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Relationship between sex hormones, markers of adiposity and inflammation in male patients with severe obesity undergoing bariatric surgery

Angelo Di Vincenzo, Marika Crescenzi, Marnie Granzotto, Roberto Vettor, Marco Rossato

Internal Medicine 3, Department of Medicine - DIMED, University Hospital of Padova, Padova 35128, Italy.

Correspondence to: Dr. Angelo Di Vincenzo, Department of Medicine, University Hospital of Padova, via Giustiniani 2, Padova 35128, Italy. E-mail: angelo.divincenzo@unipd.it

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Abstract

Aim: In males, obesity is characterized by features resembling those observed during aging, such as hypogonadism and cytokines imbalance, yet at an early age. A direct connection between the low-grade inflammatory state and sex steroid abnormalities has been proposed to explain the development of these conditions in obesity.

Methods: We evaluated the relationship between sex hormones plasma levels and metabolic and inflammatory parameters in a cohort of patients with grade III obesity (n = 24, BMI 43.4 ± 8.5 kg/m^2) undergoing bariatric surgery. Furthermore, we assessed the in vitro effects of testosterone exposure on the expression of markers of adiposity such as FABP-4, PPARγ, leptin, and adiponectin in human-derived adipocytes.

Results: A direct correlation was observed between BMI and hsCRP (P < 0.05), while testosterone plasma levels showed a statistically significant inverse correlation with hsCRP, but also with HOMA index, leptin, and von Willebrand factor concentrations (P < 0.05). In human-derived adipocytes, testosterone exposure promotes a reduction in the gene expression of adiposity markers, which is inhibited by co-exposure with the antiandrogen flutamide.

Conclusion: Our study shows a relationship between testosterone plasma levels and markers of inflammation in severe obesity, with testosterone exposure affecting adiposity biomarkers expression in humans. In light of these results, hypogonadism should be promptly identified in male patients with obesity and timely treated to reduce the

burden of the disease.

Keywords: Obesity, testosterone, inflammation, hsCRP, adipocyte, cytokines

INTRODUCTION

Obesity and metabolic syndrome are conditions associated with a constellation of hormonal abnormalities, often neglected but severely affecting the quality of life of these patients. Particularly in males, obesity is frequently characterized by low testosterone and high 17-β-estradiol plasma levels, a clinical condition defined as male obesity-secondary hypogonadism (MOSH). MOSH represents a common complication in patients with obesity and type 2 diabetes[1-3], showing an increasing prevalence with increasing BMI. Despite the underlying mechanisms remain not fully clarified yet, it is generally accepted that in obesity, the adipose tissue expansion leads to the increased activity of the enzyme aromatase, highly expressed on adipocytes, enhancing the conversion of circulating testosterone to 17-β-estradiol[4], and thus leading to the development of MOSH. However, this hypothesis still presents some limitations since, in clinical practice, the reduction of testosterone is not commonly accompanied by an increase of 17-β-estradiol[5].

When adipocytes reach the biological threshold for their expansion, they become dysfunctional, producing several pro-inflammatory mediators defined as adipocytokines, which are capable of peripherally mediating the detrimental effects of obesity, in particular on the cardiovascular system and endothelial function[6-8].

High-sensitivity C-reactive protein (hsCRP) is currently considered a biomarker for atherosclerosis outcome, but also tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and soluble interleukin-6 (IL-6)receptor seem involved in the inflammatory reaction occurring locally on the atherosclerotic plaque, and increased levels of these cytokines are usually observed in metabolic syndrome and obesity and also hypogonadism[9,10]. Fatty acid binding protein 4 (FABP-4) is another molecule locally secreted by adipocytes in the systemic circulation, and FABP4 levels are reported to increase in patients with obesity compared to lean controls. Furthermore, it has been reported that increased circulating FABP-4 is related to arterial hypertension and atherosclerosis, suggesting its potential role as a biomarker[11]. Peroxisome proliferation-activated receptor γ (PPARγ) is a nuclear receptor involved in fatty acid metabolism in the adipose tissue but also presents a wide spectrum of effects in the cardiovascular systems, and its targeting is under evaluation for the treatment of various cardiovascular conditions due to anti-inflammatory potential[12].

Beyond these effects, the adipocytokines may also be involved in the development of reproductive function abnormalities. In fact, these molecules seem to affect sex hormones plasma concentration acting at both gonadal and hypothalamic levels, altering steroid synthesis on one hand, and the normal hormonal feedback loop on the other, thus contributing to MOSH progression. Furthermore, the dysregulation of leptin signaling, as observed in obesity, may also exaggerate the abnormalities of the reproductive axis.

The clinical relevance of this bidirectional interconnection is even more evident considering that in the male, low testosterone plasma levels, as observed during androgen deprivation therapy[13], are associated with the development of metabolic abnormalities and obesity, but in addition, accumulating evidence is showing the role of hypotestosteronemia as an independent risk factor for cardiovascular disease[14]. In this regard, MOSH may represent a relevant target in the management of patients with obesity and metabolic syndrome, while it still remains an ancillary topic in the obesity research area and also in clinical practice, with obesity management guidelines rarely recommending the recognition of MOSH and its treatment in

male subjects with obesity.

In this study, we selected 24 subjects with III-grade obesity referring to our Bariatric Unit undergoing a complete clinical, andrological, biochemical, and instrumental assessment before bariatric surgery, to provide data on the relationship between sex hormones and metabolic/inflammatory parameters. In addition, we performed an *in vitro* study to evaluate the effects of testosterone exposure on the expression of biomarkers of adiposity in human-derived adipocytes.

METHODS

Patients

The research was carried out following the Declaration of Helsinki guidelines and received approval from the Ethics Committee at the University Hospital of Padova (CESC 4502/AO/18). All patients provided informed consent before participating. We evaluated 24 male obese patients referring to our Bariatric Unit - Center for the Study and Integrated Treatment of Obesity at the University Hospital of Padova. The indication for bariatric surgery was confirmed according to consensus criteria for bariatric surgery (BMI greater than 35 kg/m^2 in the presence of comorbidities or with a BMI greater than 40 kg/m^2)[15]. All subjects underwent both endocrinological and andrological assessments in an ambulatorial regimen. The assessment included clinical history with a focus on reproductive organs (previous testicle trauma, previous diagnosis of hypogonadism, erectile dysfunction, and/or infertility), andrological examination, bioumoral assessment (total testosterone, 17-β-estradiol, LH, and FSH, evaluated by commercial electrochemiluminescence immunoassay methods Elecsys 2010, Roche Diagnostics), testicular ultrasound, and sexual functions anamnesis and questionnaire (i.e., International Index of Erectile Function-5, IIEF-5).

The anthropometric assessment contemplated body weight, height, waist, and BMI. Additionally, body composition was determined with body impedance assessment (BIA) by an electrical impedance analyzer (Soft Tissue Analyzer, Akern, Pontassieve, Italy). Bioumoral assessment was performed with total blood count, serum electrolytes, urea, creatinine, TSH, FT4, transaminases, γGT, but also parameters of metabolic/cardiovascular risk such as fasting glucose, insulin, HOMA insulin resistance index, leptin, lipid profile (total cholesterol, LDL-C, HDL-C, triglycerides), high-sensitivity C reactive protein (hsCRP), IL-6, TNF-α, and von Willebrand factor (vWF). In addition, in nondiabetic subjects, an oral glucose tolerance test was performed. In diabetic subjects, glycated hemoglobin and microalbuminuria were tested.

Preadipocytes isolation, differentiation and grouping

For the *in vitro* study, we used a standardized method already described[16]. We collected subcutaneous adipose tissue (SAT) from five healthy male subjects undergoing abdominoplasty for abdominal wall laxity.

To obtain the stromal vascular fraction, SAT was treated with 1 mg/mL collagenase type II for an hour at 37 °C. Then, the cells were sown in DMEM/F12 with 10% fetal bovine serum and placed at 37 °C in a 5% CO_2 exposure. After 16-20 h, adipogenic medium consisting of DMEM/F12 with 33 μmol/L of biotin, 17 μmol/L of pantothenate, 10 μg/mL transferrin, 66 nmol/L of insulin, 100 nmol/L of dexamethasone, 1 nmol/L of triiodothyronine, 0.25 mmol/L of 3-isobutyl-1-methylxanthine (IBMX), and 10 μmol/L of rosiglitazone was added. The treatment was repeated after three days, excluding IBMX and rosiglitazone, and the medium was specifically renewed three times every week until cell differentiation. Oil-red-O staining was performed to test the adipocyte differentiation, as illustrated in Figure 1.

The fully differentiated adipocytes were exposed to different treatments. One group was stimulated with 100 nmol/L testosterone (T group), another group was treated with 200 nmol/L flutamide (an androgen

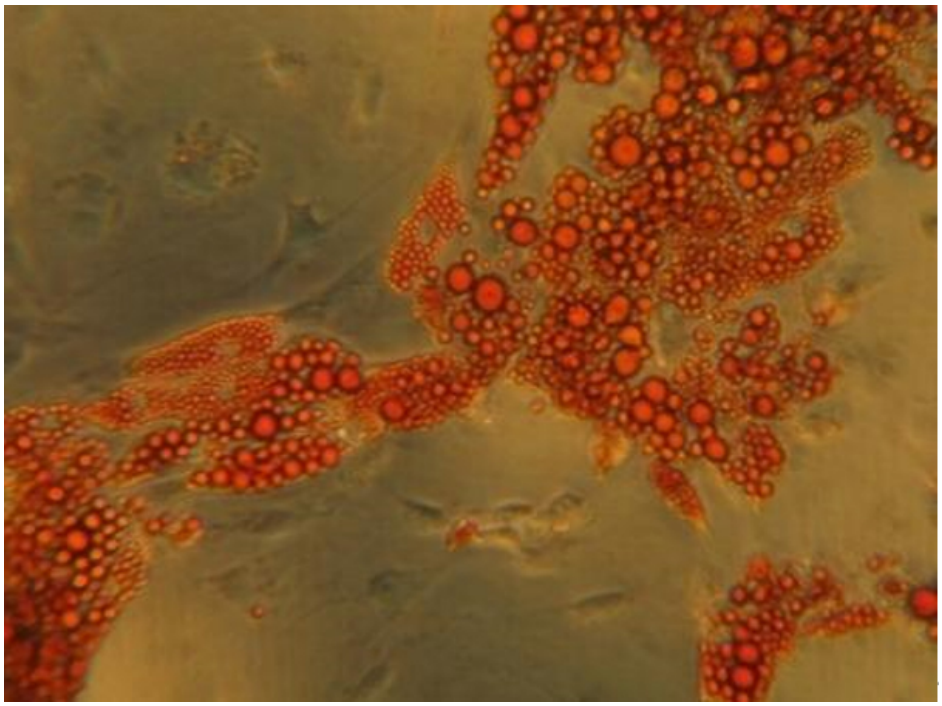

Figure 1. *In vitro* differentiation of mature human adipocytes from the stromal vascular fraction with Oil-red-O staining.

receptor blocker, Fluta group), and a third group was given a combination of both treatments (Fluta/T group). To serve as a control group, adipocytes in primary culture were exposed only to the vehicle dimethyl sulfoxide (DMSO). Each subject underwent a set of five experiments run in triplicate.

RNA extraction and real-time PCR gene expression analysis

Following a 24-hour period, the differentiated cells were rinsed using PBS buffer and an RNEasy kit from Qiagen GmbH in Germany. The quantity of RNA collected was measured using a spectrophotometer, while the quality was verified using an Agilent 2100 Bioanalyzer. First-strand cDNAs were produced from total RNA extracted using random primers and M-MLV reverse transcriptase. SYBR Green fluorophore was used to determine the gene expression levels of adipocyte markers, including Fatty Acid Binding Protein-4 (FABP-4), Peroxisome Proliferator-Activated Receptor γ (PPARγ), leptin, and adiponectin. The fluorescence change at each cycle was observed, and a threshold cycle above the background for each reaction was calculated. After each run, a melt curve analysis was performed to ensure a single amplified product for each reaction. All samples were duplicated in at least two reactions. The relative mRNA transcript levels were determined using the $2^{-\Delta\Delta ct}$ method, with Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) serving as the internal control gene.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.5.1. Pearson's or Spearman's correlations were calculated for correlation between variables after testing their normal distribution. For the in vitro study, the results are reported as mean values ± standard deviation (SD). Comparisons of data from two groups were analyzed with Student's *t*-test. Multiple comparisons were performed with ANOVA test and post hoc Tukey HSD test. The statistical significance was identified at $P < 0.05$.

RESULTS

Clinical study

The mean BMI and waist circumference of our patients was 43.4 ± 8.5 kg/m^2 and 135.6 ± 17.4 cm, respectively. The mean age was 43 ± 8. Thirteen patients were diagnosed with metabolic syndrome according to the NCEP-ATP III criteria[17]. Among the features of metabolic syndrome as defined by the NCEP-ATP III criteria, arterial hypertension was the most frequent. Dyslipidemia was identified in 11 patients (45.8%), and a diagnosis of diabetes was already present in four subjects (16.7%). Our patients showed mean testosterone plasma levels at the lower limit (10.6 ± 5.5 nmol/L), with a value below 8 nmol/L in nine subjects (37.5%). The majority of these subjects reported on the clinical history and IIEF-5 questionnaire key symptoms of overt hypogonadism. Mean 17-β-estradiol plasma levels were 124.4 ± 46.4 pmol/L, just below the upper limit Table 1.

In this cohort of subjects with severe obesity, we confirmed an inverse relationship between anthropometrical parameters and testosterone plasma levels; in particular, a significant inverse correlation was observed between total testosterone plasma levels, waist circumference, and BMI (data not shown). With regard to inflammatory cytokines, a direct relationship was observed between BMI and hsCRP levels (r = 0.4, P < 0.05), while an inverse relationship has been shown between total testosterone plasma levels and hsCRP levels (r = -0.5, P < 0.05), as reported in Figure 2.

In addition, an association was observed between total testosterone plasma levels and some metabolic parameters: in particular, inverse correlations were observed between total testosterone and HOMA index (r = -0.4; P = 0.05), and total testosterone and leptin plasma levels (r = -0.5; P < 0.05). Furthermore, an inverse relationship was also observed between testosterone and vWF [Figure 3]. On the contrary, testosterone plasma levels were not correlated with the other inflammatory markers (IL-6, TNF-α).

In vitro study

Human differentiated adipocytes were incubated in the presence of testosterone (T), flutamide (Fluta), and flutamide plus testosterone (Fluta/T), or with vehicle only (Control). As reported in Figure 4, testosterone exposure affected FABP-4, PPARγ, leptin and adiponectin gene expression, significantly reducing mRNA concentration with respect to control. The co-exposure of testosterone with flutamide, a selective antagonist of the androgen receptor, antagonized the effects of testosterone with respect to the considered markers of adiposity, with the exception of leptin expression.

DISCUSSION

In men with obesity, low testosterone and high estradiol plasma levels are traditionally considered the results of the increased aromatase activity of the expanded adipose tissue. Among fat depots, the visceral adipose tissue presents the higher aromatase expression, accounting for the role of central obesity in the development of MOSH. Once hypogonadism has developed, reduced androgen levels may affect adiposity in a bidirectional relationship[18]. Testosterone is in fact involved in the regulation of glucose homeostasis, as demonstrated by the positive effect of hormonal replacement therapy in hypogonadal subjects with obesity when testosterone treatment ameliorates metabolic abnormalities[19]. Also in type 2 diabetes mellitus, the potential of testosterone to prevent or even revert the disease in hypogonadal subjects is well established[20-22]. On the other hand, in metastatic prostate cancer, androgen deprivation therapy is associated with detrimental effects on metabolism.

In this study, we confirmed the inverse correlation between testosterone plasma levels and metabolic parameters such as the HOMA index and leptin plasma levels in patients with obesity. HOMA index is

Table 1. Baseline characteristics of study population

Baseline characteristics of patients (*n* = 24)	
Age (yy)	43 ± 8
Type 2 diabetes	4
Arterial hypertension	16
Dyslipidemia	11
OSAS	10
Body weight (kg)	134 ± 28
BMI (kg/m^2)	43.4 ± 8.5
Waist circumference (cm)	135 ± 17
Fasting glucose (mg/dL)	119 ± 58
Total Testosterone (nmol/L)	10.6 ± 5.5
17-β-Estradiol (pmol/L)	124.4 ± 46.4
Total cholesterol (mg/dL)	202 ± 33
Triglycerides (mg/dL)	160 ± 70
LDL (mg/dL)	132 ± 36
AST (U/L)	32 ± 24
ALT (U/L)	44 ± 34
SBP (mmHg)	148 ± 19
DBP (mmHg)	96 ± 10

OSAS: obstructive sleep apnea; BMI: body mass index; LDL: low-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; SBP: systolic blood pressure; DBP: diastolic blood pressure.

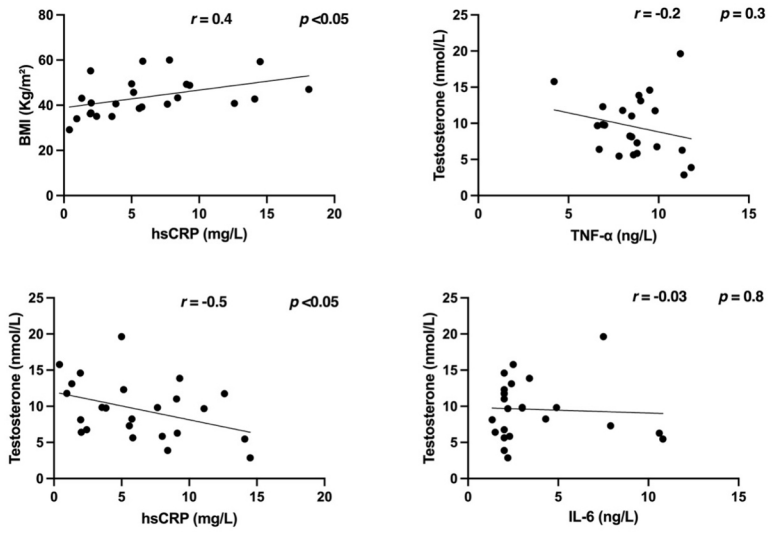

Figure 2. Relationship between hsCRP with BMI, and testosterone plasma levels with hsCRP, TNF-α, and IL-6. hsCRP: High-sensitivity C-reactive protein; TNF- α : Tumor Necrosis Factor-alpha; IL-6: Interleukin-6

commonly used for the evaluation of insulin resistance severity. Our results show that insulin resistance and total testosterone plasma levels are related, with lower levels of testosterone associated with higher HOMA

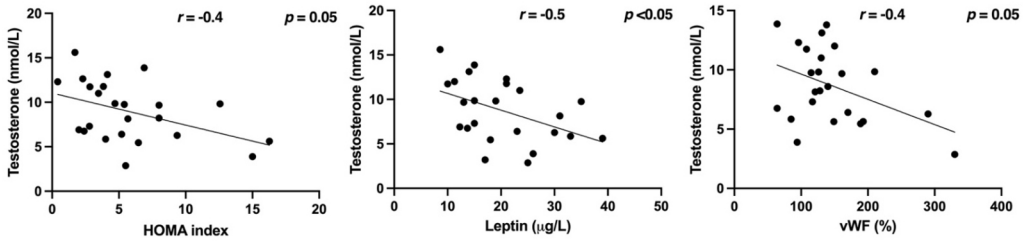

Figure 3. Relationship between testosterone plasma levels, HOMA index, leptin concentration, and vWF activity. vWF: von Willebrand factor.

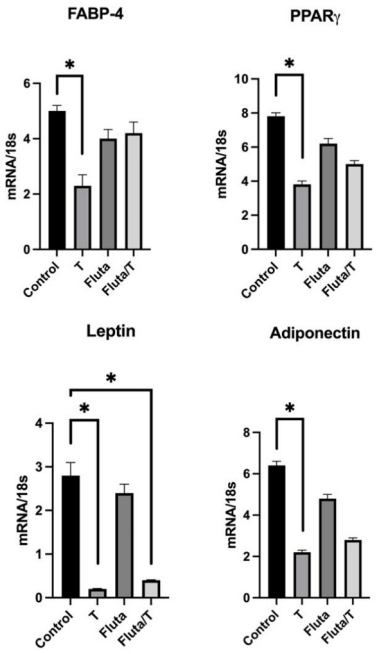

Figure 4. Testosterone negatively modulates the expression of adiposity biomarkers. The exposure of adipocytes to testosterone (T) reduced the gene expression of FABP-4, PPARγ, leptin and adiponectin, while the co-exposure with the antiandrogen flutamide (Fluta/T) antagonized its effects. A complete set of five experiments that were run in triplicate were performed on each subject. Data are expressed as means ± DS. *$P \leq 0.05$. FABP-4: Fatty acid binding protein 4; PPAR γ : Peroxisome proliferation-activated receptor γ .

scores, confirming the liaison between circulating androgens and glucose homeostasis. Beyond the hypothesis of the increased aromatase activity from the expanded adipose tissue, the pathophysiology of hypotestosteronemia in patients with metabolic syndrome and obesity is partially attributable to the hyperinsulinemic state. In fact, higher levels of insulin could have a negative impact on both hypothalamic-pituitary and testicular levels, affecting both gonadotropin release and testosterone synthesis. On the contrary, as we observed in the *in vitro* study, testosterone reduces markers of adiposity and fat mass with consequent positive metabolic effects such as amelioration of insulin sensitivity. Furthermore, we confirmed the relationship between testosterone and leptin plasma levels in subjects with severe obesity. Leptin is responsible for the development of MOSH[23], but this relationship seems bidirectional, with testosterone influencing leptin plasma levels as demonstrated by the effects of testosterone supplementation reducing the concentration of this adipokine in hypogonadal patients[24]. These effects could possibly be due to the inhibition of leptin gene expression from adipocytes, as shown in the present study.

Since the knowledge of the pathways of inflammation in the development of atherosclerosis is growing, increasing attention is focused on the role of adipose tissue dysfunction as a main player in cardiovascular diseases. High-sensitivity CRP is an inflammatory marker closely associated with abdominal obesity and metabolic syndrome, which may contribute to the progression of atherosclerosis binding to oxidized LDL in the atherosclerotic plaque, and aggravating the endothelial damage[25]. The increased levels of hsCRP in obesity may be related to the infiltration of inflammatory cells, particularly macrophages, in the expanded adipose tissue, and may drive the increased cardiovascular and metabolic risk, affecting vascular health and promoting insulin resistance. In this study, we showed that an inverse relationship is present between testosterone and hsCRP levels in severe obesity, similar to what is observed in the aging male[26,27]. Recent evidence suggests the beneficial effects of testosterone supplementation on the inflammatory markers, and some studies demonstrated the anti-inflammatory potential of testosterone supplementation in hypogonadal subjects with metabolic syndrome[28] and chronic inflammatory diseases such as psoriasis and Crohn's disease[29,30], thus promoting hormonal replacement therapy also in the cohort of male patients with obesity.

Von Willebrand factor (vWF) has a main role in thrombus formation, being indispensable for platelet aggregation. VWF is produced and released by endothelial cells in response to different stimuli, such as hypoxia, endotoxin, and cytokines. It has been proposed as a marker of residual cardiovascular risk and prognosis in patients with pre-existing vascular disease[31]. Furthermore, vWF has been recently associated with the increased risk of deep vein thrombosis in obesity[32]. In this sense, vWF might represent a peculiar cardiovascular risk factor in obesity, and we showed its relationship with testosterone levels in our cohort of subjects with severe obesity, further underscoring the possible deleterious effect of hypogonadism on vascular health. However, at this moment, the evidence about the relationship between testosterone and vWF is limited, in particular when considering the consequences of hormone replacement therapy: a study evaluated the effects of testosterone administration in female-to-male transgender subjects[33], while the recently reported data deriving from animal models showed the effects of testosterone supplementation on vWF concentration[34].

The physiological basis of the modulatory effect of testosterone on inflammatory parameters remains elusive. It is probable that, in obesity, low testosterone promotes a vicious cycle in which the increased adiposity maintains the hypogonadal state by increasing the aromatization of circulating androgens and the release of adipocytokines within the systemic circulation, leading to the development of the chronic low-grade inflammation that characterizes obesity.

Thus, testosterone may have beneficial metabolic and anti-inflammatory effects by reverting this circuit, specifically modulating the adipocyte biology, in particular inhibiting the expression of biomarkers of adiposity, resulting in a reduced adipose commitment and an improvement of metabolic and hormonal profile. Although we acknowledge the potential impact of various confounding variables and the limited sample size on our findings, taken together, these data seem to confirm the role of MOSH in patients with severe obesity and the association of low testosterone levels with a high burden of cardiovascular risk factors. However, a specific assessment for reproductive function abnormalities and MOSH detection in obesity is not usually provided according to the actual standard of care. The American Association of Clinical Endocrinologists (AACE) recommends the assessment of hypogonadism and, when required, testosterone supplementation for eligible patients with obesity and/or type 2 diabetes[35,36], while European Societies still show little awareness. MOSH is a condition very often undiagnosed and untreated in subjects with obesity, with possible relevant clinical implications. As for the other obesity-related complications, we should increase awareness of this comorbidity to guarantee a better clinical outcome for our patients.

DECLARATIONS

Authors' contributions

Made substantial contributions to the conception and design of the study and performed data analysis and interpretation: Di Vincenzo A, Rossato M

Performed data acquisition, as well as provided technical and material support: Crescenzi M, Granzotto M

Contributed to writing-review and manuscript editing: Vettor R

Read and agreed to this version of the manuscript: Di Vincenzo A, Crescenzi M, Granzotto M, Vettor R, Rossato M

Availability of data and materials

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

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital of Padova (CESC 4502/AO/18). Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable.

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