Testosterone treatment improves metabolic syndrome-induced adipose tissue derangements

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Abstract

We recently demonstrated that testosterone dosing ameliorated the metabolic profile and reduced visceral adipose tissue (VAT) in a high-fat diet (HFD)-induced rabbit model of metabolic syndrome (MetS). We studied the effects of HFD and *in vivo* testosterone dosing on VAT function and the adipogenic capacity of rabbit preadipocytes isolated from VAT of regular diet (RD), HFD, and testosterone-treated HFD rabbits. VAT was studied by immunohistochemistry, western blot, and RT-PCR. Isolated rPADs were exposed to adipocyte differentiating mixture (DIM) to evaluate adipogenic potential. Adipocyte size was significantly increased in HFD VAT compared with RD, indicating adipocyte dysfunction, which was normalized by testosterone dosing. Accordingly, perilipin, an anti-lipolytic protein, was significantly increased in HFD VAT, when compared with other groups. HFD VAT was hypoxic, while testosterone dosing normalized VAT oxygenation. In VAT, androgen receptor expression was

positively associated with mRNA expression of *GLUT4* (*SLC2A4*) (insulin-regulated glucose transporter) and *STAMP2* (*STEAP4*) (androgen-dependent gene required for insulin signaling). In testosterone-treated HFD VAT, *STAMP2* mRNA was significantly increased when compared with the other groups. Moreover, GLUT4 membrane translocation was significantly reduced in HFD VAT, compared with RD, and increased by testosterone. In DIM-exposed preadipocytes from HFD, triglyceride accumulation, adipocyte-specific genes, insulin-stimulated triglyceride synthesis, glucose uptake, and GLUT4 membrane translocation were reduced compared with preadipocytes from RD and normalized by *in vivo* testosterone dosing. In conclusion, testosterone dosing in a MetS animal model positively affects VAT functions. This could reflect the ability of testosterone in restoring insulin sensitivity in VAT, thus counteracting metabolic alterations.

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Introduction

The increased prevalence of excessive visceral obesity is closely associated with the rising incidence of cardiovascular diseases (CVD) and type 2 diabetes mellitus. The clustering of cardiovascular risk factors in visceral obesity is often referred to as metabolic syndrome (MetS), which has been recognized to be a complication of adipose tissue dysfunction. It is well known that the main biological function of adipose tissue is to manage lipid storage providing both long-term and short-term regulation and delivery of stored lipids in response to the peripheral energy need. Adipose tissue dysfunction starts when in the face of a nutritional overload there is a failure to

accommodate all nutrients. This results in leakage of nutrients that accumulate in other organs such as liver and muscle. This inappropriate accumulation negatively impacts on the normal metabolic response of these organs (Després & Lemieux 2006). How adipose tissue becomes dysfunctional is still unknown. Besides the excessive visceral deposition, several pathogenetic mechanisms are implicated, including brown to white fat transdifferentiation, mitochondrial damage and decreased mitochondrial biogenesis, an increased adipose cell size, a decreased insulin sensitivity of fat cells, and the failure of their storage function with the consequent peripheral lipotoxicity (Virtue & Vidal-Puig 2010), macrophage infiltration, and inflammation along with qualitative and/or

quantitative changes in adipokine production (Vettor *et al.* 2005), alteration in the angiogenetic process, and fibrosis development. Recently, stem cell abnormalities have also been described with alteration of the adipogenic process within adipose tissue or as an abnormal adipogenetic differentiation of stem cells residing in other organs (Aguiri *et al.* 2008, Vettor *et al.* 2009). The resulting metabolic consequences include abdominal obesity, insulin resistance, and atherogenic dyslipidemia along with a pro-thrombotic, inflammatory profile that defines the MetS.

MetS is often associated with testosterone deficiency in males (Laaksonen *et al.* 2003, Kapoor *et al.* 2005). A recent meta-analysis of the available cross-sectional studies on the association between MetS and hypogonadism found that patients with MetS had significantly lower testosterone plasma levels (about 3 nmol/l), in comparison with age-matched subjects (Corona *et al.* 2011a). In particular, androgen deficiency in men is associated with insulin resistance and obesity and testosterone treatment of hypogonadal patients improves insulin sensitivity and reduces fat mass (Corona *et al.* 2011a). Despite this evidence, the pathogenetic link and the clinical significance of MetS-associated male hypogonadism have not been completely clarified (Corona *et al.* 2011b). Epidemiological studies showed that MetS could predict the development of male hypogonadism (Niskanen *et al.* 2004, Esposito *et al.* 2008), which in turn may be a risk factor for MetS and CVD (Stellato *et al.* 2000, Oh *et al.* 2002, Corona *et al.* 2006, Kupelian *et al.* 2006, Rosano *et al.* 2007). Elevated waist circumference and hypertriglyceridemia resulted in the most important MetS determinants of hypogonadism (Corona *et al.* 2009). Thus, accumulation of visceral adipose tissue (VAT) may be regarded as the pathogenetic event linking these two conditions, even if the cause–effect relationship remains to be elucidated.

The precise role of androgens in regulating metabolism and body fat in males has been investigated in several animal studies, with the most important lesson being that derived from androgen receptor (AR) knockout (ARKO) mice (Yanase *et al.* 2008). The results from ARKO models revealed that AR plays an important role in male metabolism, by affecting the energy balance and lipolysis (Sato *et al.* 2003, Fan *et al.* 2005). A negative effect of AR activation on visceral adiposity has been clearly demonstrated in ARKO mice (Yanase *et al.* 2008). Accordingly, *in vitro* studies demonstrated that androgens inhibit adipogenic differentiation of mouse or human preadipocytes through an AR-mediated pathway (Singh *et al.* 2006, Gupta *et al.* 2008). By contrast, studies on the relationship between the androgen–AR system and insulin sensitivity produced conflicting results, showing both negative (Sato *et al.* 2003, Fan *et al.* 2005) and positive (Lin *et al.* 2005) effects.

Recently, we developed a non-genomic animal model of MetS, by exposing rabbits to a high-fat diet (HFD). Such a model essentially recapitulates the human MetS phenotype (hypertension, hyperglycemia, dyslipidemia, visceral fat

accumulation, and reduced glucose tolerance), including a condition of hypogonadotropic hypogonadism (Filippi *et al.* 2009). Interestingly, testosterone dosing in MetS rabbits not only drastically prevented the HFD-induced VAT expansion but also reduced fasting glucose and improved glucose tolerance (Filippi *et al.* 2009, Vignozzi *et al.* 2011, 2012, Morelli *et al.* 2012a,b). Using this HFD-induced MetS model, the aim of this study is to investigate the role played by testosterone in adipose tissue dysfunction in this experimental MetS condition. Along with the evaluation of both morphological and functional VAT features, the study is focused on analyzing the adipogenic capacity of VAT preadipocytes, isolated from the MetS model, treated or not *in vivo* with testosterone. In particular, the ability of preadipocytes in responding to insulin is investigated in terms of triglyceride synthesis and lipid droplet formation, mRNA expression of adipogenesis-specific genes, as well as glucose uptake and glucose transporter 4 (GLUT4) membrane localization. Hence, this rabbit model responded well to the scope of the study aimed at analyzing adipose tissue function, given the sufficient amount of visceral fat to enable different experimental procedures (whole tissue analysis, isolation of fresh cell cultures of preadipocytes sufficient to perform several *in vitro* experiments at passage one). On the other hand, the main cons of using rabbits is the unfeasibility of procedures (i.e. microcomputed tomography (micro-CT) imaging) to measure body fat distribution.

Materials and Methods

MetS rabbit model

A rabbit model of MetS has been obtained in male New Zealand White rabbits (Charles River, Calco, Lecco, Italy), exposing them to a HFD, as described previously (Filippi *et al.* 2009). Animals were randomly numbered and assigned to different groups: untreated group ($n=35$) fed a regular diet (RD) or treated group ($n=51$) fed a HFD (0.5% cholesterol and 4% peanut oil) for 12 weeks. A subset of HFD rabbits ($n=19$) was treated with i.m. injection of testosterone (30 mg/kg, weekly for 12 weeks). Blood samples were obtained from the animals via marginal ear vein at time 0 (baseline) and at week 12 in all groups. Mean arterial pressure and oral glucose tolerance test (OGTT) were measured, at the time of killing, as described previously (Filippi *et al.* 2009). Afterward, the rabbits were killed by a lethal dose of pentobarbital. VAT was harvested from the different experimental groups and appropriately processed for the subsequent analyses and preadipocyte isolation. Biochemical and hormonal serum analyses were performed as described previously (Filippi *et al.* 2009, Morelli *et al.* 2012a,b, Vignozzi *et al.* 2012). Animal handling complied with the Institutional Animal Care and Use Committee of the University of Florence, Florence, Italy, in accordance with the Italian Ministerial Law # 116/92.

Histomorphometric analysis of VAT

VAT specimens from all rabbit groups were removed, weighted, and fixed in 4% formalin in PBS. Tissues were then processed routinely for paraffin embedding, and 3 µm-thick sections were prepared for histomorphometric analysis. For each VAT sample, three to five sections were cut along the length. Hematoxylin and eosin-stained sections were used for direct microscopic examination. Negative staining controls were used in order to evaluate and subtract background. The diameter of adipocytes was measured using the Nikon Microphot-FXA microscope, considering the adipocytes more regularly spherical and using the program ImageJ.

Hypoxia detection and immunohistochemistry

VAT oxygenation was analyzed using the bio-reductive drug pimonidazole hydrochloride (Hypoxyprobe-1, 60 mg/kg), i.p. injected 1 h before killing, as described previously (Morelli *et al.* 2012a,b, Vignozzi *et al.* 2012). VAT samples were rapidly removed and fixed in 4% neutral-buffered formalin, dehydrated, and embedded in paraffin. Hypoxic cells were then easily detected by immunohistochemistry with a MAB (Hypoxyprobe-1Mab1), following the manufacturer's instruction. Computer-assisted quantification of the hypoxyprobe positivity has been made after background subtraction using Adobe Photoshop 6.0 software (Adobe Systems).

Preparation of total and membrane/cytosolic fractions for western blot analysis

VAT samples were ground in liquid nitrogen and divided into two aliquots: one for total protein extraction and the other for membrane/cytosolic preparations. Membrane and cytosolic fractions were prepared using the ProteoExtract sub-cellular proteome extraction kit (Calbiochem-Merck KGaA, Darmstadt, Germany), according to the manufacturer's instructions. Protein extracts were quantified with the BCA reagent (Pierce, Rockford, IL, USA). Equal amounts of proteins (15 µg) were loaded onto 10% SDS-PAGE and then transferred onto PVDF membrane (ImmobiloneP, Millipore Corporation, Bedford, MA, USA). After blocking in TRIS buffer saline (0.1% Tween 20) and 5% skimmed milk for 1 h, membranes were incubated with primary antibodies (anti-GLUT4 (1:1000), Upstate Biotechnology, Lake Placid, NY, USA; anti-perilipin (1:1000), anti-pAKT (1:1000), anti-AKT (1:1000), and anti-STAT1 (1:1000), Santa Cruz Biotechnology, Inc.) overnight at 4 °C followed by respective secondary antibody. Densitometry analysis of band intensity was performed using Photoshop 5.5 software (Adobe Systems, Inc. Italia srl).

Isolation and culture of rabbit preadipocytes (rPADs)

rPAD isolation was performed according to Zuk *et al.* (2001). Briefly, after dissection under sterile conditions, VAT samples

were immediately placed in serum-free (SF) DMEM/F12 (Sigma-Aldrich) supplemented with 200 µg/ml streptomycin and 200 U/ml penicillin. Then, VAT samples were washed in PBS, minced, and digested with 1 mg/ml collagenase type 2 (Sigma-Aldrich) in PBS, in a shaking water bath at 37 °C for 1 h. Collagenase type 2 was inactivated with an equal volume of DMEM containing 10% fetal bovine serum (FBS) and the pellet was collected by centrifugation at 2000 *g* for 10 min at room temperature (RT). The cellular pellet was treated with red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 10 min at RT, then centrifuged, resuspended in DMEM/10% FBS, and filtered through a 150 µm mesh filter to remove debris. The filtrate was centrifuged as detailed earlier and the cellular pellet was plated onto 100 mm cell culture dishes in complete culture medium (DMEM containing 10% FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 1 µg/ml amphotericin-B; Sigma-Aldrich). Cells were cultured at 37 °C in humidified atmosphere of 95% air-5% CO₂. After 24 h, non-adherent cells were removed, while adherent cells were washed twice with PBS and left to grow in complete medium. A sub-confluent (90% of cell culture dish) and homogeneous fibroblast-like cell population at passage 0 (P0) was obtained after 4–5 days of culture. Sub-confluent cells were trypsinized and plated onto cell culture dishes (P1). For all the experiments, only P1 culture was used, and the experiments were repeated using at least three different rPAD preparations for each experimental group.

Flow cytometry analysis

rPADs were analyzed by flow cytometry for the expression of the following conjugated MABs: CD34-PE, CD45-FITC, CD31-FITC, CD14-PE, CD90-PE, CD106-FITC (BD Pharmingen, San Diego, CA, USA), and CD105 PE (Ancell, Bayport, MN, USA), as described previously (Mazzanti *et al.* 2008). Briefly, cells were harvested in 0.25% trypsin/EDTA and washed in Hank's Balanced Salt Solution (HBSS) containing 10% FBS. After re-suspension in the flow cytometry buffer CellWASH (PBS, 2% FBS, 0.1% sodium azide; Becton Dickinson, Milan, Italy) at a concentration of 1.5×10^5 cells/100 µl, cells were incubated with the specific antibodies for 20 min. Aminoactinomycin D (7-AAD) was added to exclude dead cells from the analysis. At least 10^4 cells were acquired from each sample on FACSCalibur instrument (Becton Dickinson) and analyzed using the CellQuest software. Data are expressed as percentage of marker-positive cells over total and are reported as the mean $\pm$ s.d. of the mean of *n* different rPAD preparations from each experimental group.

In vitro adipogenic differentiation

rPADs plated in six-well (8×10^4 cells/well) or in 96-well (9×10^3 cells/well) plates were cultured until confluence. Two days after reaching confluence (time 0), the cells were stimulated with differentiation mixture (DIM) containing

5 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in 5% stripped FBS (FBS S)-supplemented DMEM for 8 days (Student *et al.* 1980). The culture medium was replaced every 48 h, and then cells were shifted to medium containing 10 g/ml insulin for 48 h. The absence of testosterone in the culture medium was verified by measuring the hormone concentration in aliquots of FBS S/DMEM. Some aliquots of FBS S/DMEM were measured after adding a known concentration of testosterone (8 nM) as internal control: the total recovery of testosterone was 8.65 ± 0.8 nM.

Qualitative and quantitative estimation of triglyceride accumulation

DIM-rPADs were stained with Oil Red O to detect qualitative accumulation of intracellular lipids (Halvorsen *et al.* 2001). Dishes were washed in PBS, and cells were fixed in 10% formalin for 1 h at RT, followed by staining with Oil Red O for 5 min. Oil Red O was prepared by diluting a stock solution (0.3 g Oil Red O in 100 ml isopropanol) with water (3:2) followed by filtration. After staining, plates were washed twice in water and photographed. Quantitative estimation of triglyceride accumulation was performed by Adipored Assay (Cambrex BioScience, Walkersville, MD, USA). Briefly, after incubation with DIM for 10 days, the medium was removed and each well carefully rinsed with 200 µl PBS. Each well was then filled with 200 µl PBS and 5 µl di Adipored, incubated at RT for 10–15 min and immediately placed in the fluorimeter for fluorescence measurement (excitation at 485 nm and emission at 572 nm). Triglyceride content was normalized on protein content and adipogenic ability was expressed as percentage change vs untreated cells.

DIM-treated rPADs, Adipored stained, were imaged immediately using a Leica DMI6000 microscope equipped with a DFC350FX camera. Images were acquired using the Leica N3 filter set and a Fluotar 20× 0.4NA long-working distance objective with correction collar. Adipored-positive cells, identified as those clearly showing lipid droplets staining, were counted using ImageJ software and expressed as percentage change compared with total cells.

Confocal microscopy

DIM-treated rPADs, Adipored stained, were imaged immediately using a Leica SP2-AOBS confocal microscope. Images were collected as z-stacks through a 63× 1.2NA water-immersion objective, sampling according to the Nyquist criterion and taking care to minimize spherical aberration by adjusting the objective correction collar. Images were then deconvolved with Huygens Professional software (SVI, The Netherlands) using the Classic Maximum Likelihood Estimation (CMLE) algorithm and a theoretical Point Spread Function (PSF). Finally, deconvolved images were quantitatively analyzed using Volocity 5 software (Perkin-Elmer, Foster City, CA, USA) to measure the number and volume of lipid droplets.

GLUT4 and SNAP23 immunolocalization

DIM-treated rPADs were cultured for 24 h in SF DMEM and then incubated for 5 min with insulin (100 nM). Then, cells were treated for 15 min with membrane marker wheat germ agglutinin, rhodamine conjugated (WGA, 1:250, Vector Laboratories, Burlingame, CA, USA) and fixed with 3.7% paraformaldehyde (pH 7.4) for 10 min. Immunostaining was performed using anti-GLUT4 (1:40; Abcam Ltd, Cambridge, UK), followed by goat anti-mouse Alexa Fluor 488 secondary antibody (A11001, 1:200; Molecular Probes), or anti-SNAP23

Table 1 Primer sequences for quantitative RT-PCR in rabbit samples

Genes	Primer sequences (5'–3')
CD90	F: TGACAGTCTTGACAGGTGTCC R: ACGCTCGTGGTGTTCTCAT
DKK1	F: GACATGCGGTGGACTCCTAT R: CAGGGGAGTTCATCAAGAA
PPARG	F: TGGGGATGTCTCATAATGCCA R: TTCCTGTCAAGATCGCCCTCG
CEBPA	F: TGGACAAGAACAGCAACGAG R: TTGTCACTGGTCAGCTCCAG
FABP4	F: GGATGGAAAATCAACCACCA R: GTGGAAGTGACCGCTTTCAT
ADPN	F: ACCAGGACAAGAACGTGGAC R: GAAGGAAGCCAGTGGAGATG
LEPT	F: GGAAAGTCCAGGATGACACC R: TCCATTGTGGACAAGCTCAG
DGAT2	F: TCCAGTGGGTCTTGTCCTTC R: CCAGCCAGGTGAAGTAGAGC
SREBP2	F: GGCGGACAAGACAAATATCA R: GTCCCATGACCAAGCTTTTCA
LPL	F: GACATTGGGGAGTTGCTGAT R: TTTCTCCCTGGAGCAGAAGA
HSL	F: AGAGCCCCCTCTAGCTCAAC R: CAAAGATTCTGCTGCTGATT
PPARA	F: GGCCTGGCCTTCTAAACATA R: TTCTTGATGACCTGCACGAG
GLUT4	F: CTTCCAGACAGCAGGGGTAG R: AGAAGCAGAGCCACGTTTCT
SNAP23	F: AGAAGAAGGCATGGACCAGA R: CTGGCTGCTTAGACACCACA
SYNT5	F: CATCCAGAGTCGACGACACA R: CGTCGATCCTCTGAATGGTT
VAMP4	F: AAGTTCAAGCGCCACCTAAA R: CCACCTGATTCTGAACATGC
VIM	F: TGCAGGAAGAGATTGCCCTT R: GAGGTCAGGCTTGAAACAT
CCND1	F: GAAGGACATCCAGCCCTACA R: AGGAAGCGGTCCAGGTAGTT
CCND3	F: GAAGCTGCATCAGGGAGAG R: GATGGCTGTGACATCTGTGG
BAX	F: CCTTTTGCTTCAGGGTTTCA R: ATCCTCTGCAGTCCATGTT

CD90, cluster of differentiation 90; DKK1, dickkopf 1; CEBPA, CCAAT enhancer binding protein α; PPARG, peroxisome proliferator-activated receptors-gamma; FABP4, fatty acid binding protein 4; ADPN, adiponectin; LEPT, leptin; DGAT2, diacylglycerol O-acyltransferase 2; SREBP2, sterol regulatory element binding protein-2; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase; PPARA, peroxisome proliferator-activated receptors-α; GLUT4, glucose transporter type 4; SNAP23, synaptosomal-associated protein 23; SYNT5, syntaxin 5; VAMP4, vesicle-associated membrane protein 4; VIM, vimentin; CCND1, cyclin D1; CCND3, cyclin D3; BAX, Bcl2-associated X protein.

(1:100; Santa Cruz Biotechnology), followed by mouse anti-goat FITC conjugate secondary antibody (1:50; Sigma–Aldrich). GLUT4/WGA or SNAP23/WGA colocalization was detected with Leica SP2-AOBS confocal microscope and calculated using the COLOC plugin of ImageJ software. Results are given for Pearson overlap algorithm.

Glucose uptake

Glucose uptake in rPADs was performed as described previously (Raimondi *et al.* 2004). After 10 days of exposure to DIM, rPADs were cultured for 24 h in SF DMEM. Insulin-dependent stimulation was evaluated after 30 min of exposure to increasing concentrations of insulin (1, 5, 10, and 50 nM) diluted in glucose-free Krebs phosphate buffer (2.5 mmol Ca²⁺, 1 mg/ml BSA). At the end of the incubation, glucose uptake was started by adding [³H]2-deoxy-D-glucose (16 µM [1 µCi/µl]; ICN Pharmaceuticals, Costa Mesa, CA, USA) for 5 min. Cells were then washed with PBS, lysed with NaOH 0.5 M, and radioactivity incorporated was measured by scintillation liquid using a B-counter (Perkin-Elmer). Data were normalized on protein content.

RNA extraction and quantitative RT-PCR analysis

Isolation of RNA from tissue and cells was performed as described previously (Morelli *et al.* 2012a,b). cDNA synthesis

was carried out using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative RT-PCR (qRT-PCR) was performed using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories) as previously reported (Morelli *et al.* 2012a,b). Specific primers for all the target genes were either previously reported (Filippi *et al.* 2009, Morelli *et al.* 2012a,b, Vignozzi *et al.* 2012) or reported in Table 1. The expression of 18S ribosomal RNA subunit, quantified with a pre-developed assay (Applied Biosystems), was chosen as reference gene and used for relative quantitation of the target genes. Amplification and detection were performed with the MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories).

Statistical analysis

Results are expressed as mean ± S.D. or S.E.M. for *n* experiments as specified. Statistical analysis was performed with one-way ANOVA test followed by Tukey–Kramer *post hoc* analysis in order to evaluate differences between groups and *P* < 0.05 was considered significant. Correlations were assessed using Spearman's method and statistical analysis was performed on Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) for Windows 15.0. Half-maximal response effective concentration (EC₅₀) values and maximal effect (*E*_{max}) values were calculated using the computer program ALLFIT (De Lean *et al.* 1978).

Table 2 Metabolic and hormonal parameters in experimental rabbits. Results are reported as mean ± S.D. of the mean

	RD (<i>n</i> =35)	HFD (<i>n</i> =32)	HFD + testosterone (<i>n</i> =19)
Total body weight (g)			
Baseline	3211.9 ± 281.6	3211.2 ± 333.7	3175.4 ± 300
Week 12	3938 ± 242 [†]	3668.7 ± 297 [†]	3804.5 ± 266.3 [†]
Blood glucose (g/l)			
Baseline	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
Week 12	1.2 ± 0.2	1.9 ± 0.4 ^{*,§}	1.4 ± 0.3 ^{*,}
OGTT (iAUC)			
Week 12	158.2 ± 33.1	226.5 ± 43 [†]	177 ± 25.3
Cholesterol (mg/dl)			
Baseline	38.2 ± 13.6	43.9 ± 13	49.5 ± 13.5
Week 12	45.3 ± 19	1398.9 ± 349.5 ^{*,§}	1244.6 ± 228.3 ^{*,§}
Triglycerides (mg/dl)			
Baseline	86.1 ± 26.6	85.4 ± 24	91.4 ± 29
Week 12	102.7 ± 29	299.8 ± 156.1 ^{*,§}	293.1 ± 115.5 ^{*,§}
MAP (mmHg)			
Week 12	92.9 ± 13	134.9 ± 21.6 [§]	113.5 ± 8.5 ^{§,}
Testosterone (nmol/l)			
Baseline	6.2 ± 8	8.7 ± 10.1	4.9 ± 3.05
Week 12	6.1 ± 4.4	1.5 ± 1.6 ^{*,§}	17.6 ± 12.5 ^{*,§,}
17β-Estradiol (pmol/l)			
Baseline	150.5 ± 43.6	146.4 ± 51	140.2 ± 40.3
Week 12	168.7 ± 53.6	307.6 ± 104.1 ^{*,§}	161.9 ± 46.1
Seminal vesicles weight (mg)			
Week 12	729.4 ± 207	491.7 ± 142 [§]	1551.5 ± 438 ^{§,}
Testis weight (g)			
Week 12	3.5 ± 0.3	3 ± 0.3 [§]	1.7 ± 0.3 ^{§,}

iAUC, incremental area under the curve of glucose blood level during OGTT; MAP, mean arterial pressure. **P* < 0.05, [†]*P* < 0.01, [‡]*P* < 0.001 vs baseline; [§]*P* < 0.001 vs RD week 12; ^{||}*P* < 0.01, [¶]*P* < 0.001 vs HFD week 12.

Figure 1 Relationship between the number of MetS components (abscissa) and the incremental area under the curve (AUC) of glucose during OGTT (A, $n=52$, ordinate), ΔT (B, $n=59$, ordinate), or ΔE_2 (C, $n=61$, ordinate), as derived from univariate Spearman's regression analysis. The relative coefficient of correlation (r) and level of significance (P) are also reported.

Results

MetS features in experimental rabbits

Table 2 shows the main physical and biochemical characteristics of the different experimental groups. No statistical differences of total body weight after 12 weeks among groups have been found (Table 2). To evaluate the effects of MetS, we designed an algorithm taking into account the presence, as a dummy variable, of one or more of the following factors: hyperglycemia, high triglyceride, high cholesterol, increased blood pressure, and visceral fat accumulation. Cutoffs for each factor were derived by the mean $\pm$ two s.d. of the analyzed parameter, as measured in RD rabbits. Positivity for three or more factors identifies MetS.

We found that glucose intolerance, as evaluated by the OGTT, increased as a function of the number of MetS components ($r=0.650$; $P<0.0001$; Fig. 1A). MetS rabbits were characterized by a sex steroid imbalance, including reduced testosterone and elevated estradiol (E_2) plasma levels, as previously published (Filippi *et al.* 2009, Morelli *et al.* 2012a,b, Vignozzi *et al.* 2012). Interestingly, ΔT (testosterone at 12 weeks – testosterone at baseline) negatively ($r=-0.558$; $P<0.0001$) while ΔE_2 (E_2 at 12 weeks – E_2 at baseline) positively ($r=0.492$; $P<0.0001$) correlated with the number of MetS components (Fig. 1B and C respectively).

Significant associations were also found between ΔT and/or ΔE_2 and the mRNA expression of several genes in VAT, as reported in Fig. 2 and Table 3. In particular, ΔT positively, and ΔE_2 negatively, correlated with the glucose transporter *GLUT4* ($r=0.430$, $P<0.0001$ and $r=-0.257$, $P<0.05$ respectively, Fig. 2A and B). By contrast, the insulin-sensitizing adipokine adiponectin (*ADPN* (*ADIPOQ*)) was highly testosterone sensitive ($r=0.464$, $P<0.0001$; Fig. 2C), while the hypoxia marker *CA9* was estrogen sensitive ($r=0.454$, $P<0.01$; Fig. 2D). Other genes involved in lipid turnover along with their relationship with sex steroid variation are summarized in Table 3. The expression of the apoptosis-related gene *BAX* was not only upregulated by ΔT ($r=0.335$, $P=0.01$; Table 3) but also tightly associated with the expression of *AR* in VAT ($r=0.375$, $P<0.005$; Table 4).

We next analyzed other associations between *AR* mRNA and the VAT expression of several genes involved in lipid turnover. As shown in Table 4, significant positive associations were found between *AR* and genes regulating adipogenesis (*PPARG*, *CEBPA*, *FABP4*, *PPARA*, *ADPN*, and *LEPT*), glucose transport and insulin signaling (*GLUT4*, *RHOA*, *ROCK2*, *VIM*, and *STAMP2*), and lipid droplet formation (*VAMP4*, *SYNT5*, and *SNAP23*). By contrast, *AR* mRNA was negatively associated with the early-induced adipogenic gene, *DKK1* (Table 4).

To further characterize the role of testosterone in MetS-related VAT dysfunctions, we treated a subset of HFD rabbits with testosterone (30 mg/kg per week) for 12 weeks. We found that testosterone dosing to HFD rabbits was able to not only restore plasma testosterone and E_2 levels (Table 2)

Figure 2 Relationship between *GLUT4* mRNA (ordinate) and ΔT (A, abscissa) or ΔE_2 (B, abscissa), as derived from univariate Spearman's regression analysis ($n=65$). (C) Relationship between *ADPN* mRNA (ordinate) and ΔT (abscissa), as derived from univariate Spearman's regression analysis ($n=60$). (D) Relationship between *CA9* mRNA (ordinate) and ΔE_2 (abscissa), as derived from univariate Spearman's regression analysis ($n=54$). The relative coefficient of correlation (r) and level of significance (P) are reported within the text. Solid lines indicate the 95% confidence interval of the regression line.

but also decrease the prevalence of MetS (from 82% in HFD to 18.2% in HFD + testosterone, $P<0.01$), by decreasing the number of components fulfilling MetS criteria (HFD = 2.54 ± 0.15 vs HFD + testosterone = 1.86 ± 0.18 , $P=0.005$). In particular, testosterone dosing dramatically reduced VAT weight (HFD = 41.51 ± 2.05 vs HFD + T = 4.37 ± 0.87 , $P<0.0001$, Fig. 3A, B, C and D). Histomorphometric analysis evidenced that adipocyte size was significantly increased in HFD rabbits, compared with the RD group ($P<0.05$), and reduced by testosterone dosing ($P<0.001$ vs RD and HFD; Fig. 3E, F, G and H). In addition, HFD VAT was hypoxic, as demonstrated by the significant increase of hypoxyprobe positivity ($P<0.0001$ vs RD; Fig. 3I, J, and L), while testosterone treatment normalized VAT oxygenation ($P<0.0001$ vs HFD; Fig. 3J, K and L).

Western blot analysis showed that the expression of the anti-lipolytic protein perilipin was significantly increased in VAT from HFD rabbits, compared with RD and testosterone-treated HFD groups ($P=0.001$, Fig. 3M). In addition, mRNA expression of *STAMP2*, a gene induced by androgens and required for normal insulin signaling, was significantly increased in VAT from testosterone-treated HFD rabbits, when compared with RD and untreated HFD groups ($P<0.02$; Fig. 4A).

To further investigate the effects of HFD and testosterone treatment on insulin signaling, we analyzed the intracellular localization of GLUT4 and the pAKT/AKT ratio in VAT protein extracts. Figure 4B shows that GLUT4 membrane translocation was significantly reduced in HFD compared with the RD group ($P=0.02$), while it was restored in VAT from testosterone-treated HFD rabbits. Similarly, HFD VAT showed a reduced pAKT/AKT ratio compared with the RD group ($P=0.03$; Fig. 4C), that was normalized by testosterone ($P<0.05$ vs HFD).

Molecular and functional characterization of visceral adipocyte precursor cells from experimental rabbits

To investigate the effects of HFD and *in vivo* testosterone treatment on VAT adipogenic capacity, we isolated rPADs from VAT of all experimental groups. Each cell preparation was characterized by flow cytometry for the expression of mesenchymal stem cell (MSC) markers and hematopoietic-monocytic contamination. The percentage of positive cells expressing MSC markers CD90, CD105, and CD106 was not statistically different among the different groups, as reported in Table 5. All rPADs were negative for endothelial (CD31), hemopoietic (CD34 and CD45), and monocytic (CD14) markers. Expression analysis by qRT-PCR showed that *CD90* (*THY1*) was the most abundant gene in all rPADs, followed by the two adipocyte commitment markers *DKK1* and *FABP4*, without statistical differences among the groups (Table 5).

The adipogenic potential of rPADs was investigated by exposing *in vitro* cells to a DIM for 10 days. The qualitative estimation by Oil Red O staining of triglyceride accumulation showed an increase in lipid droplets in the cytosol of all rPAD groups when compared with untreated cells (Fig. 5A). Quantitative analysis by Adipored assay of triglyceride

Table 3 Association between ΔT or ΔE_2 and mRNA expression lipogenic/lipolytic genes in VAT

	ΔT		ΔE_2	
	r	P value	r	P value
Lipogenic genes				
PPARG	0.343	0.007	-0.178	0.178
ADPN	0.464	<0.0001	-0.239	0.071
LEPT	0.414	0.001	-0.223	0.096
DGAT2	0.449	0.001	-0.425	0.002
SREBP2	0.474	0.003	-0.470	0.003
STAR	0.390	0.025	-0.566	0.001
Lipolytic genes				
LPL	0.508	<0.0001	-0.332	0.011
PPARA	0.275	0.032	-0.122	0.359
HSL	0.283	0.049	-0.482	<0.0001
Apoptotic gene				
BAX	0.335	0.01	-0.189	0.155

Correlation coefficients (r) and level of significance (P value) are derived from univariate analysis.

Table 4 Association between AR mRNA and VAT-specific genes

	<i>r</i>	<i>P</i> value
Adipogenesis		
<i>DKK1</i>	−0.372	0.004
<i>PPARG</i>	0.560	<0.0001
<i>CEBPA</i>	0.620	<0.0001
<i>FABP4</i>	0.610	<0.0001
<i>ADPN</i>	0.281	0.033
<i>LEPT</i>	0.308	0.020
<i>PPARA</i>	0.572	<0.0001
Glucose transport and insulin signaling		
<i>GLUT4</i>	0.291	0.026
<i>RHOA</i>	0.354	0.007
<i>ROCK2</i>	0.315	0.017
<i>STAMP2</i>	0.474	<0.0001
<i>VIM</i>	0.571	<0.0001
Lipid droplet formation		
<i>VAMP4</i>	0.535	<0.0001
<i>SYNT5</i>	0.359	0.010
<i>SNAP23</i>	0.476	<0.0001
Apoptosis		
<i>BAX</i>	0.375	0.004

Correlation coefficients (*r*) and level of significance (*P* value) are derived from univariate analysis.

content revealed a significant increase in Adipored positivity in DIM-exposed rPADs from each group when compared with the untreated counterpart, although to a different extent (Fig. 5B). In fact, while in rPADs from RD, DIM induced a 534% increase over the basal level ($P<0.0001$), the increase in rPADs from HFD was modest (66%), although still statistically significant ($P<0.05$). *In vivo* testosterone dosing partially restores responsiveness to DIM in rPAD from HFD (262% increase, $P<0.0001$). Adipored positivity in DIM-exposed rPADs from HFD was statistically different when compared with all other groups ($P<0.01$, Fig. 5B).

The percentage of Adipored-positive cells, calculated by fluorescence microscopy inspection after DIM exposure, was significantly lower in rPADs from HFD than in rPADs from RD ($P<0.01$; Fig. 6A, B and G) and rPAD from testosterone-treated HFD ($P<0.01$; Fig. 6B, C and G). By confocal microscopy, we analyzed the lipid droplet content within the single cell. As shown in Fig. 6, lipid droplets from DIM-treated rPADs from HFD showed both a reduction in the average number (Fig. 6E and H; $P<0.05$) and an increase in the size (Fig. 6E and I; $P<0.0001$), when compared with those from DIM-treated rPADs from RD (Fig. 6D, H and I). Interestingly, both number and size of lipid droplets of DIM-treated rPAD from testosterone-treated HFD were comparable to RD levels (Fig. 6F, H and I).

The responsiveness of rPADs to DIM has also been investigated in terms of expression of adipocyte-related genes (*PPARG*, *DKK1*, *FABP4*, *ADPN*, and *LEPT*). As reported in Table 6, in rPADs from RD, there was a significant induction of all the investigated genes after 10 days of exposure to DIM, when compared with the basal (time 0) expression ($P<0.01$). In rPADs from HFD, DIM exposure was able to significantly

induce *FABP4* mRNA, without changing the expression of all the other genes investigated, which was significantly reduced ($P<0.01$ vs the other groups). DIM-induced expression of all adipocyte-specific genes, with the exception of *LEPT*, was observed in rPAD from testosterone-treated HFD (Table 6). Similarly, cyclin D3 (*CCND3*) mRNA expression, which has been described to become predominant during adipogenic differentiation (Hishida *et al.* 2008), was significantly induced in all DIM-treated rPADs, with the exception of rPAD from HFD (Table 6). On the contrary, cyclin D1 (*CCND1*) mRNA, which is usually unregulated during the period of mitotic expansion that precedes adipogenic differentiation (Hishida *et al.* 2008), was significantly increased only in DIM-treated rPADs from HFD and not in the other groups ($P<0.05$, Table 6).

To better investigate whether the reduced ability of rPADs from HFD in responding to DIM was due to an impaired sensitivity to insulin, we analyzed the lipid content in DIM-treated rPADs after exposure to increasing concentrations of insulin. As shown in Fig. 7A, the percentage of triglyceride accumulation in response to insulin increased in a dose-dependent manner, with the same EC_{50} for all rPADs (shared $EC_{50}=99\pm84$ nM). However, the maximal effect of insulin was significantly reduced in rPADs from HFD compared with RD and testosterone-treated HFD, which was not different ($E_{max}=111.8\pm4.5$ vs shared $E_{max}=140\pm11$, $P<0.01$). Insulin sensitivity of rPADs was then investigated in terms of glucose uptake induced by increasing concentrations of insulin. Although insulin dose dependently stimulated glucose uptake in all rPADs with the same EC_{50} (shared $EC_{50}=3.8\pm1.6$ nM; Fig. 7B), the maximal effect was significantly reduced in rPADs from HFD ($E_{max}=111\pm12\%$) compared with the other rPADs (shared $E_{max}=219\pm15\%$, $P<0.01$).

To determine the insulin-stimulated glucose transport vesicles trafficking in DIM-treated rPADs, we examined the GLUT4 and SNAP23 membrane localization after insulin stimulation (100 nM, 5 min). Using confocal microscopy and WGA a as membrane marker, we found that in rPADs from HFD, insulin stimulation was less effective in stimulating GLUT4 and SNAP23 membrane translocation (8 ± 0.3 and $19.3\pm0.9\%$; $P<0.01$ respectively) when compared with RD (26.4 ± 5.7 and $24.2\pm0.9\%$ respectively, both $P<0.01$; Fig. 8). GLUT4 and SNAP23 membrane translocation in rPADs from testosterone-treated rabbits was comparable to those from RD (29.2 ± 0.8 and $27.1\pm0.9\%$ respectively; Fig. 8).

Discussion

This study demonstrates that in a animal model of MetS (diet-induced), VAT is not only increased in mass but it is also dysfunctional, showing morphostructural alterations, along with an impaired insulin-stimulated adipogenic ability of the preadipocyte component. *In vivo* testosterone dosing to MetS

Figure 3 Effects of testosterone treatment on VAT weight, adipocyte size, and hypoxia in experimental rabbits. (A, B, and C) Pictures show fat accumulation at the visceral level within the intestinal loops. (D) VAT weight was significantly increased in HFD rabbits ($n=32$), when compared with RD ($n=35$) and testosterone-treated HFD rabbits (HFD + T; $n=19$). (E, F and G) Representative images of hematoxylin/eosin-stained VAT sections showing different adipocyte size among experimental groups (magnification $20\times$). (H) Histomorphometric analysis of adipocyte diameter (μm) in the different experimental groups ($n=3$ for each group). (I, J, K, L) Immunohistochemical staining of hypoxyprobe adducts in VAT sections. Hypoxyprobe adducts were revealed in hypoxic cells ($\text{PO}_2 < 10 \text{ mmHg}$) of VAT transverse sections by a MAB (magnification $12.5\times$). Only scanty positive labeling is present in RD (I) and testosterone-treated HFD (K) VAT, while intense hypoxyprobe positivity is detected in HFD VAT (J). (L) Computer-assisted quantitative image analysis of three independent experiments ($n=3$ for each group). RD optical density was taken as 100%. (M) Protein expression of perilipin in VAT extracts from experimental rabbits. Representative immunoblots with anti-perilipin and anti-STAT1 primary antibodies and the corresponding graphical representation of optical density (OD) analysis of perilipin band intensity normalized over STAT1 ($n=3$ for each group) are reported. Data are mean $\pm$ S.E.M. expressed as percentage of RD values * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ vs RD, ° $P < 0.0001$ vs HFD and # $P = 0.001$ vs all groups. Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0333>.

Figure 4 Analysis of the *STAMP2* mRNA expression, GLUT4 membrane translocation, and pAKT/AKT ratio in VAT. (A) Quantitative analysis using real-time RT-PCR for *STAMP2* mRNA expression in VAT extracts from all animal groups. Data are calculated according to the comparative cycle threshold method using *18S* ribosomal RNA subunit as the reference gene for normalization and are expressed as percentage of RD values. (B) The lower panel shows the representative immunoblots with anti-GLUT4 primary antibody on membrane (m) and cytosolic (c) fractions of VAT from RD, HFD, and testosterone-treated HFD rabbits. Bar graph shows optical density analysis of membrane/cytosol GLUT4 ratio expressed as mean $\pm$ S.E.M. of at least three different samples from each group. (C) The lower panel shows the representative immunoblots with pAKT and AKT as primary antibodies on total protein extracts of VAT from RD, HFD, and testosterone-treated HFD rabbits. Bar graph shows optical density analysis of pAKT/AKT ratio expressed as mean $\pm$ S.E.M. of at least three different samples from each group. * $P < 0.05$ vs RD, ^o $P < 0.05$ vs HFD.

animals was able to counteract all the HFD-induced VAT alterations, restoring preadipocyte maturation through a clear positive effect on their insulin sensitivity. Overall, these effects of testosterone on adipose tissue functions, along with the improvement of MetS factor (fasting glucose, glucose tolerance, and hypertension), indicate a crucial role played by androgens in metabolic control. Although several previous studies explored the effect of adding testosterone *in vitro* to differentiating preadipocytes (Singh *et al.* 2003, 2006, Gupta *et al.* 2008), this is the first report on a prolonged (3 months) *in vivo* testosterone dosing showing persistent effects in *ex vivo* VAT tissue cultures.

The association of MetS with an imbalance of sex steroid plasma levels (concomitant testosterone decrease and E_2 increase) has been well established in humans (Corona *et al.* 2011a,b), as well as in the HFD-induced rabbit MetS model (Filippi *et al.* 2009). Interestingly, we found that androgenic decreased (ΔT) negatively, while estrogenic increased (ΔE_2) positively, correlated with the number of MetS components. The existence of correlations between sex hormone levels and body fat distribution could also be envisaged but not demonstrated in this study because of the technical difficulties in applying to rabbits the micro-CT system used with small rodents (Quarta *et al.* 2010). Moreover, testosterone dosing in HFD rabbits improved insulin sensitivity (reduction of glycemia, improvement of glucose tolerance, and reduction of VAT). Accordingly, ΔT was positively associated with VAT expression of *GLUT4*, and *ADPN* – two markers of insulin sensitivity – while ΔE_2 negatively associated with *GLUT4* mRNA expression. ΔE_2 also positively associated with VAT expression of the hypoxia marker *CA9*. Hypoxia of adipose tissue represents a determinant mechanism for the dysregulation of tissue function (O'Rourke *et al.* 2011) and insulin resistance (Trayhurn *et al.* 2008). In obese mice, hypoxia is localized and is not systemic, due to hypoperfusion of the rapidly expanding fat mass (Hosogai *et al.* 2007, Ye *et al.* 2007). Accordingly, morphostructural analysis of VAT clearly shows that in MetS animals, the expanded tissue is hypertrophic, characterized not only by a reduced oxygenation but also by an increased adipose cell size. Several studies in the early 1970s have well established that, compared with small adipocytes, large adipocytes exhibit reduced insulin-stimulated glucose uptake (Salans & Dougherty 1971, Smith 1971, Jacobsson & Smith 1972, Olefsky 1976). Consistent with these findings, VAT from MetS rabbits showed a reduced membrane expression of *GLUT4* (Huang & Czech 2007) and a decreased AKT signaling activation, along with an increased expression of perilipin, the main anti-lipolytic protein coating the cytosolic surface of intracellular lipid droplets (Brasaemle 2007).

One of the most striking results of our study is that, besides counteracting the HFD-induced visceral fat mass expansion, testosterone dosing completely prevented all the MetS-related VAT alterations, reducing adipocyte size and hypoxia, as well as restoring membrane *GLUT4*, pAKT/AKT ratio, and perilipin expression. In addition, ΔT positively correlated

Table 5 Characterization of rPADs at passage 1 from each experimental group. (a) Cells were labeled with surface antibodies and analyzed by flow cytometry. Data are expressed as percentage of marker-positive cells and are reported as the mean $\pm$ s.d. of n different rPAD preparations from each experimental group. No statistically significant differences were found among groups. (b) Expression of *CD90* and pre-adipocyte markers by qRT-PCR in rPADs. Data are reported as the mean (s.d.) of n different rPAD preparations from each experimental group. No statistically significant differences were found among groups

	(a) Immunophenotype by flow cytometry			(b) qRT-PCR of <i>CD90</i> and preadipocyte markers		
	CD105	CD90	CD106	<i>CD90</i>	<i>DKK1</i>	<i>FABP4</i>
RD ($n=6$)	35.3 $\pm$ 16.2	20.8 $\pm$ 10	0.3 $\pm$ 0.3	1.5 $\times 10^5$ (1.5 $\times 10^5$)	1.9 $\times 10^3$ (0.7 $\times 10^3$)	2 $\times 10^4$ (2.2 $\times 10^4$)
HFD ($n=6$)	43.7 $\pm$ 11.5	19 $\pm$ 9.8	0.4 $\pm$ 0.2	0.9 $\times 10^5$ (0.7 $\times 10^5$)	2.5 $\times 10^3$ (1.7 $\times 10^3$)	0.4 $\times 10^4$ (0.2 $\times 10^4$)
HFD+T ($n=5$)	59.7 $\pm$ 28.3	14.8 $\pm$ 7.6	0.2 $\pm$ 0.02	1.4 $\times 10^5$ (0.9 $\times 10^5$)	5.8 $\times 10^3$ (2.2 $\times 10^3$)	0.4 $\times 10^4$ (0.2 $\times 10^4$)

with the expression in VAT of several genes involved in lipid turnover, such as lipogenic (*PPARG*, *DGAT2*, *SREBP2*, and *STAR*), adipose tissue-specific (*LEPT* and *ADPN*), and lipolytic (*LPL*, *PPARA*, and *HSL* (*LIPE*)) genes. Interestingly, we also found that VAT expression of all these genes correlated with ΔE_2 , but in a negative manner, thus further indicating the deleterious role played by the MetS-related sex steroid imbalance on adipose tissue function and metabolism. However, additional investigations, such as aromatase activity/expression analysis, that could help to clarify the differences in the correlations of the sex hormones have not been performed in this study.

The mechanisms through which testosterone regulates fat mass are poorly understood (Singh *et al.* 2003, 2006, Gupta

et al. 2008). Testosterone inhibits adipocyte differentiation through the AR-mediated nuclear translocation of β -catenin and the subsequent activation of Wnt signaling (Rosen & MacDougald 2006). When activated, Wnt signaling promotes differentiation of resident MSCs into myocytes and osteocytes, while suppressing their commitment toward the adipocyte lineage and terminal differentiation by inhibiting the expression of *PPARG* and *CEBPA*, the central regulators of adipogenesis (Christodoulides *et al.* 2006, 2009). In our study in VAT, mRNA expression of both *PPARG* and *CEBPA* was tightly positively associated with *AR* expression, which also positively correlated with several genes exploring not only adipogenesis (*FABP4*, *ADPN*, and *LEPT*) but also other VAT-specific functions, such as lipid droplet formation,

Figure 5 The adipogenic response of rPADs to DIM. (A) Lipid content in untreated (left panels) and DIM-exposed (right panels) rPADs from each experimental group, as evaluated by Oil Red O staining. (B) Quantitative assessment of lipid content in untreated (gray bars) and DIM-exposed (black bars) rPADs from each experimental group, as evaluated by Adipored assay. Results are expressed as relative fluorescence unit (RFU) per microgram of protein and are reported as mean $\pm$ s.e.m. ($n=5$ for each group). * $P<0.01$ vs all other DIM-rPAD. Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0333>.

Figure 6 Analysis of the lipid droplet content in DIM-exposed, Adipored-stained rPADs from all rabbit groups. rPADs of RD (A), HFD (B), and testosterone-treated HFD (HFD+T; C) imaged by fluorescence microscopy (magnification 20×). (G) Adipored-positive cells were counted using ImageJ software and expressed as percentage change over total cells. rPADs of RD (D), HFD (E), and HFD+T; (F) imaged by confocal microscopy (magnification 63×). Images were quantitatively analyzed using Volocity 5 software to measure the number (H) and size (μm³; I) of lipid droplets within the single cell. At least eight cells were analyzed for each group. Results are reported as mean ± s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs RD; ^o $P < 0.01$; ^{oo} $P < 0.001$; ^{ooo} $P < 0.0001$ vs HFD. Full color version of this figure available via <http://dx.doi.org/10.1530/joe-12-0333>.

glucose transport, and insulin signaling. In particular, *AR* was positively associated with the expression of *SYNT5*, *SNAP23*, and *VAMP4*, which encode for proteins belonging to the SNARE complex, crucially involved in the processes of lipid droplet formation (Boström *et al.* 2007). Besides the positive association with *GLUT4*, *AR* mRNA also correlated with *RHOA*, *ROCK2*, and *VIM*, all proteins implicated in the cytoskeleton remodeling required for the insulin-stimulated intracellular trafficking of GLUT4 vesicles (Hirata *et al.* 2011, Chun *et al.* 2012). Moreover, a highly significant positive correlation was found between *AR* and *STAMP2*, whose expression is required for normal insulin signaling, as demonstrated in loss-of-function studies (Wellen *et al.* 2007). This protein is upregulated by TNF- α and by nutrient stimuli and has been characterized as a counter-regulator of inflammation and insulin resistance in mice (Waki & Tontonoz 2007, Wellen *et al.* 2007). Accordingly, *STAMP2* gene expression in VAT was significantly decreased in obese subjects, mainly in those with type 2 diabetes, and was associated with adipose tissue dysfunction (Moreno-Navarrete

et al. 2011). Interestingly, *STAMP2* is induced by androgens in androgen-regulated prostate cells (Korkmaz *et al.* 2005). Consistent with these findings, we observed a significant increase in *STAMP2* mRNA in VAT from testosterone-treated MetS rabbits, thus indicating the occurrence of an androgen-dependent activation of regulatory mechanisms linking inflammatory and nutritional signals with metabolism.

Overall, our data suggest that a direct AR-mediated action of testosterone on VAT function in the MetS model could lead to two different, apparently opposite, biological effects: i) anti-adipogenic, explaining the dramatic inhibitory effect of testosterone dosing on the HFD-induced VAT expansion (tenfold decrease) and ii) pro-adipogenic, as derived from the positive association found between VAT expression of *AR* and genes involved in lipid turnover. However, the mechanisms through which testosterone exerts these effects *in vivo* are complex and only in part clarified.

The anti-adipogenic action is in agreement with previous studies *in vitro* (Singh *et al.* 2003, 2006, Gupta *et al.* 2008) and *in vivo* (ARKO mice), clearly demonstrating the role of

Table 6 Effect of DIM on mRNA expression of adipocyte-related genes in rPADs. Relative mRNA expression of adipocyte-related genes was evaluated using qRT-PCR in untreated (time 0) and DIM-exposed rPADs from RD, HFD, and testosterone-treated HFD. Data were calculated according to comparative C_t method using 18S rRNA subunit as the reference gene for normalization. Results are reported as the mean $\pm$ s.d. of five different experiments, each performed in triplicate using a different cell preparation per group and are expressed in fold change over time 0

	RD	HFD	HFD+testosterone
Adipocyte-related genes			
<i>DKK1</i>	10 $\pm$ 7.3 [†]	1.4 $\pm$ 1.1 [*]	3.6 $\pm$ 2 [†]
<i>CEBPA</i>	2 $\pm$ 0.9 [†]	1.2 $\pm$ 0.4 [*]	2.4 $\pm$ 0.4 [†]
<i>PPARG</i>	2.3 $\pm$ 1.1 [†]	1 $\pm$ 0.7 [*]	1.8 $\pm$ 0.7 [†]
<i>FABP4</i>	19.2 $\pm$ 12.5 [†]	5.2 $\pm$ 3.3 [†]	14.3 $\pm$ 8 [†]
<i>ADPN</i>	12.4 $\pm$ 8.2 [†]	0.9 $\pm$ 0.4 [*]	3.8 $\pm$ 1.5 [†]
<i>LEPT</i>	8.5 $\pm$ 4.7 ^{††}	0.6 $\pm$ 0.3	1.1 $\pm$ 1.4
<i>CCND1</i>	0.9 $\pm$ 0.7	2.3 $\pm$ 1.6 [*]	0.3 $\pm$ 0.1
<i>CCND3</i>	2.5 $\pm$ 1.3 ^{*§}	1.3 $\pm$ 0.7	4.8 $\pm$ 2.6 ^{†,}

* $P < 0.05$; [†] $P < 0.01$ vs relative time 0; ^{††} $P < 0.01$ vs all other groups; [§] $P < 0.05$; ^{||} $P < 0.01$ vs relative *CCND1*.

androgens in decreasing visceral adiposity (Yanase *et al.* 2008). Accordingly, we found a negative association between *AR* and VAT expression of *DKK1*, which acts as an important inhibitor of Wnt signaling and is normally upregulated during the early steps of adipogenesis (Christodoulides *et al.* 2009). In addition, both ΔT and VAT *AR* mRNA positively correlated with VAT expression of the proapoptotic gene *BAX*. The promotion of BAX-mediated apoptosis by androgen and its receptor has been already reported in different cellular systems (Lin *et al.* 2006), but not fully investigated in this study. Whether the induction of apoptotic processes could be an additional mechanism through which testosterone counteracts VAT expansion in MetS rabbits remains to be demonstrated.

Concerning the mechanisms through which testosterone may exert pro-adipogenic effects, our results in isolated rPAD indicate that an androgen-dependent preservation of insulin sensitivity (and thereby of adipocyte-specific functions) is responsible for restoring normal morphological and functional VAT features. Despite VAT expansion, rPAD from HFD rabbits showed a reduced ability in responding to adipogenic DIM and in particular to insulin, as demonstrated by the reduced ability of triglycerides synthesis, glucose uptake, and GLUT4 membrane localization, as well as by the impaired induction of adipogenesis-specific genes. In rPADs from testosterone-treated HFD rabbits, all the DIM-induced adipocyte functions, including insulin-stimulated GLUT4 membrane translocation, glucose uptake, and triglyceride synthesis, were preserved and comparable to those observed in rPADs from RD rabbits. Our data are in perfect agreement with a recent study in nonhuman primates (Varlamov *et al.* 2012).

We speculate that the expansion of fat mass in experimental MetS may be ascribed to the lipid overloading, and

consequent enlargement, of mature adipocytes, likely dysfunctional, rather than to their replacement with newly synthesized and functional small adipocytes. In this regard, our *in vitro* results in isolated rPADs are indicative of a defect in preadipocyte maturation, which may impair the correct cell turnover in VAT of MetS rabbits. Accordingly, rPADs from HFD had increased *CCND1* expression after DIM induction, while *CCND3* remained unchanged. Indeed, it has been reported that *CCND1* blocks adipocyte maturation by antagonizing the function of *CEBPA* and *PPARG* and it is specifically induced only during preadipocyte clonal expansion (Fu *et al.* 2004). By contrast, prevalent expression of *CCND3*, as compared with *CCND1*, has been reported as a marker of adipogenic differentiation (Fu *et al.* 2004). Moreover, we found that in rPADs from HFD, DIM exposure

Figure 7 Insulin sensitivity of DIM-exposed rPADs. (A) Dose-response curves of the lipid synthesis in DIM-treated rPADs after exposure to increasing concentrations of insulin, as evaluated by Adipodex assay. Results are expressed in percentage of 0 nM (baseline) insulin and are reported as mean $\pm$ s.e.m. of four different experiments, each performed in triplicate and using a different cell preparation per group. The relative EC_{50} s and E_{max} values are reported in the text. * $P < 0.01$ vs E_{max} of all other groups. (B) Dose-response curves of radiolabeled (³H) glucose uptake in DIM-treated rPADs after exposure to increasing concentrations of insulin. Results are expressed in percentage over 0 nM insulin and are reported as mean $\pm$ s.e.m. of four different experiments, each performed in duplicate and using a different cell preparation per group. The relative EC_{50} s and E_{max} values are reported in the text. * $P < 0.01$ vs E_{max} of all other groups.

Figure 8 Effect of insulin (100 nM, 5 min) on GLUT4 or SNAP23 membrane immunolocalization in DIM-exposed rPADs of RD (A, E respectively), HFD plus testosterone (HFD) (B, F respectively), and HFD + T; C, G, respectively) rabbits. Immunolocalization was displayed by dual labeling of anti-WGA (membrane marker) antibody (red color or white arrowheads) and anti-GLUT4 or anti-SNAP23 (green color or white asterisk) antibodies, and colocalization is shown by merging the images (yellow color or white arrows). Percentage of GLUT4/WGA (D) or SNAP23/WGA (H) colocalization was detected with Leica SP2-AOBS confocal microscope and calculated using ImageJ software. Data are reported as mean $\pm$ S.E.M. ($n=3$). Bars = 58 μ m. * $P<0.01$ vs the other groups. Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0333>.

was not able to increase the percentage of Adipored-positive/differentiating cells, further suggesting that the adipogenic commitment in HFD VAT is impaired. Consistent with the positive effect of testosterone on adipogenesis, rPADs from testosterone-treated MetS rabbits showed a normal induction of *CCND3* by DIM, as well as a percentage of DIM-induced Adipored-positive cells comparable with that of RD cells.

An additional sign of insulin resistance in rPADs from HFD rabbits, which was normalized by testosterone treatment, is the altered DIM-induced formation of lipid droplets, as observed with confocal microscopy. In particular, reduced neo-formation (reduced number) and altered fusion processes (increased size) of lipid droplets, along with decreased SNAP23 membrane localization, were observed in rPADs from HFD, compared with rPADs from RD. Among the

proteins forming the SNARE complex, SNAP23 has also a key role in the regulation of insulin sensitivity, mediating the fusion of GLUT4 vesicles with the cell membrane (Boström *et al.* 2007). However, under conditions of dyslipidemia (high intake of fatty acids), SNAP23 is sequestered in the cytosol, where it mainly mediates the fusion of lipid droplets, with the consequent increase of their size. Interestingly, rPADs from testosterone-treated HFD rabbits were characterized by a prominent localization of SNAP23 to the membrane, as well as by lipid droplets that are reduced in size, but increased in number. This finding further demonstrates a testosterone-mediated increase in insulin sensitivity.

Overall, our observations not only reinforce the evidence of a positive effect of testosterone on insulin signaling in adipocytes but also indicates that its action is persistent, as a sort of 'metabolic memory', during *in vitro* experiments. Hence, the involvement of epigenetic mechanisms could be envisaged and should be properly investigated in future studies.

In conclusion, our study in an experimental model of MetS adds new information about the link between visceral obesity, hypogonadism, and metabolic alterations, all factors strictly associated with a potential CV risk. In particular, our results suggest that MetS rabbits are characterized by an accumulation of a dysfunctional VAT in which the normal differentiation process appears to be compromised because of impaired insulin signaling. The *in vivo* treatment with testosterone seems to preserve VAT function, through the maintenance of insulin sensitivity, which, in turn, allows preadipocyte commitment and a normal cell turnover. These effects could be seen as protective against the hypertrophic expansion of VAT that becomes dysfunctional, leading to the deleterious metabolic consequences typical of MetS.

Declaration of interest

E M, A M, S F, I C, B M, P C, T M, A C, E S, L V, R V, and G B V have nothing to declare. F S is an employee of Bayer Pharma AG, Berlin, Germany. M M is a scientific consultant for Bayer Pharma AG, Germany, Eli-Lilly Indianapolis, Indiana; Intercept Pharmaceuticals Italia Perugia, Italy.

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