

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:	<p>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.</p> <p>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. Conclusions: These exploratory data</p>	

	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 overflow and uptake of excess glucose and triglycerides into VAT, liver and arterial walls.
Response to Reviewers:	see attachment

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

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10
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27 **Abstract**

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3 28 **Purpose:** Testosterone deficiency is commonly associated with obesity, metabolic syndrome, type 2
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5 29 diabetes and their clinical consequences - hepatic steatosis and atherosclerosis. The testicular
6
7 30 feminised (tfm) mouse (non-functional androgen receptor and low testosterone) develops fatty liver
8
9 31 and aortic lipid streaks on a high-fat diet whereas androgen replete XY littermate controls do not.
10
11 32 Testosterone replacement ameliorates these effects, although the underlying mechanisms remain
12
13 33 unknown. **Methods:** We compared the influence of testosterone on the expression of regulatory
14
15 34 targets of glucose, cholesterol and lipid metabolism in muscle, liver, abdominal subcutaneous (SAT)
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17 35 and visceral adipose tissue (VAT). **Results:** Tfm mice displayed significantly reduced GLUT4 in
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19 36 muscle and glycolytic enzymes in muscle, liver and SAT but not VAT. Lipoprotein lipase required for
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21 37 fatty acid uptake was only reduced in SAT, enzymes of fatty acid synthesis were increased. Stearoyl-
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27 40 regulator nuclear receptors involved in metabolism: - Liver X receptor expression was suppressed in
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29 41 all tissues except VAT whereas PPAR γ was lower in SAT and VAT and PPAR α only in SAT.
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31 42 Testosterone replacement improved the expression (androgen receptor independent) of some targets
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33 43 but not all. **Conclusion:** These exploratory data suggest that androgen deficiency may reduce the
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35 44 buffering capability for glucose uptake and utilisation in SAT and muscle and fatty acids in SAT. This
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37 45 would lead to an overspill and uptake of excess glucose and triglycerides into VAT, liver and arterial
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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].

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74 While a growing body of evidence points towards the presence of heterogeneity regarding insulin
75 responsiveness and lipid homeostasis among different tissues [19], the mechanisms by which
76 testosterone may impart beneficial actions on insulin sensitivity and hence the development of MetS,
77 T2D and cardiovascular risk remain unknown but are likely to be tissue dependent and involve
78 multiple targets of lipid and carbohydrate metabolism. In the present exploratory study, we aim to
79 investigate whether the metabolic protective effects of testosterone act via modulation of the
80 expression of key targets involved in lipid and glucose metabolism in muscle, liver and adipose tissue
81 of cholesterol-fed Tfm mice. Specifically, we investigate key regulatory enzymes of glycolysis,
82 glycogen synthesis, pentose phosphate pathway, glucose transporters, fatty acid synthesis, fatty acid
83 uptake, cholesterol synthesis and efflux, and master regulators of metabolism (see table 1).

84 **Materials and methods**

85 *Animals*

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

98 ***Experimental design and tissue collection***

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3 99 8-week-old Tfm and XY littermate mice were fed a high-fat, high-cholesterol diet, containing 42%
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5 100 butterfat, 1.25% cholesterol and 0.5% cholate (Special Diet Services, Essex, UK) *ad libitum* for a
6
7 101 period of 28 weeks. Separate 7-week old Tfm mice were randomly assigned to one of two groups;
8
9 102 placebo group receiving a once-fortnightly intramuscular injection of 10 μ L of saline (n=14), or
10
11 103 testosterone group (n=14) receiving a once-fortnightly intramuscular injection of 10 μ L of 100mg/mL
12
13 104 testosterone esters (Sustanon100; testosterone propionate 20mg/mL, testosterone phenylpropionate
14
15 105 40mg/mL and testosterone isocaproate 40mg/mL, Organon Laboratories Ltd, Cambridge, UK),
16
17 106 providing a dose of 50mg/kg, previously shown to replace circulating levels to those of wild-type
18
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20 107 littermate mice [16]. XY littermate mice (n=14) received placebo injections (10 μ L saline). Animals
21
22 108 were caged under standard conditions in a temperature and humidity controlled room on a 12h
23
24
25 109 light:12h darkness cycle. Water and food were unrestricted throughout the study.

26
27
28 110 At the end of the experimental period which corresponded with the midway point of the fortnightly
29
30 111 injection cycle, whole blood was collected from the thoracic cavity following mid-line sternotomy
31
32 112 and severance of the thoracic aorta. Following centrifugation, serum samples were frozen at -80°C.
33
34
35 113 The liver was removed from the abdomen, skeletal muscle dissected from the quadriceps of the hind
36
37 114 legs and fat tissue collected from subcutaneous and visceral abdominal regions. The heart with
38
39 115 thoracic aorta attached was carefully dissected free from the adventitia and perfused. Tissues were
40
41 116 processed for both histological and gene and protein expression analysis and were archived for future
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44 117 analysis. Analyses were made on individual samples.

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47 118 ***Measurement of Total Testosterone and 17 β -Estradiol***

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50 119 Serum quantification of total testosterone (DRG Instruments GmbH, Marburg, Germany) and 17 β -
51
52 120 estradiol (Demeditec Diagnostics, Kiel, Germany) were measured in duplicate via ELISA
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54 121 (measurement range 0.2-16ng/mL and 3-200pg/mL respectively).

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57 122 ***Quantitative analysis of mRNA***

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

133 *Western Immunoblotting*

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

148 **Statistical Analysis**

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3 149 Results are presented as mean \pm SEM. Differences between groups with normally distributed data
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5 150 were compared using unpaired t tests without assuming consistent standard deviations of groups.
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7 151 Mann–Whitney U tests were used where data did not follow a normal distribution. Corrections for
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9 152 multiple comparisons were made using the Sidak-Bonferroni post hoc test. Significance was accepted
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11 153 at $P \leq 0.05$.

14
15 154 **Results**

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18 155 Serum testosterone levels were greatly reduced in Tfm mice (2.2 ± 1.2 nM, $p=0.03$) compared to wild-
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20 156 type equivalents (16.5 ± 4.3 nM)¹. Testosterone treatment of Tfm mice increased serum levels of
21
22 157 testosterone comparable to wild-type levels (14.7 ± 5.2 nM, $p=0.98$). 17- β estradiol levels were similar
23
24 158 between all groups, Tfm mice (94.2 ± 15.5 pMol) compared to wild-type (106.0 ± 33.9 pMol, $p=0.17$)
25
26 159 and testosterone-treated Tfm mice (135.2 ± 28.7 pMol, $p=0.99$). Animal weights and weight gain did
27
28 160 not significantly differ between groups over the duration of the 28 week feeding period but there was
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30 161 a trend towards Tfm mice gaining more weight compared to littermates by the end of the study period
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32 162 ($p=0.066$, $n=14$; Figure 1).
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37 163 **Carbohydrate Metabolism**

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40 164 Gene expression of the glycolytic regulatory gateway enzymes hexokinase (*Hk2*, *Gck*) and *Pfk* was
41
42 165 significantly lower in muscle ($p=0.012$, $p=0.032$), liver ($p=0.002$, $p=0.04$) and SAT ($p=0.009$, $p=0.03$)
43
44 166 but not in VAT of Tfm-placebo mice compared to XY littermates (Table 3). Testosterone
45
46 167 administration increased *Gck* expression ($p=0.015$) in the liver of Tfm mice but these enzymes were
47
48 168 not significantly altered in other tissues by treatment. *Glut4* was similarly decreased in muscle
49
50 169 ($p=0.015$) and SAT ($p=0.014$) of Tfm mice versus wild-type mice, with no effect of testosterone
51
52 170 treatment. Hepatic *G6pdx* was elevated in Tfm mice compared to XY mice ($p<0.001$) and testosterone
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59 ¹ As previously published [16, 18, 20]
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171 treatment showed a trend to reducing this expression in Tfm mice ($p=0.056$). All other gene targets
172 were not altered between experimental groups in the tissues investigated.

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

183

184 *Lipid Metabolism*

185 a) Cholesterol metabolism

186 Expression of cholesterol transporters, *ApoE* and *AbcA1*, were reduced in the liver of Tfm mice
187 compared to littermates ($p=0.009$, $p=0.002$). Treatment with testosterone significantly increased this
188 expression ($p=0.027$, $p=0.02$), similar to wild-type levels (Table 3). Similarly, *ApoE* was decreased in
189 SAT of Tfm mice ($p=0.01$), an effect that was abolished by testosterone administration ($p=0.015$ vs
190 Tfm P). *Srebf1* and *Srebf2* expression was significantly lower in the SAT of Tfm mice versus XY
191 littermates ($p=0.002$, $p=0.003$). Treatment with testosterone elevated these expression levels of *Srebf1*
192 ($p=0.015$) similar to those demonstrated in wild-type mice although not significantly so for *Srebf2*
193 with only a trend towards increased expression observed ($p=0.053$). In contrast to gene expression
194 data, ABCA1 protein was not significantly altered between treatment groups although mean
195 expression appeared to demonstrate a similar pattern (Figure 2). APOE protein expression matched

196 gene expression data with significantly lower levels in placebo-treated Tfm mice compared to XY
1 littermates and testosterone-treated Tfm mice (p=0.011, p=0.007 respectively).

198 b) Fatty acid metabolism

199 Visceral adipose *Scd1* expression was significantly higher in Tfm mice receiving placebo than in XY
200 littermates also receiving placebo injections (p=0.034, Table 3). Testosterone treatment of Tfm mice
201 returned expression levels to those of XY mice with a significant reduction compared to placebo-
202 treated Tfm mice (p=0.027). T test analysis similarly revealed an increase in hepatic *Scd1* expression
203 in Tfm placebo mice although not statistically significant (p=0.08). Decreased *Lpl* expression was
204 observed in SAT from Tfm mice compared to wildtype (p=0.016) although testosterone
205 administration to Tfm animals had no effect on this. Hepatic gene expression of *Fasn* and *Acaca*, the
206 key regulatory enzymes in de novo lipogenesis, were significantly increased in Tfm mice receiving
207 placebo injections compared to wild-type littermates (p=0.049, p=0.042 respectively²). Testosterone
208 treatment decreased this expression but not significantly. Gene expression of all other lipid
209 metabolism targets in liver and adipose tissue were not significantly different between animal groups.
210 Western blotting showed hepatic protein expression of FASN and ACACA to be increased in Tfm
211 mice confirming gene expression findings². Testosterone treatment significantly reduced the protein
212 expression of these enzymes versus placebo treated Tfm mice to similar levels as XY littermates.
213 No targets of fat metabolism and cholesterol homeostasis displayed altered gene expression in muscle
214 tissue from the different experimental groups.

215

216 *Master Regulators*

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

² As previously published [15]

220 p=0.024, SAT p=0.03). *Ppara* and *Pparg* were significantly reduced in SAT of Tfm mice receiving
1
2 221 placebo versus XY littermate controls (p=0.01, p=0.02 respectively). *Pparg* was also reduced in
3
4 222 visceral adipose tissue of Tfm mice (p=0.001). Testosterone treatment had no effect on the altered
5
6 223 expression of *Ppars* when compared to placebo treated Tfm mice (see Table 3).
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10 224 LXR protein expression in liver and muscle demonstrated the same pattern indicated by gene
11
12 225 expression analysis with a reduction in Tfm placebo mice compared to wild-type littermates (p=0.001,
13
14 226 p=0.01). Treatment with testosterone elevated LXR levels significantly in liver and muscle (p=0.024,
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16 227 p=0.022), to similar levels seen in placebo-treated Tfm mice (Figure 2).
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19 228 **Discussion**

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23 229 Exploratory evidence from this study suggests that testosterone has tissue-specific metabolic effects in
24
25 230 the regulation of gene targets which control glucose utilisation in liver, SAT and skeletal muscle, and
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27 231 lipid metabolism in liver and SAT. Some of these effects are, at least in part, androgen receptor-
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29 232 independent and may potentially explain some of the observed clinical benefit of testosterone in men
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31 233 with T2D and MetS.
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34 35 234 *Testosterone Effects on Expression of Targets of Glucose Metabolism*

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41 236 GLUT4 expression is known to correlate positively with insulin responsiveness and defects in
42
43 237 expression of GLUT4 have been observed in patients with T2D [24]. We have shown that there is
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45 238 decreased expression of GLUT4 in muscle and SAT in the testosterone deficient Tfm mouse.
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47
48 239 Testosterone has previously been shown to increase the expression of GLUT4 in cultured skeletal
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50 240 muscle cells, hepatocytes and adipocytes [25-27] as well as augmenting membrane translocation and
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52 241 promoting glucose uptake in adipose and skeletal muscle tissue [27]. Key enzymes involved in
53
54 242 glycolysis, PFK and HK, were significantly reduced in muscle, liver and SAT of Tfm mice. This
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56 243 supports previous studies which have demonstrated an increase in the activity of PFK and HK in
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59 244 cultured rat skeletal muscle cells and increased hexokinase activity in muscle tissue of castrated rats
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1 245 following testosterone treatment thus diminishing the raised blood levels of glucose seen in untreated
2 246 control rats [27-29]. Improved glucose utilisation in muscle, liver and SAT by testosterone may
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4 247 reduce the conversion of glucose to fat in times of excess and improve insulin sensitivity thus
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6 248 reducing lipid accumulation in these and other tissues. This clinically would be very important in
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8 249 muscle as this tissue accounts for approximately 75% of whole-body insulin-stimulated glucose
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11 250 uptake [30, 31].
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14 251 We have also demonstrated in this study that the mRNA expression of Glucose-6-phosphate
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16 252 dehydrogenase (*G6pd*), the gateway enzyme in the pentose phosphate shunt pathway, is elevated in
17
18 253 the liver of Tfm mice suggesting that glucose may also be utilised down this route during testosterone
19
20 254 deficiency. NADPH is produced by G6PD in the pentose phosphate pathway supplying reducing
21
22 255 power to contribute to fatty acid synthesis [32]. An aberrant increase of G6PD expression is present in
23
24 256 obese and diabetic subjects, and overexpression of G6PD alters lipid metabolism, impairs insulin
25
26 257 signalling and suppresses insulin-dependent glucose uptake in mouse adipocytes [32]. However, the
27
28 258 exact role of hepatic G6PD in metabolic function is unknown.
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34 35 36 260 *Testosterone Effects on Expression of Targets of Lipid Metabolism* 37 38

39 261 In the present study we demonstrate that testosterone deficiency negatively alters the expression of
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41 262 targets of lipid metabolism primarily in liver and SAT but had little effect in VAT. Decreased *Lpl* in
42
43 263 Tfm mice with low testosterone may limit the hydrolysis of lipoproteins and the subsequent uptake of
44
45 264 FFA into SAT. A previous study, however, has shown the expression of hormone sensitive lipase
46
47 265 (HSL) and LPL to be elevated in SAT of male mice with a selective adipocyte AR knockdown
48
49 266 (fARKO) [33]. These mice were fed a normal chow diet and therefore LPL increase in the absence of
50
51 267 testosterone activated AR signalling may reflect elevated subcutaneous lipid storage and decreased
52
53 268 triglyceride usage as an energy source in other tissues in times of low fat intake. Treatment of
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55 269 hypogonadal men with TRT for nine months resulted in a marked decrease in both LPL activity and
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57 270 triglyceride uptake in abdominal adipose tissue [34]. Following further investigation, although LPL
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1 271 expression or activity was not reported, the inhibition of lipid uptake after testosterone administration
2 272 was apparent in visceral (omental plus mesenteric) and retroperitoneal but increased in abdominal
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4 273 SAT suggesting that inhibition of triglyceride assimilation may direct lipid to subcutaneous fat in
5
6 274 TRT-treated men and may therefore involve altered lipase activity or expression in specific tissues
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8 275 [35], as suggested in the present study.

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12 276 Human SCD1 is a critical control point of lipid partitioning with high SCD activity favouring fat
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14 277 storage and suppression of the enzyme activating metabolic pathways that promote the burning of fat
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16 278 and decrease lipid synthesis [36]. Mice with a targeted disruption of the *Scd1* gene have very low
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18 279 levels of VLDL and impaired triglyceride and cholesterol ester biosynthesis as well as markedly
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20 280 reduced adiposity and decreased hepatic steatosis on both lean and ob / ob background despite higher
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22 281 food intake [37, 38]. In the present study we demonstrate significantly increased *Scd1* expression in
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24 282 VAT of Tfm mice and a trend towards increased expression in the liver. Beyond its role in fatty acid
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26 283 biosynthesis, SCD1 is an important factor in the pathogenesis of lipid-induced insulin resistance with
27
28 284 SCD1 deficiency up-regulating insulin-signalling components and glycogen metabolism in insulin-
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30 285 sensitive tissues [38]. This suggests that testosterone has the potential to improve both lipid and
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32 286 glucose metabolism via reducing *Scd1* expression in VAT and the liver of Tfm mice.
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37 287 Lower subcutaneous *ApoE* expression in testosterone deficient Tfm mice may be indicative of
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39 288 decreased reverse cholesterol transport delivery of lipoproteins and cholesterol from SAT to the liver
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41 289 for clearance. This difference was not apparent in VAT supporting an important depot-specific role of
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43 290 APOE in adipose tissue substrate flux and accumulation of triglyceride in these depots [39].
44
45 291 Additionally, in the present study we demonstrate that mRNA expression of *Srebf1* and *Srebf2*, key
46
47 292 transcription factors and master regulators of lipogenesis [40], were significantly decreased in SAT of
48
49 293 Tfm mice compared to testosterone treated animals and wild-type controls. Similarly, orchidectomy
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51 294 significantly reduced hepatic SREBP-1 expression in mice fed a high fat diet or normal chow, an
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53 295 effect that was ameliorated by testosterone treatment in high fat diet conditions [41]. As SREBPs are
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55 296 known to directly induce transcription of many genes needed for uptake and synthesis of cholesterol,
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297 fatty acids, triglycerides, and phospholipids [42], taken together, these data lead us to hypothesise that
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2 298 testosterone deficiency may diminish SAT metabolic function and reduce lipid storage capacity.
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5 299 Increased liver fat in Tfm mice from the present study is considered partly due to increased de novo
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7 300 lipogenesis and the expression of FASN and ACACA [17], which supported earlier studies indicating
8
9 301 that a lack of testosterone action results in hepatic lipid accumulation [41-43]. The present study
10
11 302 additionally indicates that ABCA1 and APOE, involved in cholesterol and lipoprotein efflux, are
12
13 303 reduced in the testosterone deficient state in the liver of Tfm mice. The overexpression of hepatic
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15 304 *Abca1* in transgenic mice results in a marked increase in HDL release, decreased LDL, and
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17 305 significantly reduced atherosclerosis when compared with control mice [44]. Furthermore, increased
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19 306 hepatic cholesterol content was reported in these mice as the level of expression of the ABCA1
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21 307 transporter decreased [45]. Indeed, Tfm mice from the present study have elevated total cholesterol
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23 308 and LDL compared to wild-type mice [18]. Therefore, the increased hepatic lipid accumulation in our
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25 309 Tfm mice may additionally result from absence of beneficial testosterone effects on lipid transport.
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30 *Testosterone Effects on Master Regulators of Lipid and Glucose Metabolism*

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34 311 Testosterone altered the expression of master metabolic regulators as a potential signalling mode of
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36 312 action to influence lipid and glucose regulation. Reduced expression of the nuclear receptor, liver X
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38 313 receptor (LXR), in muscle, liver and SAT of Tfm mice compared to testosterone replete animals
39
40 314 whether with or without AR function leads to the hypothesis that testosterone may increase LXR
41
42 315 signalling to exert some of its protective metabolic effects on. LXRs are key transcriptional regulators
43
44 316 of lipid and carbohydrate metabolism known to control molecular pathways including cholesterol
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46 317 efflux, glucose regulation, fatty acid synthesis and inflammation [46]. In parallel with testosterone
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48 318 associated changes in LXR expression in the present study, we saw alterations in known LXR target
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50 319 genes; *Fasn*, *ApoE*, *Abca1*, *Lpl*, *Srebp1*. Rather than inducing hepatic steatosis as with many LXR
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52 320 agonists, testosterone additionally protects against diet-induced hepatic lipid accumulation in this
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54 321 model [17]. Tfm mice also had reduced SAT and VAT expression of *Pparg* mRNA indicating a
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56 322 potential mechanism by which testosterone deficiency may lead to metabolic dysregulation and
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2 323 adverse fat distribution. Additionally, Tfm mice displayed lower SAT *Ppara* (a master regulator of
3 324 fatty acid oxidation) expression suggesting that testosterone deficiency may further inhibit lipid
4 325 regulation.
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7 326 The present study indicates that testosterone may signal, at least in part, beyond its classical nuclear
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9 327 androgen receptor (AR) to modulate targets of lipid and glucose metabolism and that these actions are
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11 328 further differentially dependent on the target tissue. Whether the AR-independent effects in this study
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13 329 are via conversion to estradiol and subsequent activation of the oestrogen receptor (ER) was not
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15 330 addressed. We have previously shown, however, that testosterone has additional actions on hepatic
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17 331 and aortic lipid accumulation in Tfm mice even with aromatase inhibition and ER blockade [16, 17].
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19 332 Further investigation is required to elucidate the AR-independent signalling mechanisms of
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21 333 testosterone action.
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27 28 29 335 *Limitations*

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32 336 The present exploratory study is limited to target expression data and while it indicates potential
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34 337 metabolic effects of testosterone, it does not directly assess metabolic function. Lack of tissue
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36 338 prevented protein analysis of SAT and VAT due to the reduced amounts of protein recoverable from
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38 339 available adipose tissue. In addition, the Tfm mouse is a model of global AR dysfunction and severely
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40 340 reduced testosterone levels from birth, therefore we cannot rule out any developmental effects of these
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42 341 factors on tissues which may influence the pathogenesis of metabolic disorders. Whilst the
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44 342 testosterone injections produce levels within the normal range, diurnal patterns are absent and
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46 343 supraphysiologic levels in the first few days are apparent with near-infraphysiologic levels towards
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48 344 the end of the interval [16]. Such administration may explain the influence of testosterone treatment
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50 345 on gene expression above and beyond that observed in wild-type controls. An additional
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52 346 orchidectomised XY littermate group receiving testosterone treatment would also allow us to control
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54 347 for pharmacological and dosing effects in animals with fully functional AR. These issues should be
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56 348 addressed in future studies.
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65350 *Conclusion*

351 We present exploratory evidence that suggests testosterone is a metabolic hormone that differentially
352 regulates the expression of key targets of lipid and glucose metabolism in a tissue-specific manner to
353 potentially reduce fat deposition in pathologically relevant locations such as liver and the arterial tree.
354 Indeed, as regional differences in the action of testosterone on subcutaneous and visceral adipose
355 function are apparent, we hypothesise that low testosterone in the Tfm mouse leads to decreased lipid
356 uptake and glucose utilisation in SAT resulting in its reduced capacity to act as a physiological
357 ‘buffer’ in times of positive energy balance. This decreased ability to store excess lipid may then
358 result in spillover into other tissues. Tfm mice have increased lipid accumulation in the aortic root and
359 liver as early manifestations of atherosclerosis and hepatic steatosis. These effects are significantly
360 reduced by testosterone replacement [17]. While this study adds support to the literature implicating
361 testosterone as a metabolic hormone, by combining expressional data from multiple metabolic tissues
362 with pathological evidence that testosterone protects against the development of hepatic steatosis and
363 atherosclerosis, we now suggest a system-wide androgenic action to offer new mechanistic insight to
364 the observed clinical benefit of testosterone in men with T2D and MetS.

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503 **Figure Legends:**

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3 504 **Figure 1; Animal weights and weight gain.** Tfm mice receiving either placebo (Tfm S) or
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5 505 testosterone (Tfm T) and wild-type XY littermates receiving placebo (XY S) had total body weight (a)
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7 506 measured at weekly intervals from the commencement of high-cholesterol diet feeding at week 8
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10 507 through to the end of the study at week 36. Weight gain (b) was calculated from starting weights of
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12 508 individual animals. No significant differences were noted between groups.

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15 509 **Table 3; Gene expression of targets of lipid and glucose regulation in muscle, liver, subcutaneous**
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17 510 **and visceral adipose tissue of Tfm mice.** Relative tissue-specific qPCR end-point analysis of selected
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19 511 genes of (a) fat metabolism, (b) cholesterol homeostasis, (c) carbohydrate metabolism and (d) master
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21 512 regulators between Tfm placebo-treated versus XY littermates placebo-treated, and Tfm placebo-
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23 513 treated versus Tfm testosterone-treated. N=11. *p<0.05, **p<0.01, ***p<0.001 versus XY placebo,
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25 514 †p<0.05, ††p<0.01 versus Tfm placebo, ^ap=0.053, ^bp=0.058, ^cp=0.056.

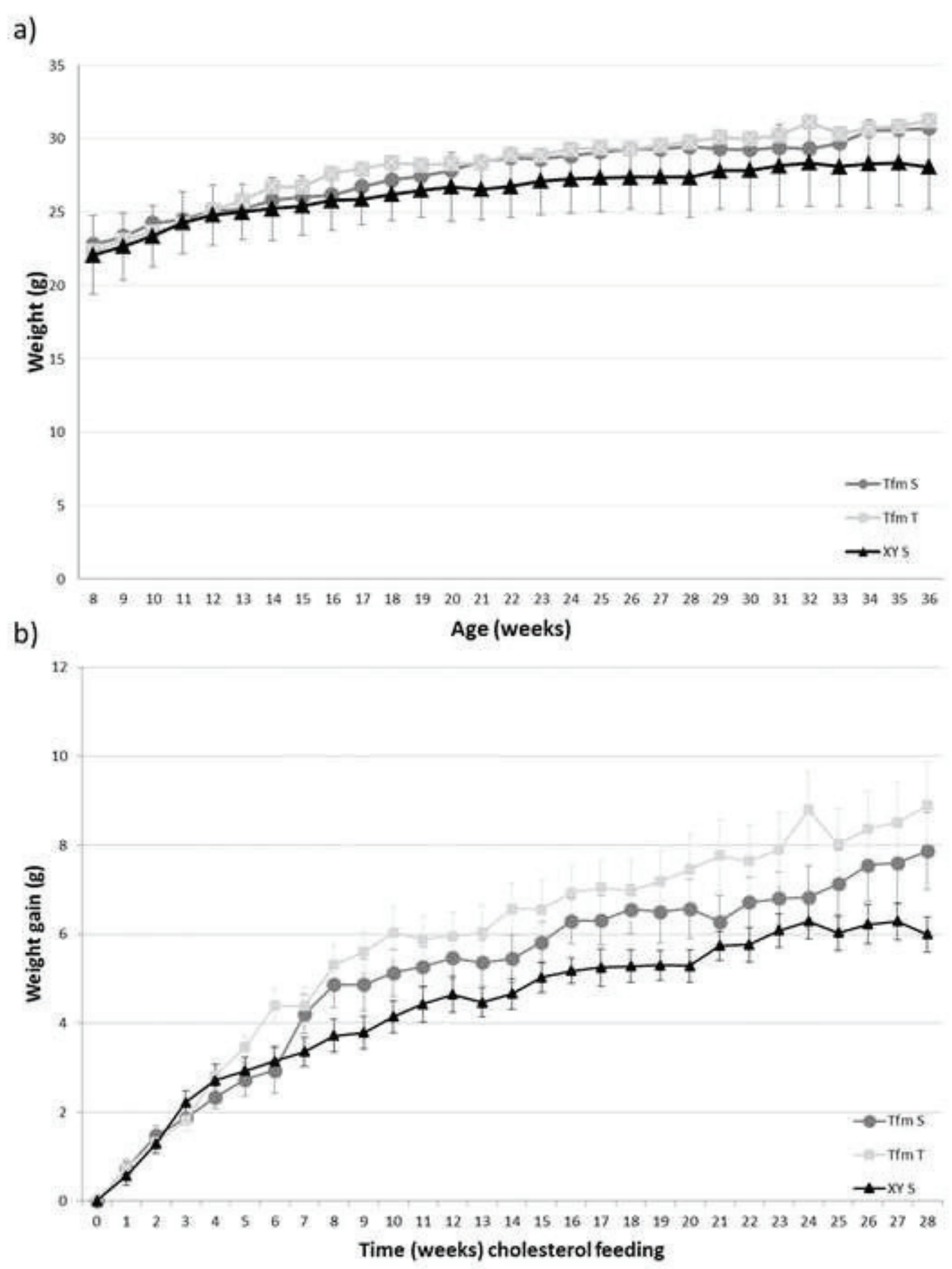
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29 515 **Figure 2; Protein expression of selected targets of lipid and glucose regulation in muscle and liver**
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31 516 **of Tfm mice.** Semi quantitative western blot analysis in (a) muscle and (b) liver of Tfm mice
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33 517 receiving either placebo (Tfm S) or testosterone (Tfm T) and wild-type XY littermates receiving
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35 518 placebo (XY S) at the end of the study period. Data are presented as densitometry arbitrary units and
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37 519 representative blot images. N=6. *p<0.05 versus XY placebo, †p<0.05 versus Tfm placebo.

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Figure 1

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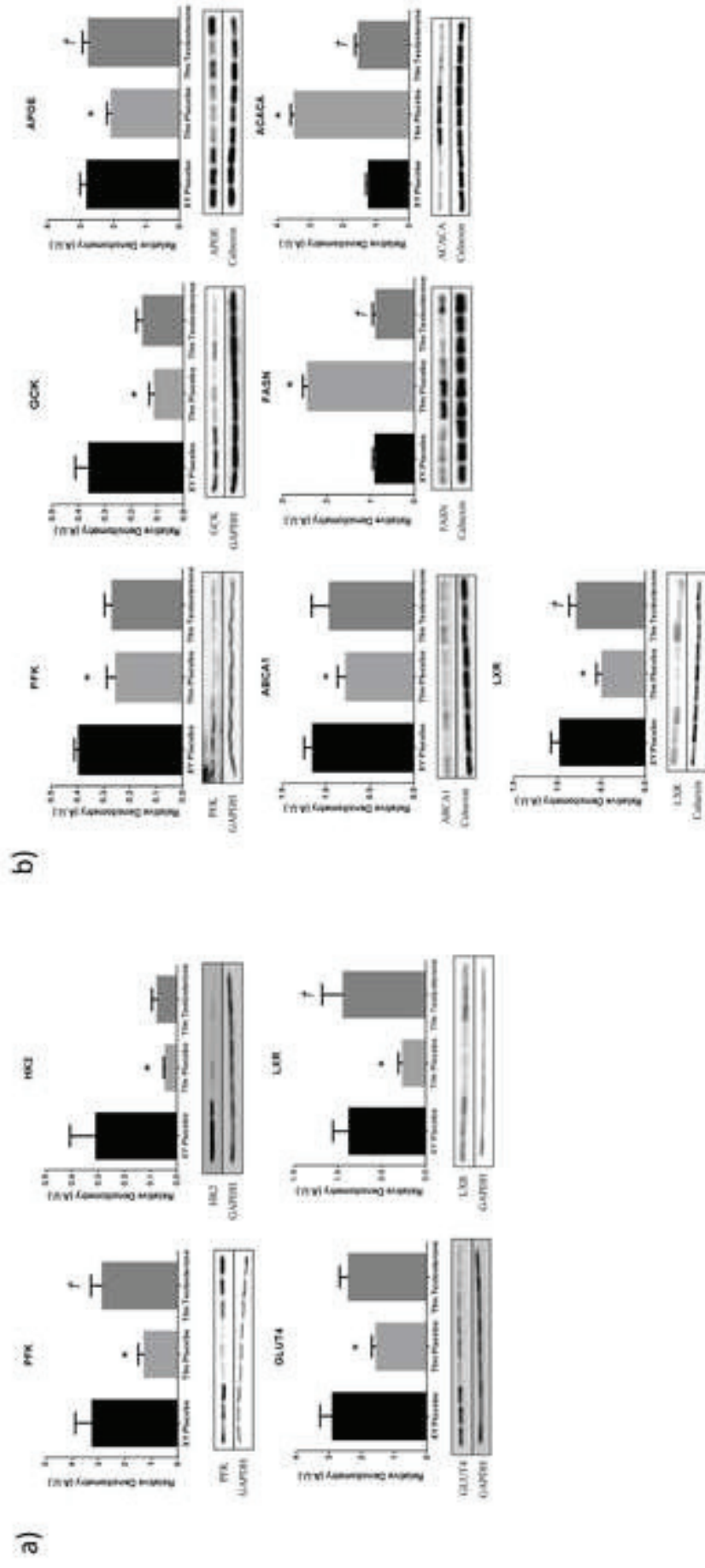


Table 1: Qiagen qPCR primers

Target	Gene	Function	Product Ref.
Fatty acid synthase	<i>Fasn</i>	Catalyses the formation of long-chain fatty acids in fatty acid synthesis	QT00149240
Acetyl coA carboxylase	<i>Acaca</i>	Essential role in regulating fatty acid synthesis	QT01554441
Stearoyl-CoA desaturase 1	<i>Scd1</i>	Catalyses a rate-limiting step in the synthesis of unsaturated fatty acids. Key enzyme in fatty acid metabolism.	QT00291151
Lipoprotein lipase	<i>Lpl</i>	Hydrolysis of triglycerides into free fatty acids	QT01750469
Hormone sensitive lipase	<i>Lipe</i>	Hydrolyses stored triglycerides to free fatty acids	QT00169057
3-hydroxy-3-methylglutaryl-CoA reductase	<i>Hmgcr</i>	Rate-controlling enzyme of the mevalonate pathway that produces cholesterol	QT01037848
Sterol regulatory element-binding protein 1	<i>Srebf1</i>	Cholesterol biosynthesis and uptake, and fatty acid biosynthesis	QT00167055
Sterol regulatory element-binding protein 2	<i>Srebf2</i>	Cholesterol biosynthesis and uptake, and fatty acid biosynthesis	QT01045870
Apolipoprotein E	<i>ApoE</i>	Lipoprotein metabolism and transport.	QT01043889
A ATP-binding cassette transporter A1	<i>Abca1</i>	Major regulator of cellular cholesterol efflux and phospholipid homeostasis	QT00165690
ATP-binding cassette transporter G5	<i>Abcg5</i>	Cellular cholesterol efflux, promote biliary excretion of sterols.	QT00157752
Insulin receptor substrate 1	<i>Irs1</i>	Transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to intracellular pathways in insulin signalling	QT00251657
Hexokinase 2	<i>Hk2</i>	Phosphorylates glucose to glucose 6-phosphate in the glycolytic pathway	QT00155582
Hexokinase 4 (Glucokinase)	<i>Gck</i>	Phosphorylates glucose to glucose 6-phosphate in the glycolytic pathway	QT00140007
Phosphofruktokinase	<i>Pfk</i>	Converts fructose-6-phosphate to fructose-1,6-bisphosphate, one of the most important regulatory enzymes of glycolysis	QT00159754
Carbohydrate-responsive element-binding protein	<i>Chrebp</i>	Activates of several regulatory enzymes of glycolysis and lipogenesis	QT00125335
Glucose transporter 4	<i>Glut4</i>	Cellular glucose transport	QT01044946
Glucose-6-phosphate dehydrogenase	<i>G6pdx</i>	Enzyme in the pentose phosphate pathway, often for tissues actively engaged in biosynthesis of fatty acids	QT01748957
Glycogen synthase	<i>Glys</i>	Converts glucose to glycogen for storage, regulating glycogen/glucose levels	QT00162099
Liver X receptor alpha	<i>Nr1h3</i>	Nuclear receptor transcription factor regulating cholesterol, fatty acid, and glucose homeostasis	QT00113729
Peroxisome proliferator-activated receptor alpha	<i>Ppara</i>	Transcription factor and major regulator of lipid metabolism	QT00137984
Peroxisome proliferator-activated receptor gamma	<i>Pparg</i>	Regulates fatty acid storage and glucose metabolism	QT00100296
Beta 2 microglobulin	<i>B2m</i>	Reference gene	QT01149547
Glyceraldehyde 3-phosphate dehydrogenase	<i>Gapdh</i>	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

Table 3

A) Carbohydrate Metabolism

Gene	Symbol	Muscle			Liver			Subcutaneous Fat			Visceral Fat		
		XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
Insulin receptor substrate 1	IRS1	1.34±0.32	1.37±0.59	1.85±0.94	1.08±0.17	1.74±0.54	1.13±0.29	–	–	–	–	–	–
Hexokinase 2	HK2	1.18±0.19	0.50±0.16*	0.54±0.10	–	–	–	1.32±0.36	0.24±0.05**	0.61±0.19^f	0.97±0.23	1.00±0.21	1.57±0.54
Hexokinase 4 (Glucokinase)	GCK	–	–	–	1.07±0.10	0.47±0.14**	0.97±0.13^f	–	–	–	–	–	–
Phosphofructokinase	PFK	1.28±0.23	0.64±0.16*	0.62±0.10	1.19±0.11	0.79±0.15*	0.77±0.06	1.76±0.68	0.16±0.05*	0.54±0.31	–	–	–
mitogen-activated protein kinase kinase 1	MAP2K1	1.22±0.22	0.65±0.19^g	1.09±0.21	1.09±0.14	1.01±0.14	1.62±0.21^f	–	–	–	–	–	–
Carbohydrate regulatory element binding protein	ChREBP	1.25±0.24	2.16±1.02	1.16±0.22	1.05±0.12	1.17±0.13	1.11±0.13	1.00±0.19	1.76±0.47	1.53±0.38	1.33±0.38	1.96±0.7	1.76±0.37
Glucose transporter 4	GLUT4	1.20±0.19	0.59±0.14*	0.71±0.13	–	–	–	1.31±0.32	0.37±0.12*	0.73±0.29	1.03±0.10	1.24±0.26	0.83±0.16
glucose-6-phosphate 1-dehydrogenase X	G6PDx	1.13±0.19	1.72±0.38	0.92±0.12	1.03±0.07	1.99±0.20***	1.45±0.18^b	1.06±0.23	2.67±1.36	1.09±0.16	1.57±0.59	2.53±0.87	1.69±0.4
Glycogen synthase	Gys1	1.14±0.15	1.01±0.31	1.82±0.72	1.18±0.18	2.13±0.65	1.56±0.22	–	–	–	–	–	–

B) Cholesterol Homeostasis

Gene	Symbol	Muscle			Liver			Subcutaneous Fat			Visceral Fat		
		XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
3-hydroxy-3-methyl-glutaryl-CoA reductase	HMGCoAr	–	–	–	1.06±0.11	3.19±2.09	1.15±0.18	1.12±0.19	0.88±0.16	1.33±0.37	–	–	–
Sterol Regulatory Element-Binding Protein	SREBPf1	1.29±0.26	0.74±0.21	1.47±0.39	1.13±0.16	1.01±0.19	1.32±0.29	1.07±0.17	0.36±0.09**	0.86±0.16^f	0.81±0.13	0.59±0.25	0.78±0.13
Sterol Regulatory Element-Binding Protein	SREBPf2	1.17±0.21	0.80±0.28	1.99±0.62	1.08±0.13	1.44±0.22	1.17±0.16	1.07±0.17	0.42±0.08**	1.12±0.33^f	0.80±0.14	0.56±0.21	0.79±0.13
Apolipoprotein E	ApoE	–	–	–	1.07±0.05	0.77±0.09**	1.14±0.11^f	1.02±0.08	0.59±0.13*	1.14±0.16^f	1.03±0.10	0.75±0.16	0.87±0.06
ATP-binding cassette transporter A1	ABCA1	–	–	–	1.05±0.04	0.71±0.09**	1.10±0.13^f	1.07±0.14	0.77±0.12	0.63±0.11	1.14±0.24	0.98±0.20	1.19±0.32
ATP-binding cassette transporter G5	ABCG5	–	–	–	1.01±0.06	0.81±0.15	1.01±0.08	–	–	–	–	–	–

C) Fat Metabolism

Gene	Symbol	Muscle			Liver			Subcutaneous Fat			Visceral Fat		
		XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
Acetyl CoA carboxylase alpha	ACACA	1.28±0.27	1.65±0.46	0.77±0.14	1.09±0.13	2.49±0.64*	1.30±0.29	1.22±0.28	1.15±0.36	1.89±0.62	1.07±0.16	1.02±0.27	0.95±0.35
Fatty acid synthase	FASN	1.52±0.43	1.48±0.56	0.56±0.14	1.15±0.17	11.42±4.93*	2.99±0.88	1.20±0.28	2.89±1.49	4.76±1.90	1.12±0.23	1.35±0.36	0.87±0.30
Stearoyl-CoA desaturase-1	SCD1	1.06±0.11	1.30±0.24	1.37±0.29	1.04±0.14	2.45±1.15	1.38±0.15	1.2±0.29	3.05±1.37	0.70±0.15	1.10±0.23	4.99±1.64*	0.94±0.15^f
Lipoprotein lipase	LPL	1.24±0.31	0.90±0.30	0.83±0.20	1.07±0.13	1.26±0.45	0.90±0.26	1.03±0.08	0.70±0.09*	0.81±0.22	1.08±0.13	0.99±0.20	2.18±0.36^f
Hormone sensitive lipase	Lipe	1.20±0.46	1.25±0.24	1.18±0.49	1.03±0.08	1.24±0.16	0.85±0.10	1.17±0.17	1.49±0.29	1.14±0.20	1.05±0.14	1.10±0.27	0.82±0.21

D) Master Regulators

Gene	Symbol	Muscle			Liver			Subcutaneous Fat			Visceral Fat		
		XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
Liver X receptor	LXR	1.35±0.29	0.62±0.12*	1.50±0.27^{ff}	1.05±0.09	0.66±0.08**	1.28±0.24^f	1.07±0.14	0.42±0.13**	1.15±0.28^f	1.27±0.35	1.00±0.30	1.44±0.48
Peroxisome proliferator-activated receptor alpha	PPARa	1.16±0.20	1.65±0.78	0.94±0.21	1.02±0.07	1.84±0.55	0.93±0.14	1.13±0.23	0.41±0.11	0.69±0.22	1.09±0.20	0.92±0.32	0.98±0.22
Peroxisome proliferator-activated receptor gamma	PPARg	1.29±0.31	1.29±0.62	0.63±0.17	1.09±0.14	0.99±0.19	1.29±0.23	1.06±0.14	0.67±0.06	0.82±0.12	1.05±0.11	0.49±0.08***	0.66±0.10