Testosterone differentially regulates targets of lipid and glucose metabolism in liver, muscle and adipose tissues of the testicular feminised mouse

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# Endocrine

**Testosterone differentially regulates targets of lipid and glucose metabolism in liver, muscle and adipose tissues of the testicular feminised mouse**

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| **Abstract:** | Purpose: Testosterone deficiency is commonly associated with obesity, metabolic syndrome, type 2 diabetes and their clinical consequences - hepatic steatosis and atherosclerosis. The testicular feminised (tfm) mouse (non-functional androgen receptor and low testosterone) develops fatty liver and aortic lipid streaks on a high fat diet whereas androgen replete XY littermate controls do not. Testosterone replacement ameliorates these effects, although the underlying mechanisms remain unknown. Methods: We compared the influence of testosterone on the expression of regulatory targets of glucose, cholesterol and lipid metabolism in muscle, liver, abdominal subcutaneous (SAT) and visceral adipose tissue (VAT). Results: Tf mice displayed significantly reduced GLUT4 in muscle and glycolytic enzymes in muscle, liver and SAT but not VAT. Lipoprotein lipase required for fatty acid uptake was only reduced in SAT, enzymes of fatty acid synthesis were increased. Stearoyl-CoA desaturase-1 that catalyses oleic acid synthesis and is associated with insulin resistance was increased in VAT and cholesterol efflux components (ABCA1, apoE) were decreased. Master regulator nuclear receptors involved in metabolism:- Liver X receptor expression was suppressed in all tissues except VAT whereas PPARy was lower in SAT and VAT and PPARα only in SAT. Testosterone replacement improved the expression (androgen receptor independent) of some targets but not all. Conclusions: These exploratory data |
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suggest that androgen deficiency could cause reduced buffering capability for glucose uptake and utilisation in SAT and muscle and fatty acids in SAT. This would lead to an overspill and uptake of excess glucose and triglycerides into VAT, liver and arterial walls.
Testosterone differentially regulates targets of lipid and glucose metabolism in liver, muscle and adipose tissues of the testicular feminised mouse

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Abstract

**Purpose:** Testosterone deficiency is commonly associated with obesity, metabolic syndrome, type 2 diabetes and their clinical consequences - hepatic steatosis and atherosclerosis. The testicular feminised (tfm) mouse (non-functional androgen receptor and low testosterone) develops fatty liver and aortic lipid streaks on a high-fat diet whereas androgen replete XY littermate controls do not. Testosterone replacement ameliorates these effects, although the underlying mechanisms remain unknown. **Methods:** We compared the influence of testosterone on the expression of regulatory targets of glucose, cholesterol and lipid metabolism in muscle, liver, abdominal subcutaneous (SAT) and visceral adipose tissue (VAT). **Results:** Tfm mice displayed significantly reduced GLUT4 in muscle and glycolytic enzymes in muscle, liver and SAT but not VAT. Lipoprotein lipase required for fatty acid uptake was only reduced in SAT, enzymes of fatty acid synthesis were increased. Stearoyl-CoA desaturase-1 that catalyses oleic acid synthesis and is associated with insulin resistance was increased in VAT and cholesterol efflux components (ABCA1, apoE) were decreased. Master regulator nuclear receptors involved in metabolism: Liver X receptor expression was suppressed in all tissues except VAT whereas PPARγ was lower in SAT and VAT and PPARα only in SAT. Testosterone replacement improved the expression (androgen receptor independent) of some targets but not all. **Conclusion:** These exploratory data suggest that androgen deficiency may reduce the buffering capability for glucose uptake and utilisation in SAT and muscle and fatty acids in SAT. This would lead to an overspill and uptake of excess glucose and triglycerides into VAT, liver and arterial walls.
47

48 Introduction

49 Evidence suggests that testosterone deficiency in men is an independent cardiovascular risk factor
50 which is associated with obesity, metabolic syndrome (MetS) and type-2 diabetes (T2D) [1, 2].
51 Insulin resistance, which is common to all of these conditions, results in diminished glucose
52 utilisation and conversion of the excess glucose into fat. Higher circulating triglycerides then lead to
53 an overspill of fat into ectopic storage in liver and arteries as well as increasing the accumulation of
54 visceral fat. The degree of insulin resistance correlates negatively with serum testosterone [3, 4].
55 Although the causality of this relationship is often debated, growing evidence indicates testosterone is
56 a metabolic multi-system player [5]. Epidemiological studies support a bidirectional relationship
57 between serum testosterone and obesity which may be explained by the hypogonadal–obesity–
58 adipocytokine hypothesis [6, 7]. Androgen deprivation therapy for the treatment of prostate cancer in
59 men, whilst reducing tumour growth also increases the risk of coronary heart disease (CHD), diabetes
60 and cardiovascular death indicating that testosterone deficiency may promote atherosclerosis [8, 9].
61 Some trials have reported that achieving a normal physiological testosterone concentration through
62 the administration of testosterone replacement therapy (TRT) improves vascular function and risk
63 factors for atherosclerosis including; reducing central adiposity, percentage body fat, fatty liver and
64 insulin resistance, and improving lipid profiles insulin sensitivity and inflammatory profiles [2, 10-
65 15].
66 A limited number of in vivo and in vitro investigations have highlighted potential molecular targets of
67 testosterone action in metabolic regulation, although a detailed analysis of tissue-specific actions
68 remains absent from the literature [2]. We have previously reported that low testosterone in the Tfm
69 mouse (which displays very low testosterone levels and non-functional androgen receptors) is
70 associated with increased lipid deposition in the aortic root and liver when mice are a fed high-
71 cholesterol diet [16-18]. Testosterone treatment to return levels to those seen in wild-type counterparts
72 significantly reduced aortic fatty steaks and hepatic lipid accumulation with an associated reduction in
73 de novo lipogenesis in the liver in Tfm mice [17].
While a growing body of evidence points towards the presence of heterogeneity regarding insulin responsiveness and lipid homeostasis among different tissues [19], the mechanisms by which testosterone may impart beneficial actions on insulin sensitivity and hence the development of MetS, T2D and cardiovascular risk remain unknown but are likely to be tissue dependent and involve multiple targets of lipid and carbohydrate metabolism. In the present exploratory study, we aim to investigate whether the metabolic protective effects of testosterone act via modulation of the expression of key targets involved in lipid and glucose metabolism in muscle, liver and adipose tissue of cholesterol-fed Tfm mice. Specifically, we investigate key regulatory enzymes of glycolysis, glycogen synthesis, pentose phosphate pathway, glucose transporters, fatty acid synthesis, fatty acid uptake, cholesterol synthesis and efflux, and master regulators of metabolism (see table 1).

Materials and methods

Animals

The Tfm mouse was used as a model of testosterone deficiency and androgen receptor (AR) dysfunction as previously described [16-18]. The loss of 17α-hydroxylase, a key enzyme necessary for testosterone synthesis, leads to serum levels of testosterone in the Tfm mouse that are severely (approximately 10-fold) reduced compared to normal XY littermate controls [20, 21]. In addition, a natural mutation in the gene encoding the AR leads to the formation of a truncated receptor protein which lacks both DNA- and steroid-binding domains rendering it non-functional [22, 23]. This model therefore allows potential AR dependent and independent effects to be investigated. All procedures were carried out under the jurisdiction of a UK Home Office project licence, governed by the UK Animals Scientific Procedures Act 1986. Mice were bred as previously described [20]. Animal numbers were calculated based on our previous investigation [16] for a significance level of 5%, and power of 90% for the primary outcome measure of lipid deposition in the aortic root (see [18]). Where available, preliminary data was used for calculation of sample numbers of individual variables.
Experimental design and tissue collection

8-week-old Tfm and XY littermate mice were fed a high-fat, high-cholesterol diet, containing 42% butterfat, 1.25% cholesterol and 0.5% cholate (Special Diet Services, Essex, UK) *ad libitum* for a period of 28 weeks. Separate 7-week old Tfm mice were randomly assigned to one of two groups; placebo group receiving a once-fortnightly intramuscular injection of 10μL of saline (n=14), or testosterone group (n=14) receiving a once-fortnightly intramuscular injection of 10μL of 100mg/mL testosterone esters (Sustanon100; testosterone propionate 20mg/mL, testosterone phenylpropionate 40mg/mL and testosterone isocaproate 40mg/mL, Organon Laboratories Ltd, Cambridge, UK), providing a dose of 50mg/kg, previously shown to replace circulating levels to those of wild-type littermate mice [16]. XY littermate mice (n=14) received placebo injections (10μL saline). Animals were caged under standard conditions in a temperature and humidity controlled room on a 12h light:12h darkness cycle. Water and food were unrestricted throughout the study.

At the end of the experimental period which corresponded with the midway point of the fortnightly injection cycle, whole blood was collected from the thoracic cavity following mid-line sternotomy and severance of the thoracic aorta. Following centrifugation, serum samples were frozen at -80°C. The liver was removed from the abdomen, skeletal muscle dissected from the quadriceps of the hind legs and fat tissue collected from subcutaneous and visceral abdominal regions. The heart with thoracic aorta attached was carefully dissected free from the adventitia and perfused. Tissues were processed for both histological and gene and protein expression analysis and were archived for future analysis. Analyses were made on individual samples.

Measurement of Total Testosterone and 17β-Estradiol

Serum quantification of total testosterone (DRG Instruments GmbH, Marburg, Germany) and 17β-estradiol (Demeditec Diagnostics, Kiel, Germany) were measured in duplicate via ELISA (measurement range 0.2-16ng/mL and 3-200pg/mL respectively).

Quantitative analysis of mRNA
Total RNA was extracted from approximately 100mg of snap-frozen tissue, reverse transcribed and cDNA (2μL) used for qPCR, using commercial SYBR green reagents (Qiagen) as described previously [17]. Primers were purchased pre-validated (QuantiTech primer assays; Qiagen), with specified amplification efficiencies of 100% (±10%) (See table 1). Primers for B-2 microglobulin (B2m) were also included and served as an internal reference control, selected as the most stable gene from a panel of commonly used reference genes (Gapdh, beta-actin, ribosomal protein 13A). Each reaction was carried out in triplicate with cycling and detection of fluorescent signal carried out using an Agilent Mx3000P QPCR System. Results were corrected for the expression of the house-keeping gene and normalised to the XY littermates as a control. Relative copy number was expressed as fold change 2-(ddCT).

**Western Immunoblotting**

In this exploratory study we selected targets that were significantly altered at the gene expression level for analysis by western blotting. Due to low concentrations of protein ascertainable from limited availability of adipose tissue, western blotting was unable to be carried out on subcutaneous and visceral samples. Protein was extracted from 200mg of mouse liver or muscle tissue as previously described [17]. In brief, 50μg of total isolated protein was separated by electrophoresis and transferred to nitrocellulose membrane (BioRad, Hertfordshire, UK). Membranes were blocked for 1 h in 5% dried semi-skimmed milk diluted in tris/glycine (TG) buffer containing 0.05% Tween 20 (TGT; BioRad, UK). Primary antibodies were incubated overnight at 4°C diluted in either 5% bovine serum albumin/TGT, 5% milk/TGT or 2.5% milk/BSA (see Table 2). Immunoreactive proteins were detected using anti-rabbit IgG HRP-linked secondary antibody (1:500, Cell Signalling) for polyclonal antibody detection or anti-mouse IgG HRP-linked secondary antibody (1:500, Cell Signalling) followed by a chemiluminescence peroxidase substrate kit (Roche, Sussex, UK). Band intensities were quantified using Genetools software (Syngene, Cambridge, UK) relative to the house-keeping protein GAPDH or Calnexin.
**Statistical Analysis**

Results are presented as mean ± SEM. Differences between groups with normally distributed data were compared using unpaired t tests without assuming consistent standard deviations of groups. Mann–Whitney U tests were used where data did not follow a normal distribution. Corrections for multiple comparisons were made using the Sidak-Bonferroni post hoc test. Significance was accepted at P ≤ 0.05.

**Results**

Serum testosterone levels were greatly reduced in Tfm mice (2.2±1.2 nM, p=0.03) compared to wild-type equivalents (16.5±4.3 nM)\(^1\). Testosterone treatment of Tfm mice increased serum levels of testosterone comparable to wild-type levels (14.7±5.2 nM, p=0.98). 17-β estradiol levels were similar between all groups, Tfm mice (94.2±15.5 pMol) compared to wild-type (106.0±33.9 pMol, p=0.17) and testosterone-treated Tfm mice (135.2±28.7 pMol, p=0.99). Animal weights and weight gain did not significantly differ between groups over the duration of the 28 week feeding period but there was a trend towards Tfm mice gaining more weight compared to littermates by the end of the study period (p=0.066, n=14; Figure 1).

**Carbohydrate Metabolism**

Gene expression of the glycolytic regulatory gateway enzymes hexokinase (Hk2, Gck) and Pfk was significantly lower in muscle (p=0.012, p=0.032), liver (p=0.002, p=0.04) and SAT (p=0.009, p=0.03) but not in VAT of Tfm-placebo mice compared to XY littermates (Table 3). Testosterone administration increased Gck expression (p=0.015) in the liver of Tfm mice but these enzymes were not significantly altered in other tissues by treatment. Glut4 was similarly decreased in muscle (p=0.015) and SAT (p=0.014) of Tfm mice versus wild-type mice, with no effect of testosterone treatment. Hepatic G6pdh was elevated in Tfm mice compared to XY mice (p<0.001) and testosterone

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\(^1\) As previously published [16, 18, 20]
treatment showed a trend to reducing this expression in Tfm mice (p=0.056). All other gene targets were not altered between experimental groups in the tissues investigated.

Protein expression of PFK in the liver of experimental animals matched gene expression data with reduced levels in Tfm placebo mice compared to wild-type (p=0.005) and no effect of treatment with testosterone (Figure 2). Muscle protein expression of PFK was reduced in Tfm mice (p=0.018) with a significant increase in expression following TRT (p=0.01). Hepatic GCK protein was also reduced in Tfm mice receiving placebo (p=0.001) as demonstrated at the gene level, however testosterone administration had no effect showing discrepancy between gene and protein expression. HK2 in muscle was also reduced at the protein level in Tfm mice (p=0.024), but there was no effect due to treatment. Muscle GLUT4 was decreased in Tfm mice compared to wildtype (p=0.037) and TRT demonstrated a trend towards increasing this expression (p=0.053). We were unable to detect G6PD protein expression in the liver of experimental animals.

Lipid Metabolism

a) Cholesterol metabolism

Expression of cholesterol transporters, Apoe and Abca1, were reduced in the liver of Tfm mice compared to littermates (p=0.009, p=0.002). Treatment with testosterone significantly increased this expression (p=0.027, p=0.02), similar to wild-type levels (Table 3). Similarly, Apoe was decreased in SAT of Tfm mice (p=0.01), an effect that was abolished by testosterone administration (p=0.015 vs Tfm P). Srehf1 and Srehf2 expression was significantly lower in the SAT of Tfm mice versus XY littermates (p=0.002, p=0.003). Treatment with testosterone elevated these expression levels of Srehf1 (p=0.015) similar to those demonstrated in wild-type mice although not significantly so for Srehf2 with only a trend towards increased expression observed (p=0.053). In contrast to gene expression data, ABCA1 protein was not significantly altered between treatment groups although mean expression appeared to demonstrate a similar pattern (Figure 2). APOE protein expression matched
gene expression data with significantly lower levels in placebo-treated Tfm mice compared to XY
littermates and testosterone-treated Tfm mice (p=0.011, p=0.007 respectively).

b) Fatty acid metabolism

Visceral adipose Scdl expression was significantly higher in Tfm mice receiving placebo than in XY
littermates also receiving placebo injections (p=0.034, Table 3). Testosterone treatment of Tfm mice
returned expression levels to those of XY mice with a significant reduction compared to placebo-
treated Tfm mice (p=0.027). T test analysis similarly revealed an increase in hepatic Scdl expression
in Tfm placebo mice although not statistically significant (p=0.08). Decreased Lpl expression was
observed in SAT from Tfm mice compared to wildtype (p=0.016) although testosterone
administration to Tfm animals had no effect on this. Hepatic gene expression of Fasn and Acaca, the
key regulatory enzymes in de novo lipogenesis, were significantly increased in Tfm mice receiving
placebo injections compared to wild-type littermates (p=0.049, p=0.042 respectively). Testosterone
treatment decreased this expression but not significantly. Gene expression of all other lipid
metabolism targets in liver and adipose tissue were not significantly different between animal groups.

Western blotting showed hepatic protein expression of FASN and ACACA to be increased in Tfm
mice confirming gene expression findings. Testosterone treatment significantly reduced the protein
erexpression of these enzymes versus placebo treated Tfm mice to similar levels as XY littermates.

No targets of fat metabolism and cholesterol homeostasis displayed altered gene expression in muscle
tissue from the different experimental groups.

Master Regulators

Gene expression of Lxr was significantly reduced in Tfm placebo mice in all tissues other than
visceral adipose (muscle p=0.032, liver p<0.001, SAT p=0.003), and testosterone administration
increased expression significantly and back to wild-type levels in these tissues (muscle p=0.008, liver

2 As previously published [15]
p=0.024, SAT p=0.03). Ppara and Pparg were significantly reduced in SAT of Tfm mice receiving placebo versus XY littermate controls (p=0.01, p=0.02 respectively). Pparg was also reduced in visceral adipose tissue of Tfm mice (p=0.001). Testosterone treatment had no effect on the altered expression of Ppars when compared to placebo treated Tfm mice (see Table 3).

LXR protein expression in liver and muscle demonstrated the same pattern indicated by gene expression analysis with a reduction in Tfm placebo mice compared to wild-type littermates (p=0.001, p=0.01). Treatment with testosterone elevated LXR levels significantly in liver and muscle (p=0.024, p=0.022), to similar levels seen in placebo-treated Tfm mice (Figure 2).

**Discussion**

Exploratory evidence from this study suggests that testosterone has tissue-specific metabolic effects in the regulation of gene targets which control glucose utilisation in liver, SAT and skeletal muscle, and lipid metabolism in liver and SAT. Some of these effects are, at least in part, androgen receptor-independent and may potentially explain some of the observed clinical benefit of testosterone in men with T2D and MetS.

**Testosterone Effects on Expression of Targets of Glucose Metabolism**

GLUT4 expression is known to correlate positively with insulin responsiveness and defects in expression of GLUT4 have been observed in patients with T2D [24]. We have shown that there is decreased expression of GLUT4 in muscle and SAT in the testosterone deficient Tfm mouse.

Testosterone has previously been shown to increase the expression of GLUT4 in cultured skeletal muscle cells, hepatocytes and adipocytes [25-27] as well as augmenting membrane translocation and promoting glucose uptake in adipose and skeletal muscle tissue [27]. Key enzymes involved in glycolysis, PFK and HK, were significantly reduced in muscle, liver and SAT of Tfm mice. This supports previous studies which have demonstrated an increase in the activity of PFK and HK in cultured rat skeletal muscle cells and increased hexokinase activity in muscle tissue of castrated rats
following testosterone treatment thus diminishing the raised blood levels of glucose seen in untreated
ccontrol rats [27-29]. Improved glucose utilisation in muscle, liver and SAT by testosterone may
reduce the conversion of glucose to fat in times of excess and improve insulin sensitivity thus
reducing lipid accumulation in these and other tissues. This clinically would be very important in
muscle as this tissue accounts for approximately 75% of whole-body insulin-stimulated glucose
uptake [30, 31].

We have also demonstrated in this study that the mRNA expression of Glucose-6-phosphate
dehydrogenase (G6pd), the gateway enzyme in the pentose phosphate shunt pathway, is elevated in
the liver of Tfm mice suggesting that glucose may also be utilised down this route during testosterone
deficiency. NADPH is produced by G6PD in the pentose phosphate pathway supplying reducing
power to contribute to fatty acid synthesis [32]. An aberrant increase of G6PD expression is present in
obese and diabetic subjects, and overexpression of G6PD alters lipid metabolism, impairs insulin
signalling and suppresses insulin-dependent glucose uptake in mouse adipocytes [32]. However, the
exact role of hepatic G6PD in metabolic function is unknown.

Testosterone Effects on Expression of Targets of Lipid Metabolism

In the present study we demonstrate that testosterone deficiency negatively alters the expression of
targets of lipid metabolism primarily in liver and SAT but had little effect in VAT. Decreased Lpl in
Tfm mice with low testosterone may limit the hydrolysis of lipoproteins and the subsequent uptake of
FFA into SAT. A previous study, however, has shown the expression of hormone sensitive lipase
(HSL) and LPL to be elevated in SAT of male mice with a selective adipocyte AR knockdown
(fARKO) [33]. These mice were fed a normal chow diet and therefore LPL increase in the absence of
testosterone activated AR signalling may reflect elevated subcutaneous lipid storage and decreased
triglyceride usage as an energy source in other tissues in times of low fat intake. Treatment of
hypogonadal men with TRT for nine months resulted in a marked decrease in both LPL activity and
triglyceride uptake in abdominal adipose tissue [34]. Following further investigation, although LPL
expression or activity was not reported, the inhibition of lipid uptake after testosterone administration
was apparent in visceral (omentum plus mesenteric) and retroperitoneal but increased in abdominal
SAT suggesting that inhibition of triglyceride assimilation may direct lipid to subcutaneous fat in
TRT-treated men and may therefore involve altered lipase activity or expression in specific tissues
[35], as suggested in the present study.

Human SCD1 is a critical control point of lipid partitioning with high SCD activity favouring fat
storage and suppression of the enzyme activating metabolic pathways that promote the burning of fat
and decrease lipid synthesis [36]. Mice with a targeted disruption of the Scd1 gene have very low
levels of VLDL and impaired triglyceride and cholesterol ester biosynthesis as well as markedly
reduced adiposity and decreased hepatic steatosis on both lean and ob / ob background despite higher
food intake [37, 38]. In the present study we demonstrate significantly increased Scd1 expression in
VAT of Tfm mice and a trend towards increased expression in the liver. Beyond its role in fatty acid
biosynthesis, SCD1 is an important factor in the pathogenesis of lipid-induced insulin resistance with
SCD1 deficiency up-regulating insulin-signalling components and glycogen metabolism in insulin-
sensitive tissues [38]. This suggests that testosterone has the potential to improve both lipid and

Glucose metabolism via reducing Scd1 expression in VAT and the liver of Tfm mice.

Lower subcutaneous Apoe expression in testosterone deficient Tfm mice may be indicative of
decreased reverse cholesterol transport delivery of lipoproteins and cholesterol from SAT to the liver
for clearance. This difference was not apparent in VAT supporting an important depot-specific role of
APOE in adipose tissue substrate flux and accumulation of triglyceride in these depots [39].

Additionally, in the present study we demonstrate that mRNA expression of Srebf1 and Srebf2, key
transcription factors and master regulators of lipogenesis [40], were significantly decreased in SAT of
Tfm mice compared to testosterone treated animals and wild-type controls. Similarly, orchidectomy
significantly reduced hepatic SREBP-1 expression in mice fed a high fat diet or normal chow, an
effect that was ameliorated by testosterone treatment in high fat diet conditions [41]. As SREBP's are
known to directly induce transcription of many genes needed for uptake and synthesis of cholesterol,
fatty acids, triglycerides, and phospholipids [42], taken together, these data lead us to hypothesise that testosterone deficiency may diminish SAT metabolic function and reduce lipid storage capacity.

Increased liver fat in Tfm mice from the present study is considered partly due to increased de novo lipogenesis and the expression of FASN and ACACA [17], which supported earlier studies indicating that a lack of testosterone action results in hepatic lipid accumulation [41-43]. The present study additionally indicates that ABCA1 and APOE, involved in cholesterol and lipoprotein efflux, are reduced in the testosterone deficient state in the liver of Tfm mice. The overexpression of hepatic Abca1 in transgenic mice results in a marked increase in HDL release, decreased LDL, and significantly reduced atherosclerosis when compared with control mice [44]. Furthermore, increased hepatic cholesterol content was reported in these mice as the level of expression of the ABCA1 transporter decreased [45]. Indeed, Tfm mice from the present study have elevated total cholesterol and LDL compared to wild-type mice [18]. Therefore, the increased hepatic lipid accumulation in our Tfm mice may additionally result from absence of beneficial testosterone effects on lipid transport.

**Testosterone Effects on Master Regulators of Lipid and Glucose Metabolism**

Testosterone altered the expression of master metabolic regulators as a potential signalling mode of action to influence lipid and glucose regulation. Reduced expression of the nuclear receptor, liver X receptor (LXR), in muscle, liver and SAT of Tfm mice compared to testosterone replete animals whether with or without AR function leads to the hypothesis that testosterone may increase LXR signalling to exert some of its protective metabolic effects on. LXRs are key transcriptional regulators of lipid and carbohydrate metabolism known to control molecular pathways including cholesterol efflux, glucose regulation, fatty acid synthesis and inflammation [46]. In parallel with testosterone associated changes in LXR expression in the present study, we saw alterations in known LXR target genes; Fasn, APOe, Abca1, Lpl, Srebf1. Rather than inducing hepatic steatosis as with many LXR agonists, testosterone additionally protects against diet-induced hepatic lipid accumulation in this model [17]. Tfm mice also had reduced SAT and VAT expression of Pparg mRNA indicating a potential mechanism by which testosterone deficiency may lead to metabolic dysregulation and
adverse fat distribution. Additionally, Tf1m mice displayed lower SAT Ppara (a master regulator of fatty acid oxidation) expression suggesting that testosterone deficiency may further inhibit lipid regulation.

The present study indicates that testosterone may signal, at least in part, beyond its classical nuclear androgen receptor (AR) to modulate targets of lipid and glucose metabolism and that these actions are further differentially dependent on the target tissue. Whether the AR-independent effects in this study are via conversion to estradiol and subsequent activation of the oestrogen receptor (ER) was not addressed. We have previously shown, however, that testosterone has additional actions on hepatic and aortic lipid accumulation in Tf1m mice even with aromatase inhibition and ER blockade [16, 17]. Further investigation is required to elucidate the AR-independent signalling mechanisms of testosterone action.

Limitations

The present exploratory study is limited to target expression data and while it indicates potential metabolic effects of testosterone, it does not directly assess metabolic function. Lack of tissue prevented protein analysis of SAT and VAT due to the reduced amounts of protein recoverable from available adipose tissue. In addition, the Tf1m mouse is a model of global AR dysfunction and severely reduced testosterone levels from birth, therefore we cannot rule out any developmental effects of these factors on tissues which may influence the pathogenesis of metabolic disorders. Whilst the testosterone injections produce levels within the normal range, diurnal patterns are absent and supraphysiologic levels in the first few days are apparent with near-infraphysiologic levels towards the end of the interval [16]. Such administration may explain the influence of testosterone treatment on gene expression above and beyond that observed in wild-type controls. An additional orchidectomised XY littermate group receiving testosterone treatment would also allow us to control for pharmacological and dosing effects in animals with fully functional AR. These issues should be addressed in future studies.
Conclusion

We present exploratory evidence that suggests testosterone is a metabolic hormone that differentially regulates the expression of key targets of lipid and glucose metabolism in a tissue-specific manner to potentially reduce fat deposition in pathologically relevant locations such as liver and the arterial tree.

Indeed, as regional differences in the action of testosterone on subcutaneous and visceral adipose function are apparent, we hypothesise that low testosterone in the Tfm mouse leads to decreased lipid uptake and glucose utilisation in SAT resulting in its reduced capacity to act as a physiological ‘buffer’ in times of positive energy balance. This decreased ability to store excess lipid may then result in spillover into other tissues. Tfm mice have increased lipid accumulation in the aortic root and liver as early manifestations of atherosclerosis and hepatic steatosis. These effects are significantly reduced by testosterone replacement [17]. While this study adds support to the literature implicating testosterone as a metabolic hormone, by combining expressional data from multiple metabolic tissues with pathological evidence that testosterone protects against the development of hepatic steatosis and atherosclerosis, we now suggest a system-wide androgenic action to offer new mechanistic insight to the observed clinical benefit of testosterone in men with T2D and MetS.

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Figure Legends:

**Figure 1; Animal weights and weight gain.** Tfm mice receiving either placebo (Tfm S) or testosterone (Tfm T) and wild-type XY littermates receiving placebo (XY S) had total body weight (a) measured at weekly intervals from the commencement of high-cholesterol diet feeding at week 8 through to the end of the study at week 36. Weight gain (b) was calculated from starting weights of individual animals. No significant differences were noted between groups.

**Table 3; Gene expression of targets of lipid and glucose regulation in muscle, liver, subcutaneous and visceral adipose tissue of Tfm mice.** Relative tissue-specific qPCR end-point analysis of selected genes of (a) fat metabolism, (b) cholesterol homeostasis, (c) carbohydrate metabolism and (d) master regulators between Tfm placebo-treated versus XY littermates placebo-treated, and Tfm placebo-treated versus Tfm testosterone-treated. N=11. *p<0.05, **p<0.01, ***p<0.001 versus XY placebo, *p<0.05, **p<0.01 versus Tfm placebo, a p=0.053, b p=0.058, c p=0.056.

**Figure 2; Protein expression of selected targets of lipid and glucose regulation in muscle and liver of Tfm mice.** Semi quantitative western blot analysis in (a) muscle and (b) liver of Tfm mice receiving either placebo (Tfm S) or testosterone (Tfm T) and wild-type XY littermates receiving placebo (XY S) at the end of the study period. Data are presented as densitometry arbitrary units and representative blot images. N=6. *p<0.05 versus XY placebo, *p<0.05 versus Tfm placebo.
| Target                                      | Gene   | Function                                                                 | Product Ref. |
|---------------------------------------------|--------|--------------------------------------------------------------------------|--------------|
| Fatty acid synthase                         | Fasn   | Catalyses the formation of long-chain fatty acids in fatty acid synthesis | QT00149240   |
| Acetyl CoA carboxylase                      | Acaca  | Essential role in regulating fatty acid synthesis                       | QT01554441   |
| Steroyl-CoA desaturase 1                    | Scd1   | Catalyses a rate-limiting step in the synthesis of unsaturated fatty acids. Key enzyme in fatty acid metabolism. | QT00291151   |
| Lipoprotein lipase                          | Lpl    | Hydrolysis of triglycerides into free fatty acids                        | QT01750469   |
| Homone sensitive lipase                     | Lipe   | Hydrolyses stored triglycerides to free fatty acids                      | QT00169057   |
| 3-hydroxy-3-methylglutaryl-CoA reductase     | Hmgcr  | Rate-controlling enzyme of the mevalonate pathway that produces cholesterol | QT01037848   |
| Sterol regulatory element-binding protein 1 | Srebf1  | Cholesterol biosynthesis and uptake, and fatty acid biosynthesis          | QT00167055   |
| Sterol regulatory element-binding protein 2 | Srebf2  | Cholesterol biosynthesis and uptake, and fatty acid biosynthesis          | QT01045870   |
| Apolipoprotein E                            | APOE   | Lipoprotein metabolism and transport.                                    | QT01043889   |
| ATP-binding cassette transporter A1         | Abca1  | Major regulator of cellular cholesterol efflux and phospholipid homeostasis | QT00165690   |
| ATP-binding cassette transporter G5         | Abcg5  | Cellular cholesterol efflux, promote biliary excretion of sterols.        | QT00157752   |
| Insulin receptor substrate 1                | Ins1   | Transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to intracellular pathways in insulin signalling | QT00251657   |
| Hexokinase 2                                | Hk2    | Phosphorylates glucose to glucose 6-phosphate in the glycolytic pathway  | QT00155582   |
| Hexokinase 4 (Glucokinase)                  | Gck    | Phosphorylates glucose to glucose 6-phosphate in the glycolytic pathway  | QT00140007   |
| Phosphofructokinase                         | PfK    | Converts fructose-6-phosphate to fructose-1,6-bisphosphate, one of the most important regulatory enzymes of glycolysis | QT00159754   |
| Carbohydrate-responsive element-binding protein | Chrebp  | Activates several regulatory enzymes of glycolysis and lipogenesis       | QT00125335   |
| Glucose transporter 4                       | Glu14  | Cellular glucose transport                                               | QT01044946   |
| Glucose-6-phosphate dehydrogenase           | G6pdx  | Enzyme in the pentose phosphate pathway, often for tissues actively engaged in biosynthesis of fatty acids | QT01748957   |
| Glycogen synthase                           | Gys    | Converts glucose to glycogen for storage, regulating glycogen/glucose levels | QT00162099   |
| Liver X receptor alpha                      | Nr1h3  | Nuclear receptor transcription factor regulating cholesterol, fatty acid, and glucose homeostasis | QT00113729   |
| Peroxisome proliferator-activated receptor alpha | Ppara  | Transcription factor and major regulator of lipid metabolism             | QT00137984   |
| Peroxisome proliferator-activated receptor gamma | Pparg  | Regulates fatty acid storage and glucose metabolism                      | QT00100296   |
| Beta 2 microglobulin                        | B2m    | Reference gene                                                           | QT01149547   |
| Glyceraldehyde 3-phosphate dehydrogenase    | Gapdh  | Reference gene                                                           | QT01658692   |
Table 2; Antibody parameters

| Antibody | Concentration | Diluent                  | Supplier       |
|----------|---------------|--------------------------|----------------|
| FASN     | 1:500         | 2.5% milk bsa in tbs     | CST            |
| ACACA    | 1:500         | 2.5% milk bsa in tbs     | CST            |
| ABCA1    | 1:250         | 2.5% milk bsa in tbs     | abcam          |
| APOE     | 1:250         | 5% milk in tbs           | abcam          |
| GCK      | 1:500         | 5% milk in tbs           | abcam          |
| PFK      | 1:250         | 0.01% milk bsa in tbs    | Proteintech    |
| GLUT4    | 1:500         | 2.5% milk bsa in tbs     | CST            |
| HK2      | 1:500         | 2.5% milk bsa in tbs     | CST            |
| LXR      | 1:500         | 1% milk bsa in tbs       | abcam          |
| G6PD     | 1:500         | 0.01% milk bsa in tbs    | Sigma          |
| GAPDH    | 1:5000        | 2.5% milk bsa in tbs     | abcam          |
| Calnexin | 1:1000        | 5% milk in tbs           | CST            |

bsa, bovine serum albumin; tbs, Tris-buffered saline; CST, Cell Signalling Technologies
### Carbohydrate Metabolism

| Gene                        | Muscle                  | Liver                  | Subcutaneous Fat            | Visceral Fat               |
|-----------------------------|-------------------------|------------------------|-----------------------------|----------------------------|
| HSL1 (HSL1 receptor substrate 1) | XY-P: 1.34±0.32, Tm-P: 1.37±0.59, Tm-P: 3.5±0.54   | XY-P: 1.74±0.54, Tm-P: 1.13±0.29 | Tm-S100: 1.32±0.16, Tm-P: 0.24±0.05** | Tm-S100: 0.61±0.13   |
| HSL2 (HSL2 kinase 2)        | XY-P: 1.18±0.19, Tm-P: 0.5±0.16, Tm-P: 0.54±0.10 | XY-P: 1.19±0.11, Tm-P: 0.79±0.15** | Tm-S100: 1.76±0.68, Tm-P: 0.16±0.05** | Tm-S100: 0.5±0.31   |
| HSL4 (HSL4 glucokinase)     | XY-P: 1.22±0.12, Tm-P: 0.65±0.19, Tm-P: 1.09±0.21   | XY-P: 1.01±0.14, Tm-P: 1.62±0.21 | Tm-S100: 1.00±0.21, Tm-P: 1.57±0.54 | Tm-S100: 0.16±0.21   |
| Phosphofructokinase         | XY-P: 1.25±0.24, Tm-P: 2.16±0.02, Tm-P: 1.16±0.22   | XY-P: 1.05±0.12, Tm-P: 1.17±0.13 | Tm-S100: 1.76±0.47, Tm-P: 1.53±0.38 | Tm-S100: 1.96±0.37   |
| Glucose transporter        | XY-P: 1.13±0.19, Tm-P: 0.59±0.14, Tm-P: 0.71±0.13   | XY-P: 1.31±0.32, Tm-P: 0.37±0.12** | Tm-S100: 1.03±0.16, Tm-P: 1.24±0.26 | Tm-S100: 0.83±0.16   |
| Glucose-6-phosphate 1-dehydrogenase | XY-P: 1.13±0.19, Tm-P: 0.59±0.14, Tm-P: 0.71±0.13   | XY-P: 1.31±0.32, Tm-P: 0.37±0.12** | Tm-S100: 1.03±0.16, Tm-P: 1.24±0.26 | Tm-S100: 0.83±0.16   |
| Glycogen synthase           | XY-P: 1.14±0.15, Tm-P: 0.10±0.31, Tm-P: 0.18±0.22   | XY-P: 1.33±0.38, Tm-P: 0.54±0.31 | Tm-S100: 1.76±0.37, Tm-P: 1.96±0.37 | Tm-S100: 1.76±0.37   |

### Cholesterol Homeostasis

| Gene                        | Muscle                  | Liver                  | Subcutaneous Fat            | Visceral Fat               |
|-----------------------------|-------------------------|------------------------|-----------------------------|----------------------------|
| 3-hydroxy-3-methylglutaryl-CoA reductase | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| Sterol Regulatory Element-Binding Protein | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| Apolipoprotein E             | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| ATP-binding cassette transporter A1 | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| ATP-binding cassette transporter G5 | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |

### Fat Metabolism

| Gene                        | Muscle                  | Liver                  | Subcutaneous Fat            | Visceral Fat               |
|-----------------------------|-------------------------|------------------------|-----------------------------|----------------------------|
| Acyl-CoA carboxylase alpha | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| Fatty acid synthase         | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| Steroyl-CoA desaturase-1    | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| Lipoprotein lipase          | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |
| Hormone sensitive lipase    | XY-P: 1.20±0.26, Tm-P: 0.74±0.21, Tm-P: 1.47±0.39   | XY-P: 1.13±0.16, Tm-P: 1.01±0.19 | Tm-S100: 1.32±0.29, Tm-P: 0.36±0.09*** | Tm-S100: 0.86±0.16   |

### Master Regulators

| Gene                        | Muscle                  | Liver                  | Subcutaneous Fat            | Visceral Fat               |
|-----------------------------|-------------------------|------------------------|-----------------------------|----------------------------|
| Liver X receptor            | XY-P: 1.35±0.29, Tm-P: 0.12±0.10, Tm-P: 1.05±0.09 | Tm-S100: 0.66±0.08** | Tm-S100: 0.12±0.39, Tm-P: 0.24±0.42 | Tm-S100: 0.05±0.08*** |
| Peroxisome proliferator-activated receptor alpha | XY-P: 1.35±0.29, Tm-P: 0.12±0.10, Tm-P: 1.05±0.09 | Tm-S100: 0.66±0.08** | Tm-S100: 0.12±0.39, Tm-P: 0.24±0.42 | Tm-S100: 0.05±0.08*** |
| Peroxisome proliferator-activated receptor gamma | XY-P: 1.35±0.29, Tm-P: 0.12±0.10, Tm-P: 1.05±0.09 | Tm-S100: 0.66±0.08** | Tm-S100: 0.12±0.39, Tm-P: 0.24±0.42 | Tm-S100: 0.05±0.08*** |