	104.32; 624.00
BMC Total (g)	2472.00	1037.00; 4333.00
Arm FM%	37.80	3.70; 65.29
Leg FM%	38.21	5.80; 64.50
Trunk FM%	41.06	6.09; 65.5
Android FM%	45.94	4.00; 71.88
Gynoid FM%	43.08	7.70; 66.53
FM%	37.81	6.00; 60.6
Arm FM (kg)	2.95	0.21; 14.81
Leg FM (kg)	9.03	0.78; 35.57
Trunk FM (kg)	14.42	1.09; 88.56
Android BLean (kg)	2.53	0.09; 13.89
Gynoid BLean (kg)	4.92	0.46; 19.02
FM (kg)	27.13	1.60; 100.85
Arm BLean (kg)	4.52	1.68; 12.48
Leg BLean (kg)	14.51	5.88; 36.02
Trunk BLean (kg)	20.33	8.48; 46.11
Android BLean (kg)	2.88	1.42; 6.68
Gynoid BLean (kg)	6.10	2.67; 15.49
TBLean (kg)	42.53	18.26; 93.03
ASSMI	1.07	0.04; 2.05
IMAT	7.24	2.48; 17.25

Descriptive table of the overall study population. Data were reported as median, minimum and maximum. Parametric values were highlighted with the ([a]). Body Mass Index (BMI), Waist Hip Ratio (WHR), Reactance (Xc), Resistance (R), Phase Angle (PA), Body Cell Mass (BCM), Hydration (HYDR), Sodium-Potassium exchange (Na/K), Total Body Water (TBW), Extracellular Water (ECW), Intracellular Water (ICW), Body Cell Mass Index (BCMI), Bone Mineral Density (BMD), Bone Mineral Content (BMC), Fat Mass (FM), Fat mass percentage (FM%), Body Lean (BLean), Total body Lean (TBLean), Appendicular Skeletal Muscle Index (ASSMI), Intramuscular Adipose Tissue (IMAT).

Table II. Descriptive characteristics of study population.

	TT genotype	A Carriers		
	Median (Min; Max)	Median (Min; Max)	Δ Median (ΔMin; ΔMax)	Δ Median %
Age (years)	43.00 (16.00; 81.00)	44.00 (16.00; 86.00)	-1.00 (0.00; -5.00)	2.33
Height (cm)	163.90 (146.00; 196.50)	163.50 (137.00; 196.00)	0.40 (9.00; 0.59)	-0.24
Weight (kg)	73.30 (33.60; 175.00)	77.00 (39.50; 185.60)	-3.70 (-5.90; -10.60)	5.05
BMI (kg/m^2)	26.47 (13.58; 59.81)	27.87 (14.24;63.70)	-1.40 (-0.66; 3.89)	5.29
Neck circumference (cm)	38.50 (29.00; 58.00)	38.00 (29.00; 100.00)	0.50 (0.00; -42.00)	-1.30
Waist circumference (cm)	84.00 (61.00; 137.00)	89.00 (29.00; 161.00)	-5.00 (32.00; -24.00)	5.95
Abdomen circumference (cm)	95.50 (56.00; 141.00)	100.50 (69.50; 156.00)	-5.00 (-13.50; 5.24)	5.24
Hip circumference (cm)	103.00 (84.00; 150.00)	105.80 (40.00; 160.00)	-2.80 (44.00;- 10.00)	2.72
WHR	0.81 (0.63; 1.16)	0.83 (0.5; 1.40)	-0.01 (0.13; -0.24)	1.84
R	527.50 (330.00; 851.00)	.00 (226.00; 778.00)	16.50 (104.00; 73.00)	-3.13
Xc	58.00 (23.00; 99.00)	54.00 (23.00; 111.00)	4.00 (0.00; -12.00)	-6,90
PA	6.10 (3.00; 26.00)	6.10 (3.40; 23.50)	0.00 (-0.80; 2.50)	0.00
BCM (kg)	26.70 (15.00; 67.00)	27.15 (15.50; 62.60)	-0.44 (-1.00; 4.10)	1.69
HYDR	73.20 (65.00; 87.00)	73.30 (65.00; 86.50)	-0.09 (0.00; 0.10)	0.14
Na/K	0.90 (0.30; 2.00)	0.90 (0.30; 1.70)	0.00 (0.00; 0.10)	0.00
TBW (L)	36.50 (25.00; 74.00)	36.90 (24.90; 88.70)	-0.39 (-0.10; -14.50)	1.10
ECW (L)	16.40 (6.00; 37.00	17.10 (6.50)	-0.70 (-0.60; -3.40)	4.27
ICW (L)	20.00 (11.00; 48.00)	20.10 (12.50; 48.50)	-0.10 (-1.80; -0.70)	0.50
BCMI	10.10 (4.90; 45.00)	10.60 (6.10; 55.40)	-0.50 (-1.20; -10.40)	4.95
BMD Head (g/cm^2)	2.24(1.46; 3.71)	2.26 (0.95; 3.26)	-0.01 (0.52; 0.45)	0.65
BMD Arm (g/cm^2)	0.79 (0.52; 1.44)	0.80 (0.48; 1.80)	-0.01 (0.04; -0.35)	1.20
BMD Leg (g/cm^2)	1.2 0(0.81; 1.81)	1.2125 (0.78; 1.69)	-0.01 (0.03; 0.12)	0.71
BMD Trunk (g/cm^2)	0.97 (0.69; 1.41)	0.978 (0.62; 1.44)	-0.01 (0.08; -0.03)	0.15
BMD Ribs (g/cm^2)	0.79 (0.56; 1.20)	0.79 (0.33; 1.26)	0.00 (0.24; -0.06)	-0.06
BMD Pelvis (g/cm^2)	1.08 (0.67; 1.87)	1.08 (0.60; 1.55)	0.00 (0.08; 0.30)	-0.46
BMD Spine (g/cm^2)	1.12 (0.77; 1.65)	1.14 (0.69; 1.70)	-0.01 (0.09; -0.05)	1.07
BMD Tot (g/cm^2)	1.16 (0.82; 1.65)	1.17 (0.80; 1.61)	-0.01 (0.03; 0.04)	1.12
Total T-score	0.4 (-3.5; 5.9)	0.50 (-2.70; 4.70)	-0.10 (-0.80; 1.20)	25.00
Total Z-score	0.5 (-2.5; 4.5)	0.40 (-2.50; 9.00)	0.10 (0.00; -4.50)	-20
BMC Head (g)	499.00 (316.00; 750.00)	508.00 (84.00; 791.00)	-9.00 (232.00; 41.00)	1.80
BMC Arm (g)	298.00 (166.00; 591.00)	288.00 (141.00;931.00)	10.00 (25.00; -340.00	-3.36
BMC Leg (g)	905.00 (151.00; 1614.00)	900.00 (284.00; 1623.00)	5.00 (-133.00; -9.00)	-0.55
BMC Trunk (g)[a]	744.00 (205.00; 1340.00)	735.00 (82.00; 1360.00)	9.00 (123.00; -20.00)	-1.21
BMC Ribs (g)	253.00 (39.00; 546.00)	246.00 (40.00; 576.00)	7.00 (-1.00; -30.00)	-2.77
BMC Pelvis(g)	294.50 (105.00; 732.00)	301.50 (87.00; 504.80)	-7.00 (18.00; 227.20)	2.38
BMC Spine (g)	197.50 (60.00; 389.00)	199.00 (96.00; 359.00)	-1.50 (-36.00; 30.00)	0.76
BMC Android (g)	44.65 (9.00; 78.00)	44.00 (5.01; 112.47)	0.66 (3.99; -34.47)	-1.47
BMC Gynoid (g)	242.13 (104.32; 624.00)	244.69 (118.71; 463.98)	-2.55 (-14.39; 160.02)	1.06
BMC Total (g)	2500.00 (1644.00; 4333.00)	2470.00 (1037.00; 3871.00)	30.00 (607.00; 462.00)	-1.20
Arm FM%	36.62 (6.66; 60.80)	38.275 (3.70; 65.29)	-1.65 (2.96; -4.49)	4.52
Leg FM%	37.70 (11.48; 64.50)	38.635 (5.80; 61.63)	-0.94 (5.68; 2.87)	2.48
Trunk FM%	39.20 (6.09; 65.50)	42.10 (6.60; 65.50)	-2.90 (-0.52; 0.00)	7.40
Android FM%	43.99 (4.00; 70.59)	46.93 (5.80; 71.88)	-2.94 (-1.80; -1.29)	6.68
Gynoid FM%	44.12 (8.23; 64.20)	45.53 (7.70; 66.53)	-1.41 (0.53; -2.33)	3.20
FM%	35.72 (9.57; 58.50)	38.45 (6.00; 60.60)	-2.73 (3.57; -2.10)	7.64
Arm FM (kg)	2.67 (0.43; 10.55	3.03 (0.21; 14.81)	-0.36 (0.22; -4.26)	13.50
Leg FM (kg)	8.55 (1.46; 33.63)	9.20 (0.78; 35.57)	-0.65 (0.68; -1.94)	7.66
Trunk FM (kg)	13.33 (1.09; 57.00)	15.20 (1.58; 88.56)	-1.87 (-0.49; -31.56)	14.03
Android FM (kg))	2.31 (0.09; 11.25)	2.69 (0.17; 13.89)	-0.37 (-0.08; -2.64)	16.23
Gynoid FM (kg)	0.5 (-2.5; 4.5)	0.40 (-2.50; 9.00)	0.10 (0.00; -4.50)	-20.00
FM (kg)	499.00 (316.00; 750.00)	508.00 (84.00; 791.00)	-9.00 (232.00; 41.00)	1.80
Arm BLean (kg)	4.49 (1.68; 11.61)	4.55 (1.88; 12.48)	-0.06 (-0.20; -0.87)	1.34
Leg BLean (kg)	14.18 (5.88; 33.23)	14.60 (6.34; 36.03)	-0.42 (-0.46; -2.80)	2.96
Trunk BLean (kg)	20.15 (8.46; 43.81)	20.36 (8.97; 46.11)	-0.21 (-0.51; -2.30)	1.04
Android BLean (kg)	2.86 (1.64; 6.68)	2.89 (1.42; 6.31)	-0.02 (0.22; 0.37)	0.77
Gynoid BLean (kg)	6.02 (3.76; 15.49)	6.15 (2.67; 14.41)	-0.13 (1.09 ;1.08)	2.16
TBLean (kg)	42.07 (18.26; 90.60)	42.905 (19.91 ;93.03)	-0.84 (-1.65; -2.43)	1.98
ASSMI	7.16 (2.74; 13.57)	7.27 (2.48; 17.25)	-0.11 (0.26; -3.69)	1.55
IMAT	0.96 (0.04; 2.05)	1.15 (0.10; 2.05)	-0.19 (-0.05; 0.00)	20.02

Descriptive table of the study population for Fat Mass and Obesity-associated gene (FTO) rs9939609 variant. Data for A carrier and homozygous TT groups were reported as median, minimum and maximum. Absolute and percentage differences (Δ) between A carrier and homozygous TT groups were reported. All the values, except the ones highlighted with the ([a]), are not parametric data. Body Mass Index (BMI), Waist Hip Ratio (WHR), Reactance (Xc), Resistance (R), Phase Angle (PA), Body Cell Mass (BCM), Hydration (HYDR), Sodium-Potassium exchange (Na/K), Total Body Water (TBW), Extracellular Water (ECW), Intracellular Water (ICW), Body Cell Mass Index (BCMI), Bone Mineral Density (BMD), Bone Mineral Content (BMC), Fat Mass (FM), Fat mass percentage (FM%), Body Lean (BLean), Total body Lean (TBLean), Appendicular Skeletal Muscle Index (ASSMI), Intramuscular Adipose Tissue (IMAT).

Table III. Descriptive characteristics of study population for FTO rs9939609 variant.

	Overall population (%)	A carriers (%)	TT genotype (%)
Phenotype classification			
UW	2.16	2.26	1.95
NW	15.20	13.15	19.22
NWO	20.45	20.08	21.17
PreOb	24.20	23.06	26.46
Ob I	17.17	18.95	13.65
Ob II	9.57	10.61	7.52
Ob III	11.26	11.88	10.03
FM%			
NW	15.60	13.56	19.60
PreOb/Ob	84.40	86.44	80.40
Gynoid FM%			
NW	6.62	6.24	7.34
PreOb/Ob	93.38	93.76	92.66
Android FM%			
NW	15.86	14.08	19.27
PreOb/Ob	84.14	85.92	80.73
PA			
Healthy	84.84	83.11	88.52
Unhealthy	15.16	16.89	11.48
BCMI			
Healthy	93.26	93.47	92.82
Unhealthy	6.74	6.53	7.18
ASMMI			
Healthy	88.39	88.63	87.90
Unhealthy	11.61	11.37	12.10
IMAT			
Healthy	13.04	11.60	15.88
Unhealthy	86.96	88.40	84.12
T-score			
Healthy	92.36	92.40	92.27
Osteopenia	6.64	6.37	7.22
Osteoporosis	1.00	1.23	0.52

Frequencies of overall population, carrier and non-carrier subjects according to study parameters classification. Total, Gynoid and Android FM% and FM%: normal weight (FM%<25% males and FM%<30% females), PreOb/Ob (FM%≥25% males and FM%≥30% females); UW (BMI<18.50 kg/m^2); NW (18.50 kg/m^2≤BMI<25 kg/m^2 or BMI≥25 kg/m^2 plus FM%<30% females and <25% Male); NWO (18.50 kg/m^2≤BMI<25 kg/m^2 plus FM%≥30% females and ≥25% males); PreOb (25 kg/m^2≤BMI<30 kg/m^2 plus FM%≥30% females and ≥25% males); Ob I (30 kg/m^2≤BMI<35 kg/m^2); Ob II (35 kg/m^2≤BMI<40 kg/m^2); Ob III (BMI≥40 kg/m^2). PA: healthy (<30 y.o. males 6≤PA≤8 and females 6≤PA≤7; 30≤y.o.≤50 males 5.5≤PA≤6 and females 5≤PA≤6; >50 y.o. males 5≤PA≤5.6 and females 4.8≤PA≤5.3), unhealthy (<30 y.o males and females PA<6; 30≤y.o.≤50 males PA<5.5 and females PA<5; >50 y.o. males PA<5 and females PA<4.8); BCMI: healthy (BCMI>10 males and BCMI>7.5 females), unhealthy (BCMI<10 males and BCMI<7.5 females); ASMMI: healthy (ASMMI>7.6 males and ASMMI>5.53 females), unhealthy (ASMMI<7.6 males and ASMMI<5.53 females); IMAT: unhealthy (IMAT>0.5), healthy (IMAT<0.5); T-score: osteoporosis (T-score≤-2.5), osteopenia (-2.5<T-score<1), healthy (T-score≥1). Body Mass Index (BMI), Fat Mass (FM), Fat Mass Percentage (FM%), Underweight (UW), Normal-Weight (NW), Normal-Weight Obese (NWO), Pre-Obese (PreOb), Obese (Ob), Phase Angle (PA), Body Cell Mass Index (BCMI), Appendicular Skeletal Muscle Index (ASSMI), Intramuscular Adipose Tissue (IMAT).

Categorizing our population for BMI and FM%, we observed a statistical significance of SNP rs9939609 among groups p=0.03) (Table V). In this classification, we also noticed significant differences for FTO carrier between NW, considered as a control group, and the other BMI classes. We observed statistical differences in FTO carrier between I grade obesity [p=0.00; OR=2.12 (1.31-3.41)], II grade obesity [p=0.01; OR=2.11 (1.21-3.67)] and III grade obesity groups [(p=0.02; OR=1.82 (1.08-3.06)] compared to NW. No differences were highlighted for UW, NOW, and PreOb phenotypes (Table V).

In our population, the pivotal role of FTO as a genetic obesity risk factor was observed also in FM tissue. In fact, the GLM analysis determined

Table IV. Anthropometric, bioimpedance and Dual Energy X-Ray Absorptiometry (DXA) analysis for FTO rs9939609 A carriers *vs.* TT genotype.

	β	S.E.	*p*
Height (cm)	0.03	0.00	0.08
Weight (kg)	-0.01	0.02	0.01*
BMI (kg/m^2)	0.45	0.02	0.01*
Neck circumference (cm)	0.00	0.01	0.98
Waist circumference (cm)	0.03	0.02	0.07
Abdomen circumference (cm)	0.04	0.01	0.00*
Hip circumference (cm)	0.02	0.01	0.15
WHR	0.01	0.97	0.32
R[a]	-0.03	0.01	0.05
Xc	-0.03	0.02	0.08
PA	-0.02	0.02	0.37
BCM (kg)	0.00	0.02	0.99
HYDR	0.00	0.00	0.97
NA/K	-0.01	0.67	0.41
TBW (L)	0.00	0.01	0.76
ECW (L)	0.01	0.02	0.42
ICW (L)	-0.01	0.02	0.59
BMD Head (g/cm^2)	-0.52	0.12	0.00*
BMD Arm (g/cm^2)	0.01	0.01	0.38
BMD Leg (g/cm^2)	-7.15	0.17	0.00*
BMD Trunk (g/cm^2)	0.00	0.01	0.79
BMD Ribs (g/cm^2)	0.00	0.01	0.94
BMD Pelvis (g/cm^2)	-1.58	0.09	0.00*
BMD Spine (g/cm^2)	-0.20	0.16	0.23
BMD Tot (g/cm^2)	-0.98	0.09	0.00*
Total T-score	0.01	0.02	0.53
Total Z-score	-0.01	0.02	0.58
BMC Head (g)	0.01	0.02	0.82
BMC Arm (g)	0.01	0.03	0.75
BMC Leg (g)	-0.01	0.03	0.77
BMC Trunk (g)[a]	-0.02	0.03	0.41
BMC Ribs (g)	-0.04	0.03	0.21
BMC Pelvis(g)	-0.01	0.50	0.48
BMC Spine (g)	-0.01	0.12	0.73
BMC Android (g)	-0.01	0.19	0.66
BMC Gynoid (g)	0.08	0.03	0.01*
BMC Total (g)	0.06	0.03	0.05
Trunk FM (kg)	0.11	0.04	0.00*
Android FM (kg)	0.11	0.04	0.01*
Gynoid FM (kg)	0.05	0.03	0.06
FM (kg)	0.09	0.03	0.00*
Arm BLean (kg)	0.00	0.01	0.75
Leg BLean (kg)	-0.01	0.01	0.34
Trunk BLean (kg)	-0.01	0.01	0.38
Android BLean (kg)	-0.01	0.02	0.62
Gynoid BLean (kg)	-0.02	0.02	0.25
TBLean (kg)	-0.01	0.01	0.36

Relationship between FTO rs9939609 A carriers and TT genotype in body composition. Statistical significance (*) were given to results with $p<0.05$ through GLM analysis. Body Mass Index (BMI), Waist Hip Ratio (WHR), Resistance (R), Reactance (Xc), Phase Angle (PA), Bone Mineral Content (BMC), Hydration (HYDR), Sodium-Potassium exchange (NA/K), Total Body Water (TBW), Extracellular Water (ECW), Intracellular Water (ICW), Bone Mineral Density (BMD), Body Cell Mass (BCM), Fat Mass (FM), Fat mass percentage (FM%), Body Lean (BLean), Total Body Lean (TBLean).

Table V. Body composition categories for FTO rs9939609 A carriers vs. TT genotype.

	β	S.E.	χ^2	*p*	OR (Min; Max)	R^2
Phenotype classification			0.03*	0.24		
UW[b+]	0.50			0.30	1.65 (0.63; 4.30)	
NWO[b+]	0.38			0.09	1.46 (0.94; 2.26)	
PreO[bb+]	0.28			0.20	1.32 (0.86; 2.01)	
Ob I[b+]	0.75			0.00*	2.12 (1.31; 3.41)	
Ob II[b+]	0.75			0.01*	2.11 (1.21; 3.67)	
Ob III[b+]	0.60			0.02*	1.82 (1.08; 3.06)	
FM%[a++]	0.47	0.18	0.01	0.01*	1.61 (1.11; 2.32)	0.20
Android FM%[a++]	0.42	0.19	0.38	0.03*	1.53 (1.04; 2.25)	0.23
Gynoid FM%[a++]	0.18	0.28	0.52	0.52	1.20 (0.69; 2.09)	0.19
PA[a++]	0.47	0.25	0.07	0.06	1.60 (0.97; 2.62)	0.03
BCMI[a++]	-0.21	0.33	0.76	0.56	0.81 (0.40; 1.65)	0.06
T-score total body			0.58	0.17		
Osteopenia[b++]	0.26			0.47	1.30 (0.64; 2.64)	
Osteoporosis[b++]	-0.74			0.51	0.48 (0.05; 4.17)	
ASMMI[a++]	0.08	0.20	0.72	0.68	1.09 (0.73; 1.62)	0.02
IMAT[a++]	0.36	0.19	0.05	0.06	1.43 (0.98; 2.07)	0.08

Body composition categories analysis among FTO rs9939609 A carriers *vs.* TT genotype. [a]Binomial Logistic Regression; [b]Multinomial Logistic Regression; [+]Reference category Normal Weight; [++]Reference category Healthy. Statistical significance (*) were given to results with $p<0.05$. All data were adjusted for sex and age. BMI: healthy (BMI<25 kg/m^2), Pre-Obese/Obese (BMI≥25 kg/m^2); Total, Gynoid and Android FM%: healthy (FM%<25% males and FM%<30% females), unhealthy (FM%≥25% males and FM%≥30% females); BMI healthy (BMI<25 kg/m^2), Pre-Obese/Obese (BMI≥25 kg/m^2); UW (BMI<18.50 kg/m^2); NW (18.50 kg/m^2≤BMI<25 kg/m^2 or BMI≥25 kg/m^2 plus FM%< 30% females and <25% Males); NWO (18.50 kg/m^2≤BMI<25 kg/m^2 plus FM%≥30% females and ≥25% males); OW (25 kg/m^2≤BMI<30 kg/m^2 plus FM%≥30% females and ≥25% males); Ob I (30 kg/m^2≤BMI<35 kg/m^2); Ob II (35 kg/m^2≤BMI<40 kg/m^2); Ob III (BMI≥40 kg/m^2). PA: healthy (<30 y.o males 6≤PA≤8 and females 6≤PA≤7; 30≤y.o.≤50 males 5.5≤PA≤6 and females 5≤PA≤6; >50 y.o. males 5≤PA≤5.6 and females 4.8≤PA≤5.3), unhealthy (<30 y.o males and females PA<6; 30≤y.o.≤50 males PA<5.5 and females PA<5; >50 y.o. males PA<5 and females PA<4.8); BCMI: healthy (BCMI>10 males and BCMI>7.5 females), unhealthy (BCMI<10 males and BCMI<7.5 females); ASMMI: healthy (ASMMI>7.6 males and ASMMI>5.53 females), unhealthy (ASMMI<7.6 males and ASMMI<5.53 females); IMAT: unhealthy (IMAT>0.5), healthy (IMAT<0.5); T-score: osteoporosis (T-score≤-2.5), osteopenia (-2.5< T-score <1), healthy (T-score≥1). Body Mass Index (BMI), Fat Mass (FM), Fat mass percentage (FM%), Underweight (UW), Normal-weight (NW), Normal-weight Obese (NWO), Pre obese (PreOb), Obese (Ob), Phase Angle (PA), Body Cell Mass Index (BCMI), Appendicular Skeletal Muscle Index (ASSMI), Intramuscular Adipose Tissue (IMAT).

a statistical significance of total FM (kg) in FTO carriers (p=0.00; β=0.09; Δ%=17.26%) compared to TT genotype. In addition, by categorizing obesity according to FM%, we noticed a statistical significance between FTO carriers and non-carriers in obesity groups compared to reference healthy subjects (p=0.01; OR=1.61 (1.11-2.32); β=0.47).

More precisely, A carriers present a significantly higher risk to have android obesity than TT genotype. In fact, the GLM analysis highlighted statistical significance between FTO A carriers and TT genotype for abdomen circumference (p=0.00; β=0.04; Δ%=5.24%), as well as for arms (p=0.01; β=0.08; Δ%=13.50%), trunk (p=0.00; β=0.11; Δ%=14.03%), and android FM (kg) (p=0.01; β=0.11; Δ%=16.23%). Therefore, we categorized our subjects for android and gynoid obesity, and then, we observed a statistical significance between FTO A carriers and TT genotype in obesity groups compared to reference healthy subjects for android FM% [p=0.03; OR=1.53 (1.04-2.25); β=0.42] but not for gynoid FM% (p≥0.05).

Finally, IMAT was observed to be higher in A carriers compared to TT genotype (p=0.00; β=0.09; Δ%=20.02%) (Table IV). However, dividing our sample for IMAT score between healthy and unhealthy subjects we did not observe any statistical significance between the FTO groups (p≥0.05) (Table V). The GLM analysis did not highlight any statistical significance between rs9939609 carriers and non-carriers for the other parameter observed, as well LBM analysis on PA, BCMI, and ASMMI (p≥0.05).

Discussion

In the last decades, obesity has become one of the most widespread medical conditions, playing an important role in the etiology of different disorders, such as type-2 diabetes and cardiovascular diseases[1]. By 2030, 20% of the world's adult population will be obese and 38% will be overweight[28]. Nonetheless, by analyzing risk factors for obesity, such as nutritional or lifestyle habits, it can be noted that medical disorder is preventable.

Independently from the risk factors, genetic plays a key role in body composition phenotype, and in the development of obesity[6]. Among the large quantity of genes involved in the development of obesity, FTO is one of the most well-known. Several variants of this gene were associated with BMI and the rs9939609[29,30]. In fact, several studies[10,31] have demonstrated that the allele A is associated with a higher BMI, and more recent studies[14,32] have also shown a good relation between this allele and both fat and lean mass.

The relationship between FTO and BMI is already shown in the Italian population, while body composition was never studied before in the same population. In view of the above data, we have conducted this study in order to analyze a possible influence of FTO rs9939609 on body composition and understand if this variant can influence not only BMI but also all the body compartments.

By analyzing our results, interesting data about BMD were found. In fact, BMD, head and lower pelvis were higher in A carriers compared to TT genotype. These data are added to other reports[33,34], in which results are uncertain. However, the association between FTO rs9939609 and BMI is confirmed in our sample. According to the descriptive statistics, A carrier is more widespread compared to TT genotype in all the three obesity grades. In fact, GLM analysis demonstrated that A carriers present a higher BMI compared to TT (p=0.01), whereas the LRM analysis showed that A carriers were associated with pre-obese/obese subjects (BMI≥25 kg/m^2). More specifically, FTO carriers were related to all the obesity groups (I grade obesity p=0.00; II grade obesity p=0.01; III grade obesity p=0.02) as highlighted by the statistical analysis we carried out. However, in view of above data and considering previous studies[27], it seems to be highly probable that, also in the Italian population, FTO rs9939609 is significantly related to body mass index.

At the same time, it is important to discuss the results we have found in body composition. BMI alone might not be considered as a good predictive value for obesity[3,35]; then, the relationship between BMI and FTO alone cannot demonstrate that this variant relates to the obesity. In our study, by categorizing obesity for FM%, we noticed that A carrier subjects with higher FM% are more widespread (86.44%) compared to TT genotype subjects (80.40%). Therefore, A carriers had a significantly greater risk of being obese for FM% classification [OR=1.61 (1.11-2.32)] than subjects in a homozygous condition, as well as total FM (kg) (Δ%= 17.26%) being significantly higher in A carriers compared to TT genotype subjects, according to previous studies[36,37] demonstrating that FTO indicates a genetic etiological factor for obesity. However, only two studies on murine models shown that FTO gene variant rs9939609 is extremely specific for FM%, leaving aside any interaction with LM[38].

To the best of our knowledge, our results verified that for the first time in humans FTO A allele is extremely specific for the FM, given that no significance was discovered with LM (p≥0.05). Indeed, it is also important to highlight that no significant results were found about the gynoid fat mass. Notably, in our population, rs9939609 is particularly specific for android obesity since it seems to be highly related to the A carriers compared to TT genotype (Δ%=16.23%). This result was confirmed when categorizing android obesity for FM%, A carriers significantly had a higher risk to have for android FM% [OR=1.53 (1.04-2.25)] than subjects in a homozygous condition. These results fully match with the results we have found regarding abdomen and trunk circumferences, which are significantly higher in A carriers (respectively Δ%=5.24% and Δ%=14.03%) than in TT genotype.

Conclusions

Altogether, the analyses we have carried out might be the baseline for further studies, which should try to understand if FTO rs9939609 particularly influences FM%. The confirmation of these results among different populations could mean that FTO rs9939609 is an actual genetic etiological factor for obesity. Moreover, further researches are necessary to confirm and understand why gynoid FM is not influenced by FTO rs9939609 whilst android mass is. Android FM is composed both of visceral and subcutaneous fat[39]. Some studies[40,41] have already demonstrated that visceral adipose tissue (VAT) is associated with cardiovascular diseases (CDVs) and at the same time, FTO rs9939609 variant was associated also with metabolic syndrome[13], type 2 diabetes[42], and response to diet therapy[43-45].

Furthermore, it is well known that FTO gene variant rs9939609 contributes to the regulation of energy homeostasis and metabolic rate, even if the association between brain FTO levels and food intake is somewhat controversial: a reduction in FTO expression levels in the arcuate nucleus of rats increases food intake and enhanced expression decreasing food intake[38]. However, it has been demonstrated that both dietetic treatment and interaction diet-gene influence the FM%, whilst FTO alone did not demonstrate the same effect[43].

Our study analyses body composition data on Caucasian Italian population for the first time, putting an effort on body fat distribution related to FTO gene variant rs9939609.

Further analyses should be encouraged in order to find any possible relations between VAT and FTO rs9939609 variant, through a more specific and precise methodology, like abdominal computed tomography scan, along with a genotype analysis. If this association is confirmed, new interesting implications would come up.

Ethics Approval and Consent to Participate

All participants enrolled in the study approved their participation studying and signing the informed consent, carried out in accordance with the Helsinki Declaration of 1975 as revised in 1983. All the enrolled subjects signed an informed consent, and the study protocol was approved by the Ethical Committee of the Calabria Region Center Area Section (Register Protocol No. 146 17/05/2018).

Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

GM, PG draft the manuscript; GC, SF, MGT performed the experiments and collected data; GB analyzed the data; AC, AP, FF reviewed the text; LDR, ADL conceived and designed the experiments, had primary responsibility for the final content. All the authors read and approved the final manuscript. All the authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Conflict of Interests

No conflicts of interest, financial or otherwise are declared by the authors.

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