Nonalcoholic steatohepatitis as a novel player in metabolic syndrome-induced erectile dysfunction: an experimental study in the

Molecular and Cellular Endocrinology 384 (2014) 143–154

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Nonalcoholic steatohepatitis as a novel player in metabolic syndrome-induced erectile dysfunction: An experimental study in the rabbit

Linda Vignozzi [a], Sandra Filippi [b], Paolo Comeglio [a], Ilaria Cellai [a], Erica Sarchielli [c], Annamaria Morelli [c], Giulia Rastrelli [a], Elena Maneschi [a], Andrea Galli [d], Gabriella Barbara Vannelli [c], Farid Saad [e], Edoardo Mannucci [f], Luciano Adorini [g], Mario Maggi [a,*]

[a] Sexual Medicine and Andrology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Italy
[b] Interdepartmental Laboratory of Functional and Cellular Pharmacology of Reproduction, Department of Neuroscience, Drug Research and Child Care, University of Florence, Florence, Italy
[c] Department of Experimental and Clinical Medicine, University of Florence, Italy
[d] Gastroenterology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Italy
[e] Global Medical Affairs Men's Healthcare, Bayer Pharma AG, Muellerstrasse 178, Berlin, Germany
[f] Diabetes Section Geriatric Unit, Department of Critical Care, University of Florence, Italy
[g] Intercept Pharmaceuticals, 18 Desbrosses Street, New York, NY 10013, USA

ARTICLE INFO

Article history:
Received 8 November 2013
Received in revised form 8 January 2014
Accepted 14 January 2014
Available online 31 January 2014

Keywords:
TNFα
Hypogonadism
Testosterone
OCA
Infliximab

ABSTRACT

A pathogenic link between erectile dysfunction (ED) and metabolic syndrome (MetS) is now well established. Nonalcoholic steatohepatitis (NASH), the hepatic hallmark of MetS, is regarded as an active player in the pathogenesis of MetS-associated cardiovascular disease (CVD). This study was aimed at evaluating the relationship between MetS-induced NASH and penile dysfunction. We used a non-genomic, high fat diet (HFD)-induced, rabbit model of MetS, and treated HFD rabbits with testosterone (T), with the selective farnesoid X receptor (FXR) agonist obeticholic acid (OCA), or with the anti-TNFα mAb infliximab. Rabbits fed a regular diet were used as controls. Liver histomorphological and gene expression analysis demonstrated NASH in HFD rabbits. Several genes related to inflammation (including TNFα), activation of stellate cells, fibrosis, and lipid metabolism parameters were negatively associated to maximal acetylcholine (Ach)-induced relaxation in penis. When all these putative liver determinants of penile Ach responsiveness were tested as covariates in a multivariate model, only the association between hepatic TNFα expression and Ach response was confirmed. Accordingly, circulating levels of TNFα were increased 15-fold in HFD rabbits. T and OCA dosing in HFD rabbits both reduced TNFα liver expression and plasma levels, with a parallel increase of penile eNOS expression and responsiveness to Ach. Also neutralization of TNFα with infliximab treatment fully normalized HFD-induced hypo-responsiveness to Ach, as well as responsiveness to vardenafil, a phosphodiesterase type 5 inhibitor. Thus, MetS-induced NASH in HFD rab-

Abbreviations: ED, erectile dysfunction; MetS, metabolic syndrome; NASH, nonalcoholic steatohepatitis; CVD, cardiovascular disease; T, testosterone; FXR, farnesoid X receptor; OCA, obeticholic acid; RD, regular diet; Ach, acetylcholine; T2DM, type 2 diabetes mellitus; NAFLD, nonalcoholic fatty liver disease; PDU, penile doppler ultrasound; LUT, lower urinary tract; PAS, periodic acid-Schiff; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; IL-12, interleukin-12; RORγt, retinoic-acid-receptor-related orphan receptor γt; ET-1, endothelin-1; ETA, endothelin receptor A; ETB, endothelin receptor B; αSMA, alpha smooth muscle actin; ROCK1, ROCK2, Rho-associated protein kinase type 1 and 2; RhoA, Ras homolog gene family, member A; TGFβ, transforming growth factor β; TIMP1 and TIMP2, tissue inhibitor of metalloproteinases-1 and -2; MMP2, MMP9, metalloproteinases-2 and -9; Cyp7A1, cholesterol 7 alpha-hydroxylase; SREBP1, sterol regulatory element-binding protein 1; VAMP4, vesicle-associated membrane protein 4; BSEP, bile salt export pump; SHP, small heterodimer partner; PLPA2, phospholipase A2; FN1, fibronectin 1; ADPN, adiponectin; AR, androgen receptor; IL-6, IL-8, IL-10, interleukin 6, interleukin 8, interleukin 10; MCP-1, monocyte chemoattractant protein-1, COX-2, inducible cyclooxygenase; CD68, macrophage marker; TLR2, TLR4, toll-like receptor 2 and 4; TNFα, tumor necrosis factor α; IL1β, interleukin-1β; GATA3, GATA-binding protein 3; AUC, area under the curve; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; VAT, visceral adipose tissue; TNFR1, TNFα receptor; CC, corpora cavernosa; PDE5, phosphodiesterase type 5; PKG1, protein kinase G 1; GCa1, GCb1, guanylate cyclase subunit a1 and b1.

* Corresponding author. Address: University of Florence, Chief of Sexual Medicine and Andrology, Department of Experimental and Clinical Biomedical Sciences, Viale Pieraccini, 6, Florence 50139, Italy. Tel.: +39 0554271415; fax: +39 0554271413.
E-mail address: mario.maggi@unifi.it (M. Maggi).

http://dx.doi.org/10.1016/j.mce.2014.01.014

bits plays an active role in the pathogenesis of ED, likely through TNFα, as indicated by treatments reducing liver and circulating TNFα levels (T or OCA), or neutralizing TNFα action (infliximab), which significantly improve penile responsiveness to Ach in HFD rabbits.

1. Introduction

Metabolic syndrome (MetS) is a constellation of medical conditions, including centrally distributed obesity, decreased high-density lipoprotein cholesterol, elevated triglycerides, elevated blood pressure, and hyperglycaemia. MetS is recognized as a driver of the current epidemics of both type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD).

MetS, in the male, is also involved in the development of several non-metabolic diseases, including hypogonadism and erectile dysfunction (ED) (Corona et al., 2011a,b; Traish et al., 2011). A link between ED and MetS has also been envisaged, because both conditions are related to high odds of developing cardiovascular (CV) events (Corona et al., 2011b). In addition, in MetS patients, an important pathogenic component of ED is the associated hypogonadism (Corona et al., 2011a). Data from a consecutive series of more than 800 patients with sexual dysfunction indicate that patients with MetS have a component-dependent higher prevalence of ED and poorest penile Doppler ultrasound (PDU) parameters (Corona et al., 2006). However, no difference in terms of ED severity or PDU parameters was observed when MetS patients were stratified according to the presence of hypogonadism (Corona et al., 2006). Hence, the influence of factors other than hypogonadism, impairing hemodynamic mechanisms at both penile and systemic vascular bed levels, has been suggested.

Penile erection is the end result of a complex neurovascular process in which nerves, endothelium of sinusoids and blood vessels, and smooth muscle cells in the corpora cavernosa (CC) are involved. Indeed, it is well established that the balance between contractant and relaxant factors modulates the degree of smooth muscle tone of the CC and determines the functional state of the penis: flaccidity or erection. The most important and specific pathway for penile erection is the nonadrenergic/noncholinergic signaling, which through the release of a labile gas, nitric oxide (NO), leads to erection. Formation of NO is strictly controlled by the activity of NO synthase (NOS) isoenzymes: endothelial (eNOS) and neuronal (nNOS). NO activates the soluble guanylyl cyclase (GC), which increases 3',5'-cyclic guanosine monophosphate (cGMP) levels, thus regulating the activity of cGMP-dependent protein kinase (PKG) and calcium channels that affect the relaxation of CC smooth muscle. The main hydrolytic enzyme involved in cGMP breakdown, thus leading to penile flaccidity is the phosphodiesterase type 5 (PDE5). In human (Morelli et al., 2004) and rabbit (Morelli et al., 2013) penis expression of PDE5 is at least one-log unit higher than in other tissues. Impaired NO bioactivity is considered a major pathogenic mechanism leading to erectile dysfunction (Vignozzi et al., 2005).

Nonalcoholic fatty liver disease (NAFLD) is considered as the hepatic hallmark of MetS (Marchesini et al., 2003). The term NAFLD covers a spectrum of histological findings ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), the most severe form of NAFLD. Epidemiological studies indicate that NASH patients are at higher risk for CVD, independently from underlying cardiometabolic risk factors (Targher et al., 2008). In patients with NAFLD, the severity of liver injury and inflammation is strongly associated with increased CV and atherogenic risk (Alkhouri et al., 2010). This suggests that NAFLD is not merely a marker of MetS, but may also actively contribute to the pathogenesis of MetS-associated CVD, most probably through the release of pro-atherogenic inflammatory factors. This concept could be inferred to link NAFLD to ED.

The current study addressed this issue by taking advantage of a non-genomic, high fat diet (HFD)-induced, animal model of MetS that closely resembles the human MetS phenotype (Filippi et al., 2009; Vignozzi et al., 2011, 2012a; Maneschi et al., 2012; Maneschi et al., 2013). This model is characterized by hyperglycaemia, glucose intolerance, hypercholesterolemia, hypertriglyceridemia, hypertension, increased visceral fat mass, hypogonadotropic hypogonadism, lower urinary tract (LUT) abnormalities (Vignozzi et al., 2012a), penile alterations (Filippi et al., 2009; Vignozzi et al., 2011) and NASH (Maneschi et al., 2013). The primary goal of our analysis was to evaluate the relationship between liver pathology and penile dysfunction in the course of HFD-induced MetS. The data indicate that MetS-induced NASH plays an active role in the pathogenesis of ED in HFD rabbits, likely via TNFα. We have recently demonstrated that both testosterone (T; Filippi et al., 2009; Maneschi et al., 2012) or obeticholic acid (OCA; Vignozzi et al., 2011; Maneschi et al., 2013) supplementation to HFD rabbits were able to normalize not only several MetS features, including visceral adipose tissue dysfunction and insulin resistance, but also HFD-induced penile alterations, including hypo-responsiveness to acetylcholine (Ach). Hence, here, we tested whether these treatments can ameliorate also HFD-induced liver alterations, as well as TNFα circulating level. To test the effect of TNFα neutralization, a subgroup of HFD animals has been treated with the selective anti-TNFα mAb, infliximab. Interestingly, treatments which reduce liver and circulating TNFα levels, such as T and the FXR agonist OCA, or which neutralize TNFα action such as infliximab, significantly improve penile responsiveness to acetylcholine (Ach) in this model.

2. Material and methods

2.1. Chemicals

Phenylephrine (Phe) HCl, sodium nitroprusside (SNP), acetylcholine (Ach), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Testosterone (T) supplementation was performed using T enanthate (250 mg; supplied by Bayer-Schering Pharma, Berlin, Germany). Obeticholic acid (OCA), a farnesoid-X receptor agonist, was supplied by Intercept Pharmaceuticals (New York, USA). Vardenafil, a selective phosphodiesterase type 5 inhibitor (PDE5i) was supplied by Bayer Schering Pharma AG, Global Drug Discovery (Wuppertal, Germany). Infliximab, an anti-TNFα chimeric mAb (Remicade, 100 mg, was from Janssen Biologics B.V. Einsteinweg 101 2333 CB Leiden, The Netherlands).

2.2. Animal model

The MetS rabbit model has been obtained by feeding adult male rabbits a high fat diet (HFD; $n = 48$) for 12 weeks, as previously described (Filippi et al., 2009). A first subgroup of HFD rabbits ($n = 28$) was treated with intramuscular injections of testosterone (30 mg/kg/week), as previously described (Filippi et al., 2009; Vignozzi et al., 2012a). A second subset of HFD rabbits ($n = 18$) was treated

with the FXR agonist OCA (10 mg/kg/day for 5 days a week, by oral gavage), as previously described (Maneschi et al., 2013). An additional subset ($n = 7$) of HFD rabbits was treated with the anti-TNFα monoclonal antibody, infliximab (5 mg/kg/week, i.v.). Rabbits fed a regular diet (RD) were taken as controls ($n = 79$).

2.3. Evaluation of MetS parameters

Blood samples for glucose, total cholesterol, triglycerides, testosterone and 17β-estradiol were obtained via marginal ear vein at baseline and at week 12 in all groups, early in the morning after an overnight fasting. Mean arterial pressure (MAP) and visceral adipose tissue weight were determined, according to Filippi et al. (2009). 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 ± two S.D. of the analyzed parameter, as measured in RD rabbits according to (Maneschi et al., 2012). Positivity for three or more factors identifies MetS.

2.4. Isolation of organs

Rabbits were sacrificed with a lethal dose of pentobarbital and specimens of several organs [corpora cavernosa (CC), liver, visceral fat (VAT), hypothalamus, bladder, prostate, testis, skeletal muscle, and epididymis] were harvested and processed for subsequent analyses.

2.5. Liver histomorphological analysis

Frozen sections were cut in a cryostat and fixed in 4% paraformaldehyde for 20 min at room temperature (RT). Masson's trichrome staining was performed following the manufacturer's instructions (Bio-Optica, Milan, Italy). To evaluate lipid accumulation, the sections were treated for 2–5 min with isopropanol and stained with Oil Red O for 20 min (Maneschi et al., 2013). A combination of Periodic Acid-Schiff (PAS) with Giemsa staining was performed following the manufacturer's instructions (Sigma–Aldrich, Milan, Italy) to visualize intact glycogen-positive hepatocytes and nuclei of infiltrating cells, respectively.

2.6. RNA extraction and quantitative RT-PCR

Isolation of total RNA from tissues was performed using TRIzol reagent (Life Technologies, Paisley, UK) and/or RNeasy Mini Kit (Qiagen, Hilden, Germany), both according to the manufacturers' instructions. cDNA synthesis was carried out using the iScriptTMcDNA Synthesis Kit purchased from Bio-Rad Laboratories (Hercules, CA). Quantitative real-time RT-PCR (qRT-PCR) analysis was performed according to the fluorescent methodology, using SsoFastTMEvagreen® Supermix (Bio-Rad Laboratories). Amplification and detection were performed with the MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories) using the following thermal cycler conditions: 40 cycles at 95 °C for 30 s and 60 °C for 1 min. Specific PCR primers for rabbit target genes were designed on rabbit sequences available at NCBI GeneBank (http://www.ncbi.nlm.nih.gov) or Ensemble Genome (http://www.ensembl.org), or based on homology to the human sequence, as reported previously. Primers were purchased from Life Technologies. A detailed list of the primers used is reported in Table 1. The expression of PDE5 was quantified with a predeveloped (Hs00153649_m1; Life Technologies). The expression of the 18S ribosomal RNA subunit, quantified with a predeveloped assay (Hs99999901_s1; Life Technologies), resulted the most stable and optimal for data normalization in comparison with two other widely used housekeeping genes, GAPDH and β-microglobulin. It was therefore chosen as the reference gene and used for relative quantitation of the target genes. Data analysis was based on the comparative threshold cycle (Ct) method, according to Filippi et al. (2009).

2.7. In vitro contractility studies

Corpora cavernosa (CC) samples were immediately placed and maintained in cold Krebs solution and in vitro contractility experiments were performed in organ chambers, according to (Filippi et al., 2009). Briefly, rabbit CC strips were vertically mounted under 1.8 g resting tension in organ chambers containing 10 mL of Krebs solution at 37 °C, gassed with 95% O_2 and 5% CO_2 at pH 7.4, and allowed to equilibrate for at least 90 min. Changes in isometric tension were recorded on a chart polygraph (Battaglia Rangoni, Casalecchio di Reno, Bologna, Italy). The degree of contractile response induced by Phe (100 μM) was taken as 100% and the relaxant effect induced by different drug (Ach and SNP) concentrations was referred to this value. Drug cumulative concentrations were added to the bath, at 3–7 min intervals, in order to obtain concentration-relaxant effect curves. Relaxant response to SNP was measured after a 30-min pre-treatment with a fixed concentration (100 nM) of the PDE5i vardenafil.

2.8. Quantification of serum TNFα

Serum TNFα content was assayed using a commercially available rabbit ELISA Kit (Cloud-Clone Corp. Houston, TX, USA) following the manufacturer instructions and analyzed by an ELISA plate reader (Victor3 1420 multilabel counter, Perkin Elmer, MA, USA) at 450 nm wave length.

2.9. Immunohistochemistry for TNFα in corpora cavernosa sections

CC sections were incubated overnight at 4 °C with a primary anti-TNFα antibody (infliximab 1:100 vol/vol, Dako Cytomation, Copenhagen, Denmark). The sections were rinsed in PBS and incubated with a biotinylated secondary antibody and then with a streptavidin–biotin-peroxidase complex (Ultravision large volume detection system anti-polyvalent, Lab Vision, Fremont, CA, USA). The reaction product was developed with 30,30-diaminobenzidine tetrahydrochloride as chromogen (Sigma–Aldrich). Control experiments were performed by omitting the primary antibody. The slides were evaluated and photographed using a Nikon Microphot-FXA microscope. Computer-assisted quantification of TNFα staining was performed after background subtraction using the Adobe Photoshop 6.0 Software (Adobe Systems).

2.10. Statistical analysis

Results are expressed as means ± S.E.M. for n experiments as specified. The statistical analysis was performed with a one-way ANOVA test followed by the Tukey–Kramer post hoc analysis in order to evaluate differences between groups, and $p < 0.05$ was considered significant. When data were non-normally distributed, statistical differences were calculated with Kruskal–Wallis test and Mann–Whitney *U*-test was used for comparisons between groups. Correlations were assessed using Spearman's method, and the statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS, Inc.) for Windows 20.0. Stepwise multiple linear regressions were applied for the multivariate analysis, whenever appropriate.

Table 1
List of target genes and the relative primers.

Adiponectin (ADPN)	F	ACCAGGACAAGAACGTGGAC
	R	GAAGGAAGCCAGTGGAGATG
Androgenreceptor (AR)	F	CCGTAACTTGCATGTGGATG
	R	GCTGTACATCCGGGACTTGT
Bile Salt Export (BSEP)	F	GAACCTCCAATGGCTGTTGT
	R	ATTAACAGCGGCACCCATAG
Macrophage marker (CD68)	F	ACTCCAAGCCCAGATTCAGA
	R	CCATAGGGGAAGGAGAGGAG
Inducible cyclooxygenase (COX2)	F	AGTGTGCGATGTGCTCAAAC
	R	AAAAGCAGCTCTGGGTCAAA
Cholesterol 7 alpha-hydroxylase (CYP7A1)	F	GCAGGACTTCCCATTCACAT
	R	CGAAGGTGGAGAGTGTGTCA
Endothelin-1 (ET1)	F	AGCGAGTAGCAGCTCCAAAG
	R	CCTGAGCCTGTCAGTGCATA
Endothelin receptor type A (ETA)	F	AGGGGTGAACAGCACAAAAC
	R	ATGTTCACTGAGGGCAATCC
Endothelin receptor type B (ETB)	F	CGTCTGCACCTGCTGAAATA
	R	AACACGAGGCAGGATACCAC
Fibronectin 1 (FN1)	F	CCTGCACCAAGAATTGGTTT
	R	TACGATCGGAGCGTCTCTTT
Farnesoid X receptor (FXR)	F	CCCCAAGTTCAACCACAGAT
	R	CCAGATGCTCTGTCTCCACA
Glucose 6-phosphatase (G6Pase)	F	GGTGCTGGACACCGACTACT
	R	TTTTTCTTCCCCCGAAAGAT
Th2 lymphocytes transcription factor (GATA3)	F	AGGCAGGGAGTGTGTGAACT
	R	CGTCGTGGTCTGACAGTTTG
Guanylate cyclase soluble subunit alpha-3 (GCSa3)	F	AGTTGTGCAGGCCAAGAAGT
	R	AGCATGGTGATGACTTGCAG
Guanylate cyclase soluble subunit beta-3 (GCSb3)	F	CAGGAGCTGGAGATCCTCAC
	R	TGTCTCAGCTCATTGGCAAC
Interleukin-1β (IL-1β)	F	CCACAGTGGCAATGAAAATG
	R	AGAAAGTTCTCAGGCCGTCA
Interleukin-6 (IL-6)	F	GAACAGAAAGGAGGCACTGG
	R	CTCCTGAACTTGGCCTGAAG
Interleukin-8 (IL-8)	F	CTCTCTTGGCAACCTTCCTG
	R	TTGCACAGTGAGGTCCACTC
Interleukin-10 (IL-10)	F	AGAACCACAGTCCAGCCATC
	R	TTTTCACAGGGGAGAAATCG
Interelukin-12 (IL-12)	F	TGCAGATGAAGCCATTGAAG
	R	ACGAATCTTGGCATCCTTGT
Monocytechemoattractant protein-1 (MCP-1)	F	CACCCGGACACCCTCTACTA
	R	CACATATGCCCAAATTTCCA
Matrix metalloproteinase-2 (MMP2)	F	CTGCAGGGCAGCGGTCACAG
	R	TACGGAAGTGCAGGTGCGGC
Matrix metalloproteinase-9 (MMP9)	F	CCCGACCCGAGCTGACTCCA
	R	CACACCAGAGGCGCCCATCC
Endothelial nitric oxide synthase (eNOS)	F	GCACAGTGATGGCAAAGAGA
	R	TCGAGGGACACCACATCATA
Neuronal nitric oxide synthase (nNOS)	F	CAACATCGCCGTTCTCTACA
	R	GGAGTGATACTGCCCGACAT
Phosphoenolpyruvatecarboxykinase (PEPCK)	F	CTGTGCACATCCCAACTCTC
	R	CCAGTTGAAGGCCTCGTAAA
Perilipin	F	AATGTGCATAGTGCCAACCA
	R	ACGTGACTCGATGTGCTCAG
cGMP-dependent protein kinase (PKG1)	F	TGGATGACGTTTCCAACAAA
	R	CACTATGTGGCGCTTCTTGA
Phospholipase A2 (PLPA2)	F	AAGTACACCCCGCTGCCTGC
	R	ACGCCTGCAGCCATGACCAC
Peroxisome proliferator-activated receptor α (PPARα)	F	GGCCTGGCCTTCTAAACATA
	R	TTCTTGATGACCTGCACGAG
Peroxisome proliferator-activated receptorγ (PPARγ)	F	TGGGGATGTCTCATAATGCCA
	R	TTCCTGTCAAGATCGCCCTCG
Ras homolog gene family, member A (RhoA)	F	CCCTCCTCATCGTCTTCAGT
	R	GTCGATGGAGAAGCACATGA
Rho-associated protein kinase 1 (ROCK1)	F	CGGAAGTGAACTCGGATTGT
	R	TCCAAATGCACCTCTACCAA
Rho-associated protein kinase 2 (ROCK2)	F	CTACGGACGGGAATGTGACT
	R	TGTTAAGAAGGCGCAGATGA
Th17 lymphocytes transcription factor (RORγt)	F	GGGCTTCATACCACCTTGAA
	R	GTGCTCTGGGCCTATCTCTG
Small heterodimer partner (SHP)	F	TGCCCAGCATACTCAAGAAG
	R	CAGGTAGGCGTATTCCTTGG
α-Smooth muscle actin (αSMA)	F	ACTGGGACGACATGGAAAAG
	R	TACATGGCTGGGACATTGAA
Sterol regulatory element-binding transcription factor 1 (SREBP1)	F	CACAGGAGCCACAATGAAGA
	R	GAACGGTAGCGCTTCTCAAT

Transforming growth factor-beta (TGFβ1)	F	GCTAATGGTGGACAGCAACA
	R	GCTGCTCCTGCTGTAACTTG
Tissue inhibitors of metalloproteinases 1 (TIMP1)	F	CCTTGGGGCATGCCACGGAT
	R	CGTTCCGCAGTTGTCCAGCGA
Tissue inhibitors of metalloproteinases 2 (TIMP2)	F	GTCCCTGGACGTGGGAGGCA
	R	GGATCATGGGGCAGCGCGAG
Toll-like receptor 2 (TLR2)	F	CCGCGGGTTCCCCAGGTTG
	R	GGATCTGGAGCGCCCATCGC
Toll-like receptor 4 (TLR4)	F	GCGGGTGGAGCTGTATCGCC
	R	CTTGGGTTCAGCCGGGCAGG
Tumor necrosis factor α (TNFα)	F	GTCTTCCTCTCTCACGCACC
	R	TGGGCTAGAGGCTTGTCACT
Tumor necrosis factor receptor (TNFR)	F	CTTGCACAGTGGACCATGAC
	R	ACACGGTGTCCTGACTCTCC
Vesicle-associated membrane protein 4 (VAMP4)	F	AAGTTCAAGCGCCACCTAAA
	R	CCACCTGATTCTGAACATGC

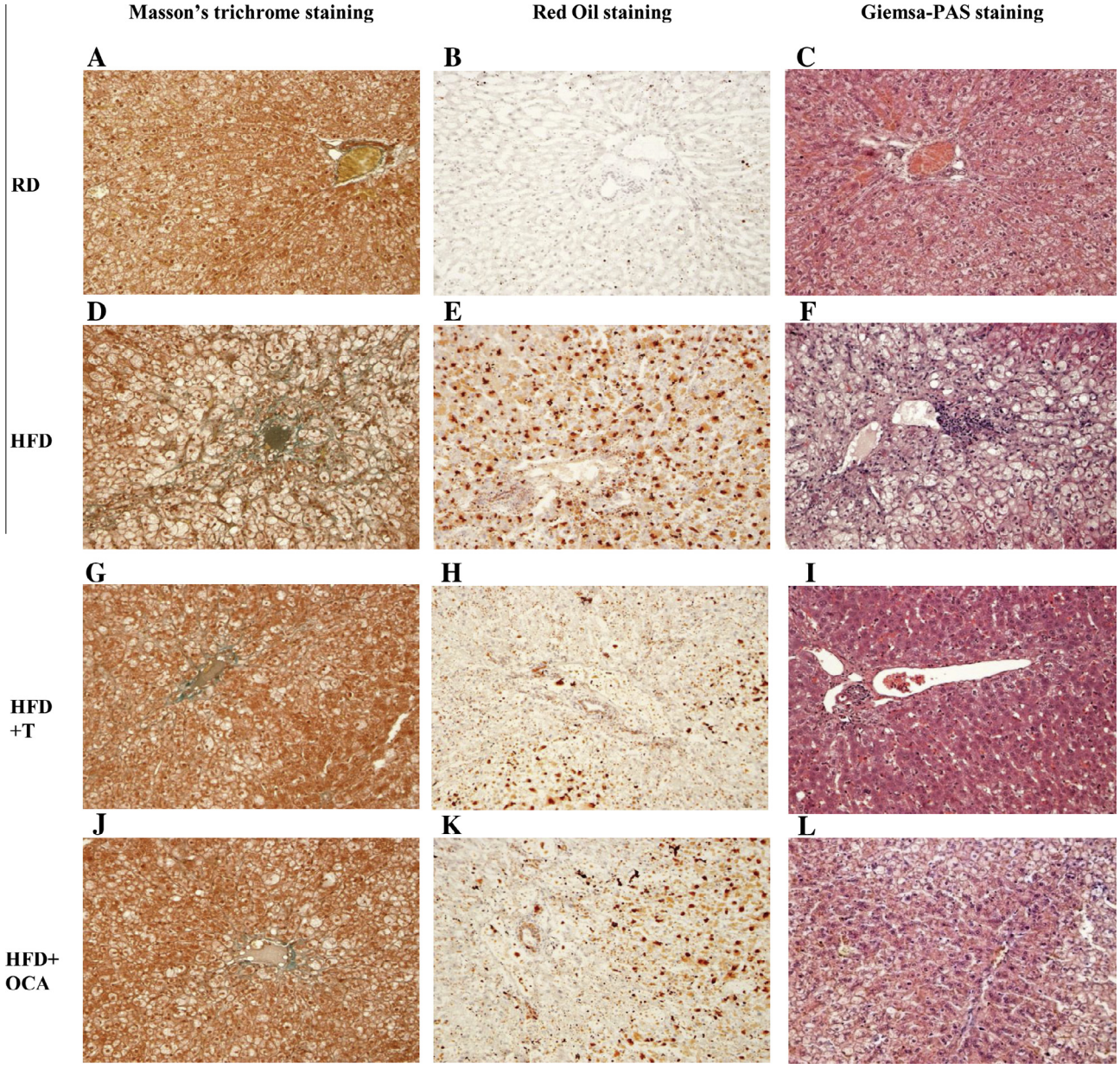

Fig. 1. Treatment with testosterone or OCA ameliorates liver pathology. Liver sections from RD (A–C), HFD (D–F), HFD + T (G–I), and HFD + OCA (J–L) treatments. Panels A, D, G, and J show Masson's trichrome staining of liver sections from RD (A), HFD (D), testosterone (T)-treated HFD (G) and OCA-treated HFD rabbits (J), respectively. Panels B, E, H, and K show lipid accumulation in liver sections, as revealed by Oil Red O staining. Panels C, F, I, and L, show PAS-Giemsa staining of liver sections. Original magnifications 10×.

Table 2
Effect of feeding a high fat diet (HFD) on liver gene expression.

	Mean ± SEM	p
Inflammation-related genes		
TNFα	360.22 ± 51.69	<0.0001
IL-6	282.21 ± 41.37	<0.0001
MCP-1	2122.37 ± 406.21	<0.0001
COX2	876.32 ± 273.83	<0.0001
IL-8	1883.21 ± 382.05	<0.0001
IL-10	985.62 ± 100.65	<0.0001
IL-12	68.11 ± 4.67	0.002
IL-1β	111.74 ± 14.24	0.436
Stellate cell activation-related genes		
TGFβ	368.60 ± 42.47	<0.0001
RhoA	259.78 ± 30.21	<0.0001
ROCK 1	133.10 ± 12.94	0.143
ROCK 2	117.22 ± 12.26	0.681
αSMA	437.89 ± 72.65	<0.0001
ET1	132.58 ± 11.93	0.029
ETA	355.33 ± 53.89	<0.0001
ETB	197.27 ± 20.08	<0.0001
Fibrosis-related genes		
TIMP1	1029.32 ± 166.95	<0.0001
TIMP2	874.11 ± 146.28	<0.0001
MMP2	2057.52 ± 485.26	<0.0001
MMP9	900.54 ± 247.94	<0.0001
FN1	110.71 ± 15.51	0.557
Bile acid metabolism		
FXR	132.98 ± 10.79	0.019
SHP	187.21 ± 40.97	0.169
CYP 7A1	301.20 ± 72.97	0.003
BSEP	82.93 ± 7.87	0.120
Immune response-related genes		
CD68	1140.20 ± 119.57	<0.0001
GATA3	159.11 ± 28.69	0.272
RORγt	81.88 ± 12.75	0.030
TLR2	733.97 ± 134.44	<0.0001
TLR4	356.38 ± 46.28	<0.0001
Steatosis and intermediate metabolism-related genes		
PPARγ	341.44 ± 32.91	<0.0001
PPARα	69.01 ± 6.96	<0.0001
PLPA2	128.65 ± 17.35	0.413
ADPN	295.51 ± 70.20	0.100
CD 36	98.23 ± 12.00	0.500
Perilipin	260.37 ± 68.64	0.007
PEPCK	54.19 ± 6.03	<0.0001
G6Pase	41.19 ± 5.73	<0.0001
SREBP1	205.52 ± 39.48	0.001
VAMP4	92.40 ± 12.40	0.260
eNOS	126.86 ± 13.89	0.327
AR	126.82 ± 13.98	0.527

Data were obtained by quantitative RT-PCR, expressed as percentage of RD and statistically compared to RD. Statistical differences between groups were calculated with Mann–Whitney *U*-test for comparison of non-normally distributed parameters. $p < 0.05$ was considered significant.

2.11. Animal handling

Animal handling complied with the Institutional Animal Care and Use Committee of the University of Florence, Florence, Italy, in accordance to the Italian Ministerial Law # 116/92 and according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

3. Results

3.1. HFD induces liver pathology typical of NASH

Feeding a high fat diet (HFD) had profound effects on liver histology (Fig. 1). In HFD liver sections, abundant fat accumulation was evident with a specific staining for lipid accumulation (Oil Red O). Masson's trichrome staining showed a marked collagen deposition, forming fibrotic septa around the centrilobular vein and within the perilobular space, at sites where fatty degeneration of hepatocytes (similar to the ballooning of hepatocytes in human NASH) was evident. Periodic acid-Schiff (PAS)/Giemsa staining identified marked mononuclear cell infiltrates, visible in the portal system space.

Feeding rabbits a HFD has also marked effects on gene transcripts in liver homogenates, as determined by qRT-PCR (Table 2). We found that HFD-induced significant decreases in PPARα, a gene involved in triglycerides metabolism, in two gluconeogenesis-associated genes (PEPCK, G6Pase), and in inflammatory genes (IL-12, RORγt). HFD induced a significant increase in the expression of several genes related to stellate cell activation (ET-1, ETA, ETB, αSMA, RhoA, TGF-β), fibrosis (TIMP1, TIMP2, MMP2, MMP9), bile acid metabolism (FXR, Cyp7A1), cholesterol metabolism (SREBP1), steatosis (PPARγ, perilipin, VAMP4) and inflammation (IL-6, MCP-1, IL-8, COX-2, IL-10, CD68, TLR2, TLR4), including TNFα. Circulating levels of TNFα were increased by over one-log unit in HFD compared to RD rabbits (RD = 5.45 ± 4.9 pg/mL; HFD = 83.9 ± 23.8 pg/mL, $p < 0.0001$).

Table 3
Association between hepatic genes and relaxant responsiveness to Ach.

	Ach 3 μM			AUC for Ach		
	r	p	n	r	p	n
Inflammation-related genes						
TNFα	−0.618	<0.0001	35	−0.636	0.048	10
IL6	−0.496	0.002	35	−0.723	0.018	10
MCP-1	−0.656	<0.0001	37	−0.673	0.033	10
IL8	−0.645	<0.0001	31	−0.821	0.023	7
COX-2	−0.662	<0.0001	36	–	–	–
IL10	−0.735	<0.0001	31	−0.667	0.050	9
IL1β	−0.398	0.027	31	−0.881	0.004	8
Immune response-related genes						
CD68	−0.794	<0.0001	34	−0.700	0.036	9
GATA3	−0.482	0.005	32	–	–	–
TLR2	−0.618	0.001	25	–	–	–
TLR4	−0.513	0.006	27	–	–	–
Stellate cell activation-related genes						
ETA	−0.464	0.007	33	–	–	–
ETB	−0.497	0.003	33	–	–	–
αSMA	−0.514	0.003	32	−0.762	0.028	8
RhoA	−0.405	0.017	34	−0.717	0.030	9
TGFβ	−0.644	<0.0001	33	−0.745	0.021	9
Fibrosis-related genes						
TIMP1	−0.724	<0.0001	32	−0.762	0.028	8
TIMP2	−0.485	0.012	26	–	–	–
MMP2	−0.588	0.002	26	–	–	–
MMP9	−0.571	0.003	25	–	–	–
Intermediate metabolism-related genes						
SREBP1	−0.475	0.005	34	–	–	–
PEPCK	0.463	0.007	33	–	–	–
G6Pase	0.424	0.014	33	–	–	–
PPARγ	−0.566	<0.0001	37	–	–	–
PPARα	0.409	0.012	37			

Correlations coefficients (*r*) and level of significance (*p*-value) are derived from univariate analysis. n indicates the number of samples; hyphen-minus (−) indicates absence of a significant correlation.

3.2. Relationship between liver pathology and penile dysfunction

We next tested whether the hepatic expression of the aforementioned genes was associated with changes in penile responsiveness to several relaxant agents. We analyzed association with the area under the relaxant dose–response curve (AUC) for: (i) Ach (or its maximal responsiveness at 3 μM), (ii) the ROCK inhibi-

Table 4
Association between families of hepatic genes and relaxant responsiveness to Ach as derived by multivariate analysis.

	Adj. r	p
Inflammation-related genes		
TNFα	−0.612	0.043
IL6	−0.085	0.632
MCP-1	−0.184	0.389
COX2	−0.152	0.695
IL8	0.136	0.786
IL10	−0.224	0.243
IL1β	−0.097	0.593
Stellate cell activation-related genes		
TGFβ	−0.571	0.086
RhoA	−0.091	0.748
αSMA	0.169	0.572
ETA	−0.381	0.073
ETB	0.101	0.691
Fibrosis-related genes		
TIMP1	−0.446	0.012
TIMP2	−0.267	0.210
MMP2	0.654	0.095
MMP9	−0.837	0.064
Immune response-related genes		
CD68	−0.971	<0.0001
GATA3	−0.315	0.462
TLR2	0.443	0.410
TLR4	0.054	0.807
Intermediate metabolism-related genes		
PEPCK	0.322	0.025
PPARγ	−0.765	<0.0001
PPARα	0.455	<0.0001
G6Pase	−0.023	0.858
SREBP1	−0.091	0.359

Correlations coefficients (r) and level of significance (p-value) were derived from multivariate analysis.

torY-27632, (iii) the NO-donor SNP (in the presence or absence of a fixed vardenafil concentration, 100 nM). Spearman correlation analysis showed no significant associations between all the tested liver genes and AUC of both Y27632 and SNP (with and without vardenafil, data not shown). Conversely, several genes related to inflammation (TNFα, IL-6, IL-1β, MCP-1, IL-8, IL-10, COX-2), immune response (CD68, TLR2, TLR4, GATA3), activation of stellate cells (RhoA, TGFβ, αSMA, ETA, ETB), fibrosis (TIMP1, TIMP2, MMP2, MMP9), and lipid metabolism (SREBP1, PPARγ) were negatively associated to maximal Ach-induced relaxation (Table 3). A positive association was found among penile responsiveness to Ach and liver PPARα, PEPCK, and G6Pase mRNA (Table 3). Similar results were observed when the Ach AUC was evaluated (Table 3). Considering these correlations, a multivariate analysis was performed to verify the specific relationships of individual gene expression with Ach-induced maximal response. These genes were therefore introduced, as covariates, in a series of iterative linear regression analyses. Adjusted regression coefficients are reported in Table 4. The genes that retained a positive association with penile maximal Ach response were PEPCK and PPARα, whereas TNFα, CD68, TIMP1, PPARγ resulted negatively associated.

3.3. Treatment with testosterone or OCA ameliorates liver pathology, reduces TNFα levels and enhances penile responsiveness to Ach

The effects of testosterone or OCA treatments in HFD rabbits on liver histology and gene expression are reported in Figs. 1 and 2, respectively. Both treatments ameliorated hepatic fat accumulation and inflammation (Fig. 1), down-regulating TNFα and up-regulating IL-10 gene expression in liver homogenates (Fig. 2A). Both treatments restored HFD-induced eNOS down-regulation and Ach hypo-responsiveness in the penis (see Table 5).

Increasing numbers of MetS components were progressively associated with increased hepatic TNFα expression ($r = 0.442$, $p < 0.001$, $n = 91$; Fig. 2B) and reduced penile maximal responsiveness to Ach ($r = -0.553$, $p < 0.001$, $n = 54$; Fig. 2C). Interestingly, hepatic TNFα expression was negatively associated with penile Ach responsiveness ($r = -0.544$, $p < 0.001$, $n = 61$, Fig. 2D), which retained statistical significance even after adjusting for confounding factors, such as VAT weight and T level (Adj. $r = -0.566$, $p < 0.001$). When other putative liver determinants of penile Ach responsiveness, as derived from the previous modeling (CD68, TIMP1, PEPCK, PPARγ, PPARα, see Table 3), were introduced as covariates in a multivariate model, only the association between TNFα and Ach response was confirmed (Adj. $r = -0.364$, $p = 0.020$, $n = 45$). Conversely, no association was observed in the expression of inflammation-related genes (TNFα, IL-6, IL-8, MCP-1) in visceral adipose tissue and penile responsiveness to Ach (data not shown).

Interestingly, T significantly blunted, whilst OCA reduced without reaching statistical significance, circulating TNFα levels (Fig. 3A). Both treatments significantly reduced liver TNFα expression (Fig. 3B). Circulating levels of TNFα in all the experimental groups were significantly related to TNFα mRNA expression level within the liver ($r = 0.360$, $p = 0.036$, $n = 34$), but not with expression in other tissues, as VAT, hypothalamus, skeletal muscle, prostate, seminal vesicles (data not shown). The quantitative evaluation of TNFα and its receptor (TNFR1) gene expression in a wide panel of rabbit tissues are shown in Fig. 3C. VAT expresses the highest density of both TNFα and its receptor. In the penis, the relative abundance of TNFα and TNFR1 was rather low, and similar to other urogenital tissues. Neither TNFα (see Fig. 3D) nor TNFR1 (not shown) mRNA penile expression was affected by HFD. Similar results were obtained in T- or OCA-treated groups (Fig. 3D). However, when penile TNFα protein was considered, we found by computer-assisted quantitative immunohistochemistry that the relative levels were increased by at least five fold in HFD rabbits (Fig. 3G). A scanty staining for TNFα was observed in penile sections from RD rabbits (Fig. 3E), whereas in the HFD rabbits there was a marked positivity in both endothelial and smooth muscle cells of the vascular bed and cavernous spaces (Fig. 3F).

3.4. Infliximab treatment restores penile responsiveness to relaxant stimuli

To test the hypothesis that TNFα is involved in HFD-induced Ach hypo-responsiveness in the penis, we treated HFD rabbits with the anti-TNFα mAb infliximab (5 mg/kg/week). Infliximab treatment significantly reduced visceral fat accumulation (HFD = 40.7 ± 1.9 g; HFD + infliximab = 25.3 ± 5 g, $p < 0.02$) and estrogen levels (HFD = 307 ± 38.4 pmol/L; HFD + infliximab = 253.3 ± 114.5 pmol/L, $p < 0.02$). Conversely, infliximab treatment did not normalize HFD-induced low testosterone plasma level (HFD = 1.59 ± 0.31 nmol/L; HFD + infliximab = 0.9 ± 0.21 nmol/L, $p = 0.559$). No significant effects of infliximab dosing were observed in any of the hepatic genes examined (data not shown).

In order to determine whether infliximab treatment could ameliorate penile function, the in vitro relaxant response of isolated penile strips to Ach, SNP and SNP + vardenafil was assessed. Infliximab administration fully restored HFD-induced hypo-responsiveness to Ach (Fig. 4A). Maximal responsiveness to Ach (3 μM; Fig. 4A inset) also showed a similar trend, being normalized by infliximab. We then tested penile responsiveness to increasing concentrations of SNP, in the presence or absence of a fixed concentration (100 nM) of vardenafil. In the absence of vardenafil, SNP-induced relaxant response did not exhibit significant between-group variations, according to Kruskal–Wallis test ($p = 0.097$; not shown). On

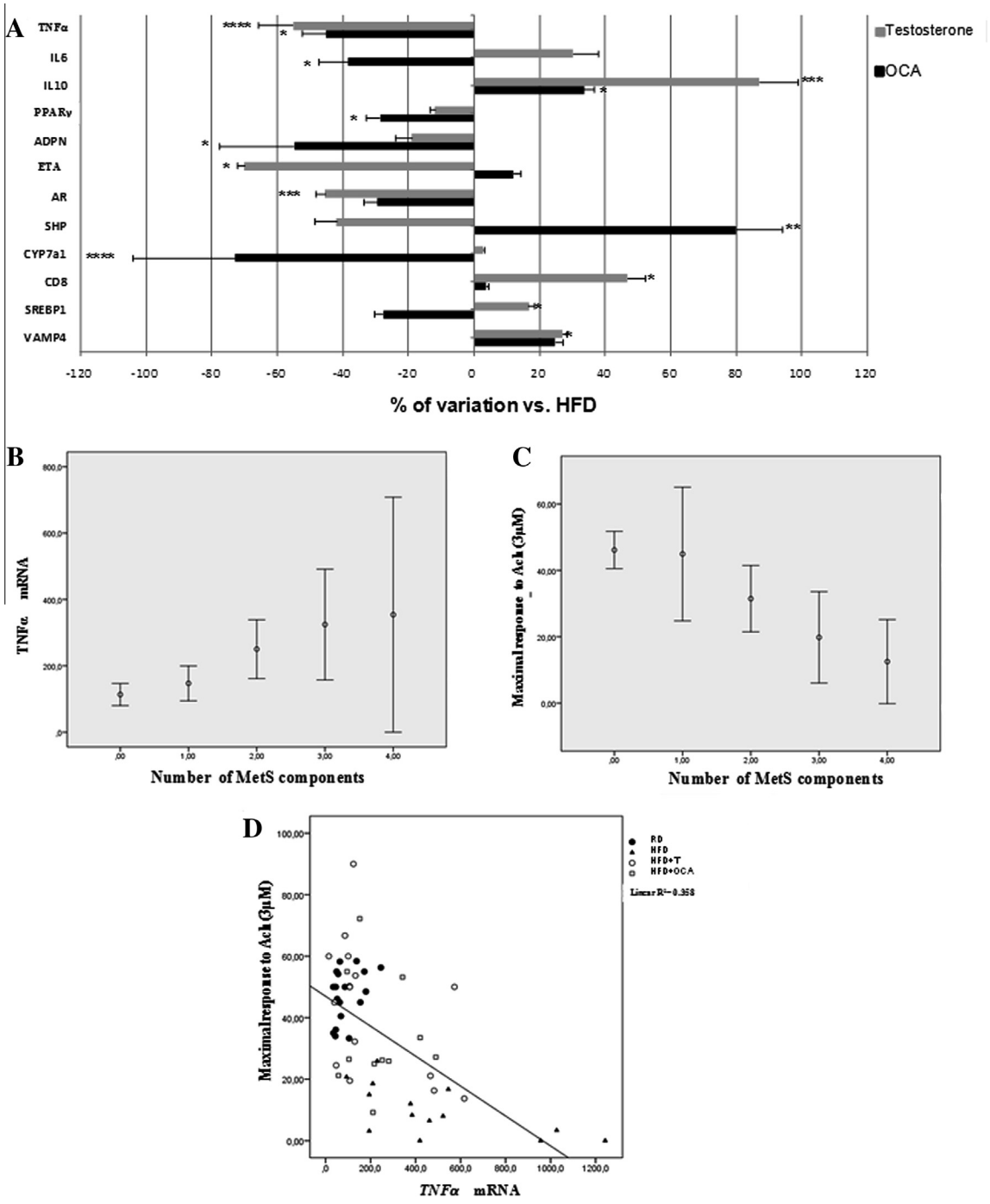

Fig. 2. Treatment with testosterone or OCA reduces TNFα levels and enhances penile responsiveness to Ach. (A) OCA-(black bars) and testosterone-(T, grey bars) on liver genes Data were expressed as percentage of variation vs. HFD-induced expression. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ vs. HFD. (B–C) Relationship between the number of MetS components (abscissa) and the liver mRNA expression of TNFα (B) or penile maximal responsiveness to Ach (3 μM; C). (D) Relationship between liver TNFα mRNA expression (abscissa) and penile responsiveness to Ach (3 μM, ordinate), as derived from univariate Spearman's regression analysis.

Table 5
Expression of eNOS and responsiveness to acetylcholine in CC from the different experimental groups.

	eNOS mRNA	AUC Ach	Maximal response to Ach (% of Phe-induced contractile response)
RD	100.0 ± 10.6	460.7 ± 32.9	46.8 ± 2.4
HFD	49 ± 6.7***	104.4 ± 16.1***	9.8 ± 1.6***
HFD + T	102.2 ± 18.5°	333.9 ± 32.6°°°**	41.2 ± 4.9°°°
HFD + OCA	162.0 ± 35.0°°	278.9 ± 39.9°°°*	34.7 ± 4.6°°°*

$^{*}p < 0.05$, $^{**}p < 0.01^{***}$, $p < 0.001$ vs. regular diet (RD); $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs. high fat diet (HFD). T: testosterone (T); obeticholic acid (OCA); endothelial nitric oxide synthase (eNOS); AUC: area under the curve; Ach: acetylcholine; Phenylephrine (Phe).

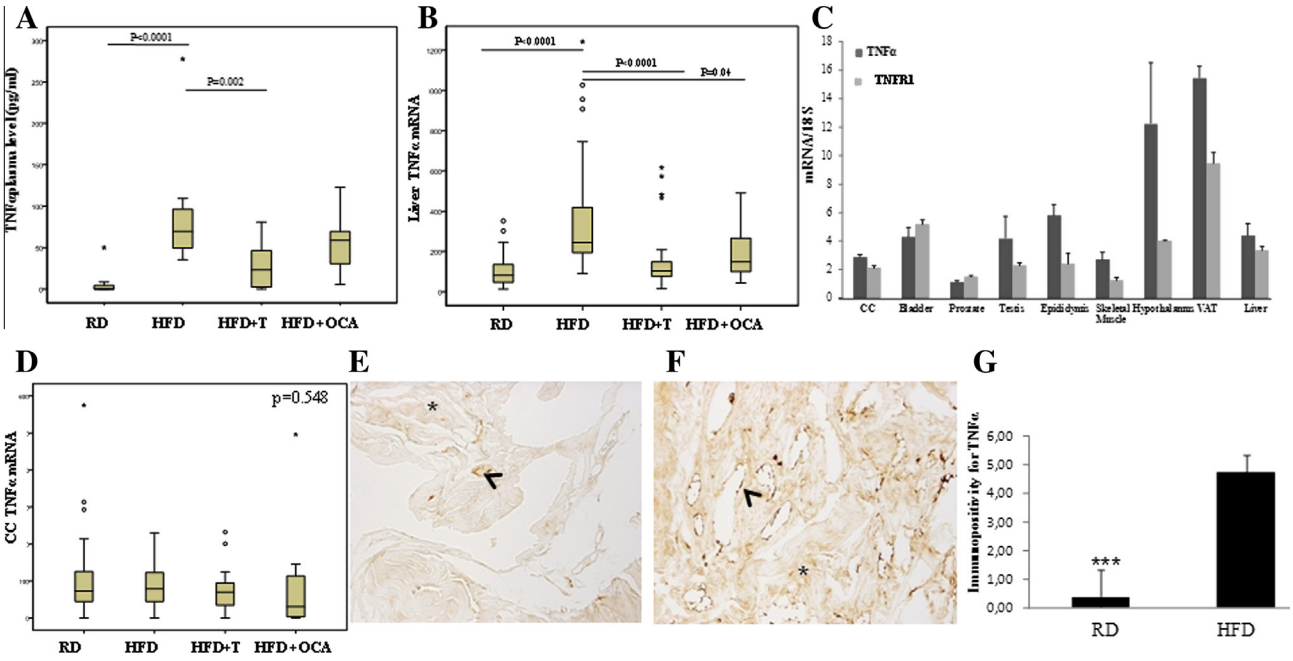

Fig. 3. Effects of HFD, and testosterone (T) or OCA treatment on TNFα. (A) Serum TNFα levels in the different experimental groups. Data are reported as mean ± SEM(n = 10 rabbits/group). (B) Liver TNFα mRNA expression (expressed as percentage of RD values). (C) TNFα and TNFR1 mRNA in several rabbit tissues (CC: penile corpora cavernosa; VAT: visceral adipose tissue), reported as mean ± SEM (3 samples/group). (D) TNFα mRNA in rabbit penis, reported as the mean SEM (n samples/group; RD n = 46; HFD n = 38; HFD + T n = 28; HFD + OCA n = 17). *Note:* TNFα expression was not significantly different at Kruskal–Wallis test (p value is reported within the panel). (E–G) Immunolocalization of TNFα in endothelium (arrowheads) and smooth muscle cells (asterisks) of rabbit CC (E: RD; F: HFD) (magnification, 10×). (G) Computer-assisted densitometry of TNFα positivity (at least 3 rabbits/group). $^{***}p < 0.01$ vs. HFD.

the contrary, as shown in Fig. 4B, responsiveness to SNP in the presence of vardenafil was reduced in HFD rabbits ($p < 0.05$ vs. RD), and restored, up to the RD level, in infliximab-treated rabbits.

Infliximab treatment in HFD rabbits increased penile eNOS mRNA expression (HFD = 49 ± 6.7; HFD + Infliximab = 104.4 ± 30.8; $p < 0.05$) up to the level of RD group ($p = 0.790$ vs. RD). In contrast, the penile expression of PDE5, PKG1, GCa3, GCb3 and nNOS was not affected by infliximab treatment (not shown). Among genes that in penis were down-regulated by infliximab there are TNFα itself and TIMP2 (HFD = 84.5 ± 11.4; 104.7 ± 15.6; HFD + infliximab = 26.7 ± 18.3; 48 ± 9.7, respectively, all $p < 0.05$).

4. Discussion

Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) are the hepatic counterpart of MetS, a condition often associated with erectile dysfunction (ED). An active role for liver alterations on ED pathophysiology has been hypothesized (Burra et al., 2010), but never demonstrated. We have previously reported that feeding rabbits a high fat diet (HFD) induces a syndromic condition that essentially recapitulates human MetS, along with associated morbidities, as hypogonadotropic hypogonadism (Filippi et al., 2009; Vignozzi et al., 2011, 2012a; Maneschi et al., 2012) and fatty liver disease (Maneschi et al., 2013). In the HFD rabbit model, we now show that MetS-dependent liver alterations are tightly associated with an impaired penile responsiveness to acetylcholine (Ach), the main physiological relaxant stimulus. We also provide evidence that the cytokine TNFα could bridge the impaired Ach-induced relaxation within penis and liver alterations. Indeed, in HFD rabbits treatments able to normalize TNFα liver expression and circulating levels (T and OCA), or to neutralize TNFα action (infliximab), significantly improve penile responsiveness to Ach.

Fatty liver disease in our experimental model of MetS is characterized by a diffuse lipid accumulation within hepatocytes and by fibrosis and mononuclear infiltration within the central vein spaces, indicating progression of NAFLD towards overt NASH. Accordingly, hepatic gene expression analysis indicates that markers of stellate cell activation (such as αSMA), fibrosis (such as TIMP1) and inflammation (such as TLR2 and TLR 4, the M1 macrophage marker CD68, and several chemokines and cytokines) were all markedly up-regulated in MetS rabbit livers. Our results are in keeping with other models of HFD-induced steatohepatitis in rabbits (Ogawa et al., 2010). Interestingly, the vast majority of these hepatic genes were negatively related to penile responsiveness to Ach. By iterative modeling of families of multivariate analyses, we found that TNFα expression in the liver is the main determinant of reduced relaxant response to Ach in penis. Feeding a HFD induced a striking increase not only in TNFα liver expression, but also in its circulating levels, fifteen-fold higher than in RD.

TNFα is a cytokine crucially involved in NAFLD progression to NASH (Tilg and Diehl, 2000). Interestingly, an increased plasma level of TNFα has been linked mechanistically to the endothelial dysfunction underlying many CVDs, including type 2 diabetes, atherosclerosis, and ED (Carneiro et al., 2010). Indeed, acute intra-arterial infusion of TNFα in humans was demonstrated to substantially reduce endothelium-dependent relaxation and NO production (Chia et al., 2003). Vlachopoulos first linked increased inflammatory biomarkers to ED (Vlachopoulos et al., 2006). Blood levels of TNFα, along with other pro-inflammatory (IL-6, IL-1β) and prothrombotic factors, were significantly increased in ED patients and negatively related to sexual performance (Vlachopoulos et al., 2006). Similar results were also found in patients with type 2 diabetes mellitus (Araña Rosaínz Mde et al., 2011) and in patients with obstructive sleep apnea syndrome (Matos et al., 2013). Animal models involving genetic manipulation of TNFα, have shown peculiar alterations of erectile function. Transgenic animals overexpressing TNFα showed erectile dysfunction (Hayward et al., 2007). Conversely, TNFα knockout mice exhibited enhanced penile NO-dependent relaxation, as well as increased eNOS expression

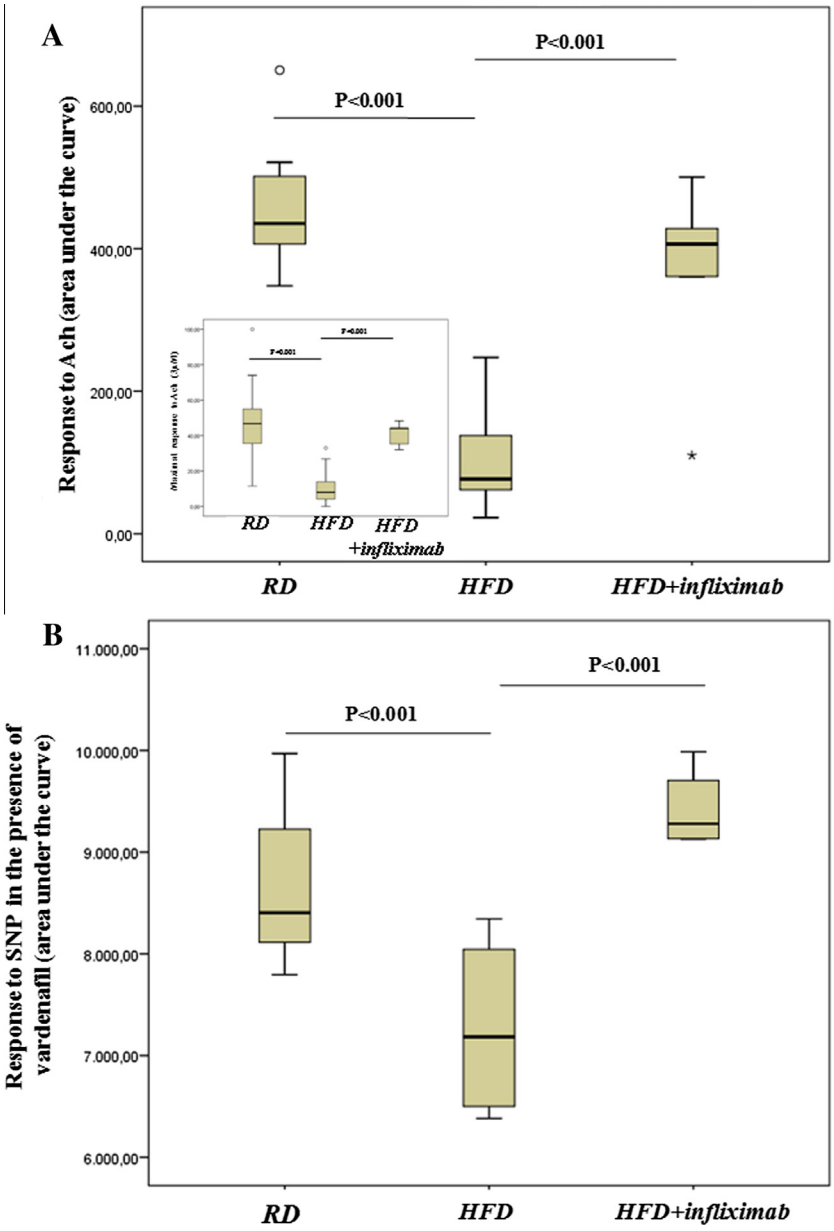

Fig. 4. Infliximab treatment restores penile responsiveness to relaxant stimuli. (A) Endothelium-dependent relaxation to increasing concentrations of Ach (1 nM–10 μM) strips expressed as area under the curve (AUC) or maximal responsiveness to Ach (3 μM, inset) in penis. Data are reported as mean ± SEM (at least five experiments/group). (B) Relaxation response to increasing concentration of SNP (1 nM–100 μM) in the presence of vardenafil (100 nM). Data are expressed as AUC and reported as mean ± SEM (at least four experiments/group). Symbols outside the boxes represent outliers.

(Carneiro et al., 2009). These findings suggest that TNFα directly induces an endothelial injury in different vascular beds. Interestingly, in endothelial cells TNFα directly suppressed eNOS expression by inhibiting either its gene promoter activity (Anderson and Rahmutula, 2004) or its mRNA stability (Yoshizumi et al., 1993).

Two distinct receptors mediate the effects of TNFα, TNFR1 and TNFR2. TNFR1 has a ubiquitous distribution (endothelial and smooth muscle cells), whereas TNFR2 expression is mostly confined to cell of hematopoietic origin (Ryffel and Mihatsch, 1993). TNFR1 selectively mediates the majority of the toxic and pro-atherogenic effects of TNFα (Zhang et al., 2010) and is also expressed by the penis (present study and Carneiro et al., 2010; Long et al., 2012). However, the relative abundance of TNFα and TNFR1 mRNA in rabbit penis was rather low and similar to other urogenital tissues. In addition, in penis, feeding a HFD induced a significant increase in the distribution of TNFα, but not in its mRNA expression. These findings suggest that penis is more a target than a source of TNFα, especially in MetS, in which TNFα plasma levels are significantly elevated.

Increased visceral adiposity, hypogonadism (Corona et al., 2011a,b), and NASH (Targher and Day, 2011), are all regarded as potential determinants in the pathogenesis of MetS-related CVDs. In MetS, both VAT and liver have been proposed as major source of TNFα (Traish et al., 2009). In the present study, we found no association between VAT expression of genes related to inflammation, including TNFα, and penile responsiveness to Ach. In line with these results, in a HFD rat model, Li et al. demonstrated that omentectomy (the surgical removal of the whole omentum and its constituent fat) did not decrease serum inflammatory factors (Li et al., 2013). We should recognize that metabolic complications, as those described in this MetS model, tend to co-segregate and parallel in

their progression. Hence, the NAFLD and endothelial/erectile dysfunction end points may represent only concomitant phenomena, associated with increased systemic TNFα, which can derive from multiple sites (Traish et al., 2009). However, we found only a significant association between circulating levels of TNFα and its hepatic, but not other tissues (VAT, skeletal muscle and others), gene expression, indicating that liver is one of the main source of TNFα.

Our previous studies (Filippi et al., 2009; Vignozzi et al., 2011; Maneschi et al., 2012, 2013) have shown that both T and OCA dosing in HFD rabbits significantly: (i) ameliorate lipid accumulation and liver inflammation; (ii) decrease TNFα liver expression as well as circulating TNFα plasma levels; and (iii) normalize impaired Ach responsiveness and eNOS gene expression levels in the penis.

To further investigate the relative contribution of TNFα in MetS-related ED, a subgroup of HFD animals were dosed with infliximab (5 mg/kg weekly),a chimeric mAb used to treat autoimmune diseases, as it binds to TNFα inhibiting its action. Therefore, treatment with infliximab has been aimed at neutralizing systemic TNF α, which can derive from a series of tissues, including liver. Infliximab did not normalize T level, but significantly increased eNOS expression and normalized Ach responsiveness in penis. Similar effects of infliximab on penile function have already been observed in a rat model of HFD/STZ type 2 diabetes (Long et al., 2012). Conversely, infliximab treatment did not affect either the relaxant response to SNP (a NO donor, which bypasses endogenous NO formation), or the expression of genes downstream NO signaling (GCa3, CGb3, PKG1). It is plausible therefore, as already demonstrated in other vascular districts (Chia et al., 2003), that TNFα exerts a selective detrimental effect on endothelium-dependent, but not endothelium-independent, relaxation also in the penis. Another important observation of the present study is that neutralization of TNFα by infliximab completely normalized HFD-induced hyporesponsiveness to the PDE5 inhibitor vardenafil. However, we did not find any significant effect of infliximab dosing on penile PDE5 mRNA expression. Hence, the increased responsiveness to PDE5 inhibitor in infliximab-treated rabbits could be related to an increased enzymatic activity or NO availability. We recently demonstrated that variations in PDE5 enzymatic activity, rather than in its mRNA expression, were responsible for changes in responsiveness to PDE5 inhibitors (Vignozzi et al., 2012b).

In the HFD-induced MetS model we previously showed that OCA treatment decreases HFD-induced TNFα gene and protein up-regulation, as well as lipid accumulation (Maneschi et al., 2013). Moreover, in a double-blind placebo-controlled study, a 6-weeks treatment with OCA in patients with type 2 diabetes mellitus and NAFLD not only decreased insulin resistance determined by a 2-stage hyperinsulinemic–euglycemic insulin clamp, a key determinant in the transition of NAFLD to NASH (Adorini et al., 2012), but also significantly reduced circulating levels of transaminases and γ-glutamyl transferase, and reduced liver fibrosis markers (Mudaliar et al., 2013).

An anti-inflammatory effect of androgens has previously been reported in preclinical studies of human and rabbit prostate (Vignozzi et al., 2012a, 2013, 2012c). We now extend this evidence to the liver, suggesting that treating hypogonadism could blunt liver inflammation, thus counteracting the development of MetS-associated CVD. Androgen deficiency and low circulating T levels have been demonstrated to be independently associated with NAFLD/NASH (Kim et al., 2012; Völzke et al., 2010). Recently, several reports demonstrated that raising serum T concentrations to normal levels in hypogonadal patients by treatment with parenteral T, along with improving metabolic profile, significantly reduces the levels of liver transaminases (Haider et al., 2010; Traish et al., 2013), and circulating TNFα levels (Kalinchenko et al., 2010). In a randomized, double-blind, placebo-controlled study, Hoyos and colleagues showed that 18-week treatment with T significantly reduces liver fat content, as well as decreased arterial stiffness (Hoyos et al., 2012). The impact of androgens upon liver has been studied also by using KO animal models. Interestingly both hepatic androgen receptor KO mice (Lin et al., 2008) and 5α-reductase type 1 KO mice (Dowman et al., 2013) developed greater hepatic steatosis and inflammation than the relative wild type mice.

We recently demonstrated that T supplementation in HFD-MetS animals, prevented hypertrophic and dysfunctional expansion of VAT, restoring lipid droplet handling and insulin sensitivity in adipocytes (Maneschi et al., 2012). We now propose this anti-inflammatory effect on the liver as an additional mechanism by which T could improve metabolic control.

In conclusion, our study demonstrates that increased liver and plasma TNFα levels, associated with HFD-induced NASH, have a direct, detrimental effect on penile pro-erectile mechanisms. This is further supported by the observation that the ED induced in this model of MetS is significantly reduced by treatments, like T and OCA, able to inhibit TNFα liver expression and plasma levels, or to neutralize TNFα activity (such as infliximab), which normalize penile endothelium-dependent relaxation.

Acknowledgements

The study was supported by PRIN (Programmi di Ricerca di Rilevante Interesse Nazionale) funds by the Italian Minister of University, Research and Instruction (Prot Number: 2009WLNXNT_002), by FIRB (Programma Futuro in Ricerca) funds by the Italian Minister of University, Research and Instruction (Prot Number: RBFR10VJ56_002).

References

Adorini, L., Pruzanski, M., Shapiro, D., Farnesoid, X., 2012. Receptor targeting to treat nonalcoholic steatohepatitis. Drug Discov. Today 17, 988–997.
Alkhouri, N., Tamimi, T.A., Yerian, L., Lopez, R., Zein, NN., Feldstein, AE., 2010. The inflamed liver and atherosclerosis: a link between histologic severity of nonalcoholic fatty liver disease and increased cardiovascular risk. Dig. Dis. Sci. 55, 2644–2650.
Anderson, H.D., Rahmutula, D., 2004. Gardner DG Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. J. Biol. Chem. 279, 963–969.
Araña Rosaínz Mde, J., Ojeda, MO., Acosta, JR., Elías-Calles, LC., González, NO., Herrera, OT., et al., 2011. Imbalanced low-grade inflammation and endothelial activation in patients with type 2 diabetes mellitus and erectile dysfunction. J. Sex Med. 8, 2017–2030.
Burra, P., Germani, G., Masier, A., De Martin, E., Gambato, M., Salonia, A., et al., 2010. Sexual dysfunction in chronic liver disease: is liver transplantation an effective cure? Transplantation 89, 1425–1429.
Carneiro, F.S., Sturgis, L.C., Giachini, F.R., Carneiro, Z.N., Lima, V.V., Wynne, B.M., et al., 2009. TNF-alpha knockout mice have increased corpora cavernosa relaxation. J. Sex Med. 6, 115–125.
Carneiro, F.S., Webb, R.C., Tostes, R.C., 2010. Emerging role for TNF-α in erectile dysfunction. J. Sex Med. 7, 3823–3834.
Chia, S., Qadan, M., Newton, R., Ludlam, C.A., Fox, K.A., 2003. Newby DE Intra-arterial tumor necrosis factor-alpha impairs endothelium-dependent vasodilatation and stimulates local tissue plasminogen activator release in humans. Arterioscler. Thromb. Vasc. Biol. 23, 695–701.
Corona, G., Mannucci, E., Schulman, C., Petrone, L., Mansani, R., Cilotti, A., et al., 2006. Psychobiologic correlates of the metabolic syndrome and associated sexual dysfunction. Eur. Urol. 50, 595–604.
Corona, G., Rastrelli, G., Morelli, A., Vignozzi, L., Mannucci, E., Maggi, M., 2011a. Hypogonadism and metabolic syndrome. J. Endocrinol. Invest. 34, 557–567.
Corona, G., Rastrelli, G., Vignozzi, L., Mannucci, E., Maggi, M., 2011b. Testosterone, cardiovascular disease and the metabolic syndrome. Best Pract. Res. Clin. Endocrinol. Metab. 25, 337–353.
Dowman, J.K., Hopkins, L.J., Reynolds, G.M., Armstrong, M.J., Nasiri, M., Nikolaou, N., et al., 2013. Loss of 5α-reductase Type 1 accelerates the development of hepatic steatosis but protects against hepatocellular carcinoma in male mice. Endocrinology 154 (12), 4536–4547.
Filippi, S., Vignozzi, L., Morelli, A., Chavalmane, A.K., Sarchielli, E., Fibbi, B., et al., 2009. Testosterone partially ameliorates metabolic profile and erectile responsiveness to PDE5 inhibitors in an animal model of male metabolic syndrome. J. Sex Med. 6, 3274–3288.
Haider, A., Gooren, L.J., Padungtod, P., Saad, F., 2010. Improvement of the metabolic syndrome and of non-alcoholic liver steatosis upon treatment of hypogonadal

elderly men with parenteral testosterone undecanoate. Exp. Clin. Endocrinol. Diabetes 118, 167–1671.
Hayward, M.D., Jones, B.K., Saparov, A., Hain, H.S., Trillat, A.C., Bunzel, M.M., et al., 2007. An extensive phenotypic characterization of the hTNFalpha transgenic mice. BMC Physiol. 10, 7–13.
Hoyos, C.M., Yee, B.J., Phillips, C.L., Machan, E.A., Grunstein, R.R., Liu, P.Y., 2012. Body compositional and cardiometabolic effects of testosterone therapy in obese men with severe obstructive sleep apnea: a randomized placebo-controlled trial. Eur. J. Endocrinol. 167, 531–541.
Kalinchenko, S.Y., Tishova, Y.A., Mskhalaya, G.J., Gooren, L.J., Giltay, E.J., Saad, F., 2010. Effects of testosterone supplementation on markers of the metabolic syndrome and inflammation in hypogonadal men with the metabolic syndrome: the double-blinded placebo-controlled Moscow study. Clin. Endocrinol. 73, 602–612.
Kim, S., Kwon, H., Park, J.H., Cho, B., Kim, D., Oh, S.W., et al., 2012. A low level of serum total testosterone is independently associated with nonalcoholic fatty liver disease. BMC Gastroenterol. 12, 69–73.
Li, Y., Liu, L., Wang, B., Wang, J., Chen, D., 2013. Simple steatosis is a more relevant source of serum inflammatory markers than omental adipose tissue. Clin. Res. Hepatol. Gastroenterol., pii:S2210-7401(13)00180-0.10.1016/j.clinre.2013.08.006.
Lin, H.Y., Yu, I.C., Wang, R.S., Chen, Y.T., Liu, N.C., Altuwaijri, S., et al., 2008. Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor. Hepatology 47, 1924–1935.
Long, T., Liu, G., Wang, Y., Chen, Y., Zhang, Y., Qin, D., 2012. TNF-α, erectile dysfunction, and NADPH oxidase-mediated ROS generation in corpus cavernosum in high-fat diet/streptozotocin-induced diabetic rats. J. Sex Med. 9, 1801–1814.
Maneschi, E., Morelli, A., Filippi, S., Cellai, I., Comeglio, P., Mazzanti, B., et al., 2012. Testosterone treatment improves metabolic syndrome-induced adipose tissue derangements. J. Endocrinol. 215, 347–362.
Maneschi, E., Vignozzi, L., Morelli, A., Mello, T., Filippi, S., Cellai, I., et al., 2013. FXR activation normalizes insulin sensitivity in visceral preadipocytes of a rabbit model of MetS. J. Endocrinol. 218, 215–231.
Marchesini, G., Bugianesi, E., Forlani, G., Cerrelli, F., Lenzi, M., Manini, R., et al., 2003. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 37, 917–923.
Matos, G., Hirotsu, C., Alvarenga, T.A., Cintra, F., Bittencourt, L., Tufik, S., et al., 2013. The association between TNF-α and erectile dysfunction complaints. Andrology 1, 872–878.
Morelli, A., Filippi, S., Mancina, R., Luconi, M., Vignozzi, L., Marini, M., Orlando, C., Vannelli, G.B., Aversa, A., Natali, A., Forti, G., Giorgi, M., Jannini, E.A., Ledda, F., Maggi, M., 2004. Androgens regulate phosphodiesterase type 5 expression and functional activity in corpora cavernosa. Endocrinology 145, 2253–2263.
Morelli, A., Comeglio, P., Filippi, S., Sarchielli, E., Vignozzi, L., Maneschi, E., Cellai, I., Gacci, M., Lenzi, A., Vannelli, G.B., Maggi, M., 2013. Mechanism of action of phosphodiesterase type 5 inhibition in metabolic syndrome-associated prostate alterations: an experimental study in the rabbit. Prostate 73, 428–441.
Mudaliar, S., Henry, R.R., Sanyal, A.J., Morrow, L., Marschall, H.U., Kipnes, M., et al., 2013. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. Gastroenterology 145, 574–582.
Ogawa, T., Fujii, H., Yoshizato, K., Kawada, N., 2010. A human-type nonalcoholic steatohepatitis model with advanced fibrosis in rabbits. Am. J. Pathol. 177, 153–165.
Ryffel, B., Mihatsch, M.J., 1993. TNF receptor distribution in human tissues. Int. Rev. Exp. Pathol. 34, 149–156.
Targher, G., Day, C.P., 2011. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease. Hepatology 53, 375–386.
Targher, G., Bertolini, L., Rodella, S., Lippi, G., Franchini, M., Zoppini, G., et al., 2008. Day CP NASH predicts plasma inflammatory biomarkers independently of visceral fat in men. Obesity 16, 1394–1399.
Tilg, H., Diehl, AM., 2000. Cytokines in alcoholic and nonalcoholic steatohepatitis. N. Engl. J. Med. 343, 1467–1476.
Traish, A.M., Feeley, R.J., Guay, A., 2009. Mechanisms of obesity and related pathologies: androgen deficiency and endothelial dysfunction may be the link between obesity and erectile dysfunction. FEBS J. 276, 5755–5767.
Traish, A.M., Miner, M.M., Morgentaler, A., Zitzmann, M., 2011. Testosterone deficiency. Am. J. Med. 124, 578–587.
Traish, A.M., Haider, A., Doros, G., Saad, F., 2013. Long-term testosterone therapy in hypogonadal men ameliorates elements of the metabolic syndrome: an observational, long-term registry study. Int. J. Clin. Pract. http://dx.doi.org/10.1111/ijcp.12319.
Vignozzi, L., Corona, G., Petrone, L., Filippi, S., Morelli, A.M., Forti, G., Maggi, M., 2005. Testosterone and sexual activity. J. Endocrinol. Invest. 28, 39–44.
Vignozzi, L., Morelli, A., Filippi, S., Comeglio, P., Chavalmane, A.K., Marchetta, M., et al., 2011. Farnesoid X receptor activation improves erectile function in animal models of metabolic syndrome and diabetes. J. Sex Med. 8, 57–77.
Vignozzi, L., Morelli, A., Sarchielli, E., Comeglio, P., Filippi, S., Cellai, I., et al., 2012a. Testosterone protects from metabolic syndrome-associated prostate inflammation: an experimental study in rabbit. J. Endocrinol. 212, 71–84.
Vignozzi, L., Filippi, S., Morelli, A., Comeglio, P., Cellai, I., Sarchielli, E., et al., 2012b. Testosterone/estradiol ratio regulates NO-induced bladder relaxation and responsiveness to PDE5 inhibitors. J. Sex Med. 9, 3028–3040.
Vignozzi, L., Cellai, I., Santi, R., Lombardelli, L., Morelli, A., Comeglio, P., et al., 2012c. Anti-inflammatory effect of androgen receptor activation in human benign prostatic hyperplasia cells. J. Endocrinol. 211, 31–43.
Vignozzi, L., Gacci, M., Cellai, I., Santi, R., Corona, G., Morelli, A., Rastrelli, G., et al., 2013. Fat boosts, while androgen receptor activation counteracts, BPH-associated prostate inflammation. Prostate 73, 789–800.
Vlachopoulos, C., Aznaouridis, K., Ioakeimidis, N., Rokkas, K., Vasiliadou, C., Alexopoulos, N., et al., 2006. Unfavourable endothelial and inflammatory state in erectile dysfunction patients with or without coronary artery disease. Eur. Heart J. 27, 2640–2648.
Völzke, H., Aumann, N., Krebs, A., et al., 2010. Hepatic steatosis is associated with low serum testosterone and high serum DHEAS levels in men. Int. J. Androl. 33, 45–53.
Yoshizumi, M., Perrella, M.A., Burnett Jr, J.C., Lee, M.E., 1993. Tumor necrosis factor down-regulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ. Res. 73, 205–209.
Zhang, L., Connelly, J.J., Peppel, K., Brian, L., Shah, S.H., Nelson, S., et al., 2010. Aging-related atherosclerosis is exacerbated by arterial expression of tumor necrosis factor receptor-1: evidence from mouse models and human association studies. Hum. Mol. Genet. 19, 2754–2766.