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Original article

Serum sex steroids and steroidogenesis-related enzyme expression in skeletal muscle during experimental weight gain in men

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Abstract

Objectives. – Low-circulating testosterone is associated with development of type 2 diabetes in obese men. In this study, we examined the effects of experimental overfeeding and weight gain on serum levels of sex hormones and skeletal muscle expression of steroidogenic enzymes in healthy men with (FH+) and without (FH–) a family history of type 2 diabetes.

Methods. – Following a 3-day lead in energy balanced diet, FH+ ($n=9$) and FH– men ($n=11$) were overfed by 5200 kJ/day (45% fat) for 28 days. Body weight, fasting glucose, insulin, sex steroid, sex hormone binding globulin (SHBG) levels, insulin sensitivity (hyperinsulinaemic-euglycaemic clamp) and body fat (DXA) were assessed in all individuals at baseline and day 28, and sex steroidogenesis-related enzyme expression in *vastus lateralis* biopsies was examined in a subset ($n=11$).

Results. – Body weight, fat mass and fasting insulin levels were increased by overfeeding ($P<0.01$) and insulin was increased significantly more in FH+ men ($P<0.01$). Serum sex hormone binding globulin (SHBG) and 5α-dihydrotestosterone (DHT) were reduced with overfeeding ($P<0.05$), and serum testosterone and DHT were reduced to a greater extent in FH+ men ($P<0.05$). Overfeeding reduced mRNA expression of 3β-hydroxysteroid dehydrogenase (HSD) and 17βHSD ($P\leq 0.007$), independently of group. 5α-Reductase (SRD5A1) mRNA expression was not changed overall, but a time by group interaction was observed ($P=0.04$).

Conclusion. – Overfeeding reduced SHBG and muscle expression of enzymes involved in the formation of testosterone in skeletal muscle. Men with a family history of T2DM were more susceptible to deleterious outcomes of overfeeding with greater reductions in serum testosterone and DHT and greater increases in markers of insulin resistance, which may contribute to increased risk of developing type 2 diabetes.

Keywords: Insulin resistance; Overfeeding, Serum testosterone; Steroidogenic enzymes

1. Introduction

Obesity, and particularly visceral adiposity in conjunction with reduced muscle mass, is associated with the metabolic syndrome and an increased risk of type 2 diabetes mellitus (T2DM) [1]. In men, obesity is associated with reduced serum sex hormone binding globulin (SHBG) and testosterone concentrations while those of serum estradiol and estrone are increased [2]. A meta-analysis of 52 studies showed that men in the lowest quartile for serum testosterone or SHBG had higher risk of metabolic syndrome versus men in the highest quartile [3]. In the majority of these studies, the relationship holds following adjustment with BMI, although typically adjustments were not made for visceral adiposity. Low serum testosterone also independently predicts progression to T2DM in overweight men [4,5]. Thus, increasing evidence supports a role for low testosterone in pro-

Abbreviations: T2DM, Type 2 diabetes mellitus; FH, Family History; DHEA, Dehydroepiandrosterone; DHEA-S, Dehydroepiandrosterone and its sulfate derivate; DHT, 5α-Dihydrotestosterone; 3β-HSD, 3β-Hydroxysteroid dehydrogenase; 17β-HSD, 17β-Hydroxysteroid dehydrogenase; DHT, 5α-Dihydrotestosterone; SRD5A1, 5α-reductase type 1.

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moting insulin resistance and type 2 diabetes, although causal evidence is lacking.

Obese men with T2DM also have lower serum levels of dehydroepiandrosterone (DHEA) and its sulfate derivate (DHEA-S) [6,7]. DHEA is primarily produced by the adrenal gland, but several studies have demonstrated that other tissues, including human and rodent skeletal muscle can produce some DHEA [8–10]. DHEA is converted to testosterone by 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD. DHEA-S is a terminal steroid that cannot be further biotransformed into active steroid metabolites. Testosterone is converted in a tissue-specific manner to either 5α-dihydrotestosterone (DHT), a more potent ligand for the androgen receptor by 5α-reductase (SRD5A) [11], or oestradiol by aromatase. There are two isoforms of SRD5A (designated type 1 and type 2) that exhibit tissue-specific expression patterns with the type 1 isoform broadly distributed and the type 2 isoform found predominantly in male sex-accessory tissue, such as epididymis, the prostate and seminal vesicles. Aromatase is also found in extragonadal sites, including brain, bone and adipose tissue and its activity in adipose tissue is elevated in obesity [12]. In skeletal muscle, metabolic enzymes 3β-HSD, 17β-HSD, and SRD5A1 as well as synthesis of DHT and testosterone from DHEA have all been reported [10,13]. In skeletal muscle, reduced levels of DHEA and SRD5A1 are observed in obese men with T2DM and in rats fed with a high sucrose diet, which can be reversed with DHEA treatment [6,7]. DHEA treatment also activates the insulin signalling pathway and reduces blood glucose levels in streptozotocin-induced diabetic mice [14]. These studies suggest that local sex steroidogenesis may have direct effects on insulin sensitivity, at least in rodents.

First-degree relatives of individuals with a family history of T2DM (FH+) are at increased risk of developing T2DM [15,16] and have a greater tendency towards insulin resistance [16,17], central adiposity [18], inflammation [19], and reduced mitochondrial function [20,21]. Low serum testosterone is also associated with greater risk of developing metabolic syndrome in men with a family history of type 2 diabetes [22]. The effects of short term experimental overfeeding on testosterone and other sex steroid hormone levels as well as local muscle steroidogenesis in men with and without a family history of T2DM has not previously been tested. Here, we hypothesised that overfeeding would reduce serum testosterone levels as well as local expression of muscular steroidogenesis-related enzymes 3βHSD, 17β-HSD and SRD5A1, and that these effects would be greater in men with a family history of T2DM.

2. Methods

2.1. Subjects and metabolic tests

This study protocol was approved by the Human Research and Ethics Committee at Saint-Vincent's Hospital, Sydney and subjects provided informed written consent before commencement of the study, which were conformed to the standards set by the Declaration of Helskinki. The original study design is described in detail previously [23], and included 20 women and 20 men. This analysis includes all 20 men that were studied in the original cohort, and 9 reported at least one first-degree relative with type 2 diabetes [23]. Subjects were excluded if their weight had changed by > 2 kg in the preceding 6 months, if they exercised more than 60 min per week, if they were taking medications known to affect insulin sensitivity, lipid metabolism or blood pressure, or if they had a personal history of type 2 diabetes or cardiovascular disease. Insulin sensitivity was measured as the glucose infusion rate (GIR) necessary to maintain euglycaemia for the final 30 min of a 2 h hyperinsulinaemic clamp (60 $mUm^{-2}min^{-1}$) and was adjusted for fat free mass by dual X-ray absorptiometry (Hologics, USA), and abdominal fat distribution and liver fat was assessed by computed tomography (GE Healthcare) as previously described [23].

2.2. Diets

Estimated energy requirements were calculated for each participant using equations previously generated by doubly labeled water and intake balance techniques [24]. A trained dietitian then planned individual menus for the participants. All foods were provided at baseline energy requirements with a nutrient composition of 30% of energy as fat, 15% as protein and 55% as carbohydrate for 3 days prior to baseline metabolic testing. All foods were also provided from day 0–3 and day 25–28 of the study. On days 3–25 of overfeeding, participants were instructed to consume their regular diets and were provided with high fat snacks to achieve an intake of 5200 KJ/day above baseline. They were required to complete a checklist every day, reporting which snacks were consumed, complete 3-day diet diaries once before study commencement and twice during the overfeeding phase, and to meet with the study dietitian weekly. Diets were analysed for macronutrients and fatty acid composition using FoodWorks 2007 based on the Australian foods database (Xyris Software, QLD, Australia).

2.3. Gene expression

Muscle biopsies were carried out according to previously described techniques in a subset of men ($n = 11$, 4 FH–and 7 FH+) [25]. Total tissue RNA was isolated using Trizol according to previously described studies [25] and the quality and amounts were assessed by nanodrop. Total tissue RNA was reverse transcribed by omniscript reverse transcriptase using a Quantitec cDNA synthesis kit according to the manufactures instructions (Qiagen, California, USA). qPCR was carried out using a PCR thermal cycler with Fast Universal Master Mix according to the manufactures recommendations (Applied Biosystems, CA). The gene pecific primers for 3β-HSD [Hs04194787_g1], 17β-HSD type 3 [Hs00609319_m1], SRD5A1 type 1 [Hs00602691_mH] were purchased (Applied Biosystems, CA). Results are normalised to GAPDH [Hs02758991_g1], which was not changed by overfeeding and was not significantly different between groups.

Table 1
Anthropometric and metabolic responses to overfeeding in men with (FH+) and without (FH–) a family history of type 2 diabetes.

	FH–		FH+		*P* value		
	Baseline	28 days	Baseline	28 days	Time	Group	Time by group
Weight (kg)	81.3 ± 12.9	83.8 ± 12.5	81.2 ± 7.3	84.9 ± 7.8	0.001	0.9	0.2
BMI (kg/m^2)	25.1 ± 3.5	25.9 ± 3.4	25.8 ± 2.4	26.9 ± 2.3	0.001	0.2	0.3
Total body fat (%)	27.1 ± 0.1	28.5 ± 0.1	31.3 ± 0.1	32.5 ± 0.1	0.002	0.9	0.8
Subcutaneous fat (cm^2)	210 ± 96	227 ± 90	235 ± 48	258 ± 61	0.001	0.5	0.4
Visceral fat (cm^2)	84 ± 53	92 ± 44	107 ± 62	122 ± 59	0.02	0.3	0.5
Glucose (mmol/L)	4.7 ± 0.4	4.6 ± 0.2	4.5 ± 0.2	4.6 ± 0.2	0.6	0.4	0.1
Insulin (pmol/L)	70.8 ± 32.3	71.2 ± 22.8	66.1 ± 11.1	91.2 ± 21.6	0.01	0.4	0.01
HOMA-IR	2.09 ± 1.01	2.05 ± 0.72	1.83 ± 0.31	2.62 ± 0.66	0.02	0.6	0.01
GIR (μmol/kgFFM/min)	60.4 ± 24.8	51.0 ± 20.5	45.2 ± 11.1	43.2 ± 13.9	0.08	0.12	0.3

Means ± SD; outcomes were analysed with linear mixed effects models; HOMA-IR: homeostasis model of assessment; GIR: glucose infusion rate.

2.4. Biochemical analysis

Glucose was analysed using a glucose oxidase electrode (YSI Life Sciences, OH, USA). Fasting serum insulin and leptin were assayed by radio-immunoassay (Linco Research, St Charles, MO, USA) and serum testosterone (T), 5α-dihydrotestosterone (DHT), estrone (E1) and 17β-estradiol (E2) were measured by a validated stable-isotope dilution LC–MS/MS [26]. HDL-cholesterol and triacylglycerol were evaluated by enzymatic colorimetry (Roche, IN, USA) and LDL was calculated by the Friedewald equation.

2.5. Statistical analysis

All values are expressed as means ± SEM. Outcomes were analysed with linear mixed effects models using maximum likelihood estimation. The models included fixed effects for group, time as a categorical variable, and the group by time interaction. Relationships between sex steroid hormone concentrations and serum insulin, blood glucose levels, and mRNA expression were determined using Pearson correlation coefficients.

3. Results

There were no detectable differences in any of the baseline characteristics examined between groups. Overfeeding led to weight gain, increased body fat, increased visceral and subcutaneous fat, fasting serum insulin and HOMA-IR (Table 1) and a trend towards reduced insulin sensitivity by hyperinsulinaemic-euglycaemic clamp ($P = 0.08$, Table 1). The increase in insulin and HOMA-IR was greater in men with a family history of T2DM (Table 1).

3.1. Serum sex hormones

At baseline, there was no difference in the serum levels of the sex hormones between groups. Serum SHBG was significantly reduced by overfeeding, independently of group ($P < 0.001$, Fig. 1). Serum DHT was also reduced by overfeeding ($P = 0.03$), and to a greater extent in those with a family history of type 2 diabetes ($P < 0.05$, Fig. 1) and although, serum testosterone did not change overall, a time by group interaction was observed ($P = 0.04$, Fig. 1). There was no change in serum estradiol or estrone with overfeeding or by group, although weight gain at 28 days was inversely correlated with the changes in serum estrone ($r^2 = –0.23$, $P = 0.03$). The change in SHBG was also inversely correlated with the change in body weight ($r^2 = –0.22$, $P = 0.03$, Fig. 2A) and visceral fat mass ($r^2 = –0.47$, $P = 0.002$, Fig. 2B).

3.2. Markers of muscle steroidogenesis

Expression levels at baseline and in response to overfeeding are given in Fig. 3. SRD5A1 expression was not changed by overfeeding, but a group by time interaction was observed ($P = 0.04$) with FH+ men decreasing and FH– men increasing expression of SRD5A1. Expression of 3BHSD and 17BHSD mRNA was significantly reduced by overfeeding ($P < 0.007$), independently of group.

4. Discussion

Low levels of serum testosterone and SHBG are associated with increased risk of metabolic syndrome in men [3] and low testosterone is also associated with increased risk of type 2 diabetes [4,5]. Furthermore, weight loss is associated with a proportional increase and weight gain with a proportional decrease in serum testosterone and SHBG over 4-year follow-up in men [27]. However, it is not clear whether change in sex hormones are an early event in response to nutritional overload and contribute to the development of insulin resistance and metabolic syndrome, or are a later consequence of increased adiposity and the insulin-resistant state. In this study, and as previously reported [28,29], overfeeding induced weight gain and increased fasting insulin and HOMA-IR, and to a greater extent in men with a family history of type 2 diabetes. This study therefore provides a unique opportunity to study potential contributors to insulin resistance.

SHBG regulates the biological action of sex hormones. In this study, and as shown following 100 days of overfeeding previously [30], SHBG levels were significantly reduced by overfeeding, and this response was similar in men with and without a family history of type 2 diabetes. We also observed that the reduction in SHBG was in proportion to the amount

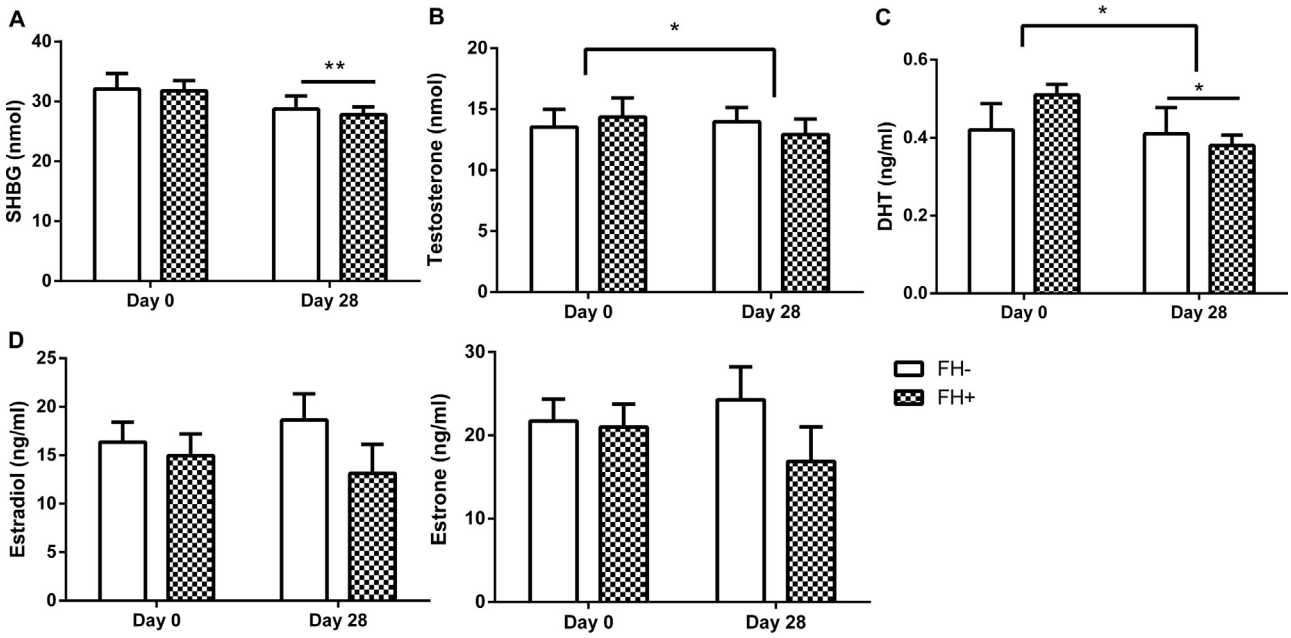

Fig. 1. Serum (A) sex hormone binding globulin, (B) testosterone, (C) 5α-dihydrotestosterone (DHT), (D) estradiol (E) estrone at baseline and 28 days of overfeeding in individuals with and without a family history (FH) of type 2 diabetes. $^{*}P < 0.05$, $^{**}P \leq 0.001$.

of fat gained, and in particular, with the amount of visceral fat gained. This study therefore suggests that the change in SHBG is more closely aligned to changes in abdominal adiposity rather than insulin resistance per se. SHBG is synthesized primarily in the liver, and some studies suggest that increased liver lipid may inhibit SHBG secretion [31]. Whilst liver density was reduced in this study, indicating an increase in liver fat with overfeeding, we did not observe relationships between these variables in this study.

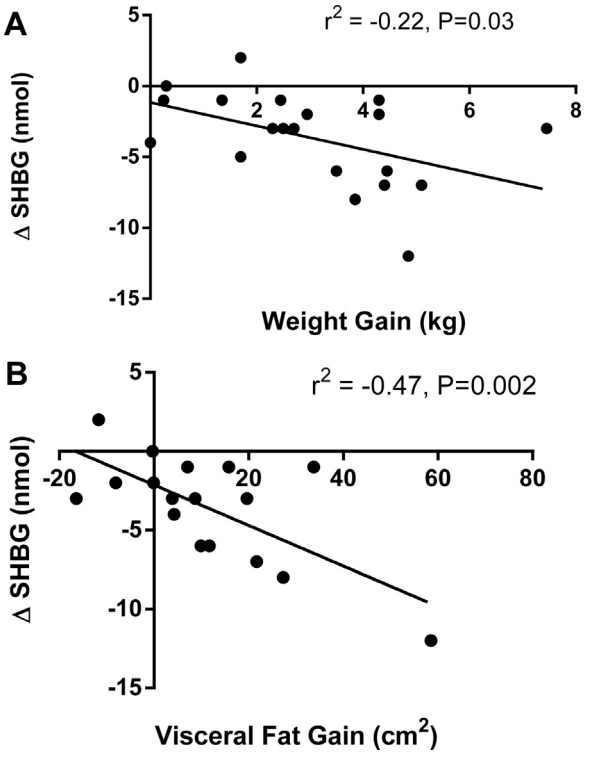

Fig. 2. Correlations between the reduction in SHBG concentrations and (A) weight gain and (B) visceral fat mass gain in response to 2 days of overfeeding.

As also reported in a previous overfeeding study [30], serum testosterone levels were not altered by 28 days of overfeeding. However, reductions in serum testosterone were reported following prolonged high sucrose overfeeding and weight gain in rats [6] and spontaneous weight gain over 4-year follow-up in men [27]. Potentially, these differences may be explained by the length of the weight gain protocols and the younger age of the participants in these overfeeding studies. Of importance, we observed a divergence in the serum testosterone and DHT responses to overfeeding, according to whether or not a family history of T2DM was present. This greater reduction in testosterone, and the more biologically active DHT, in response to overfeeding in FH+ individuals is intriguing, and potentially may contribute to increased risk of type 2 diabetes in this population. Moreover, this may be linked with the greater increase in insulin resistance in FH+, although no direct correlations were observed between the change in testosterone or DHT and the change in fasting insulin or insulin sensitivity by clamp. The mechanisms underlying greater susceptibility to metabolic consequences of over-nutrition in FH+ individuals remains unclear, but may be attributable, at least in part, to more slightly more weight gain in FH+ men.

Serum estrone and estradiol increase with increasing age and obesity in men [32,33] and estradiol levels decrease following weight loss induced by either surgery or caloric restriction and generally in proportion to the total amount of weight lost [34]. In this study, serum estrone and estradiol were not altered by short term overfeeding and no differences were reported according to family history of type 2 diabetes.

We also investigated the effects of overfeeding on enzymes required for the synthesis of sex steroids in skeletal muscle. These enzymes are required for the formation of testosterone

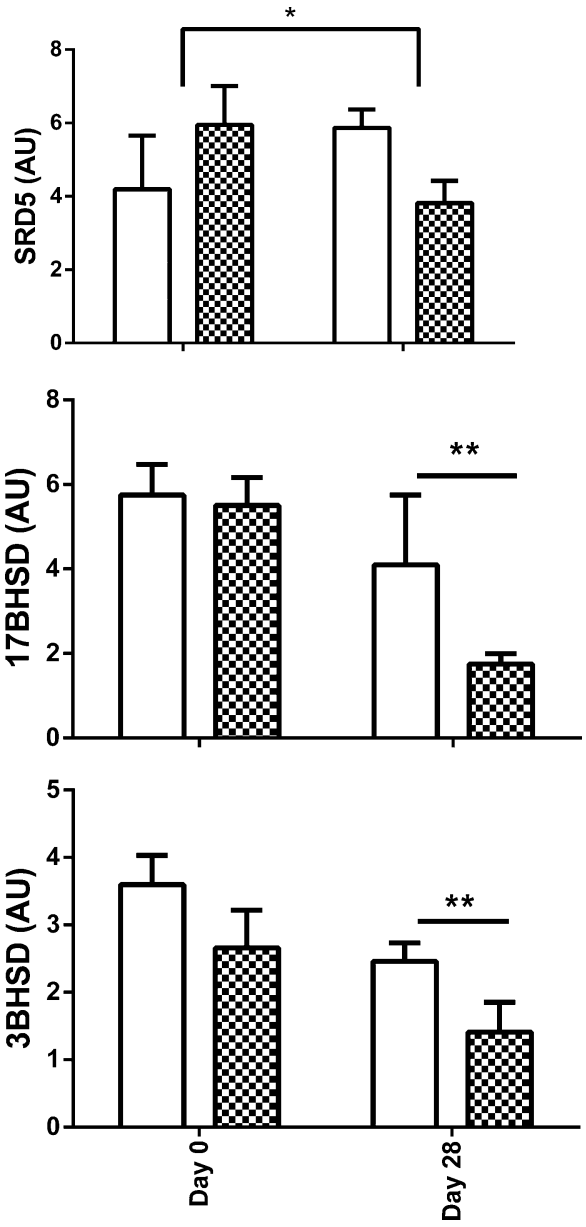

Fig. 3. Gene expression levels of SRD5A1, 3BHSD and 17BHSD in *vastus lateralis* muscle at baseline and 28 days of overfeeding in a subset of individuals with ($n = 7$) and without ($n = 4$) a family history of type 2 diabetes. **Time effect $P \leq 0.007$; *Time by group interaction $P = 0.04$.

from DHEA, and therefore, reduced expression of these enzymes may reduce local muscle testosterone concentrations, but are not customarily reflective of expression occurring in the gonads, or systemically. In this study, overfeeding reduced muscular steroidogenesis-related enzymes, 17β-HSD and 3β-HSD mRNA content in both groups and may therefore reduce local testosterone availability in skeletal muscle. However, it is important to note that the origins of the sex steroid hormones in skeletal muscle remains unclear, and may be from systemic endocrine delivery, paracrine secretion by surrounding adipocytes, or from local intracrine synthesis. In this study, individuals with a family history of T2DM also had a greater reduction in expression of SRD5A1 in skeletal muscle in response to overfeeding, potentially contributing to reduced local DHT. In mouse, DHEA-S or testosterone treatment increased SRD5A1 protein content in muscle, and this was correlated with increased insulin sensitivity as assessed by GLUT-4 translocation [10]. Furthermore, low dose testosterone increased insulin-induced phosphorylation of mTOR, S6 kinase and IRS-1 in differentiated L6 myotubes [35]. Together, this work suggests that increasing local steroidgenesis, in muscle, may improve insulin signalling. However, it should be noted that DHEA-S treatment primarily increases serum estradiol in men [36] and insulin sensitivity was not altered following chemical castration in men [37], suggesting that testosterone mediates any changes in insulin sensitivity indirectly via effects on muscle metabolism or lipolysis, although changes in local muscle steroidogenesis were not examined in that study.

In conclusion, overfeeding reduced SHBG and muscle expression of enzymes involved in the formation of testosterone in skeletal muscle. Men with a family history of T2DM were more susceptible to deleterious outcomes of overfeeding with greater reductions in serum testosterone and DHT and greater increases in markers of insulin resistance, which may contribute to increased risk of developing type 2 diabetes.

Author contributions

KS performed experiments, analysed data and wrote the manuscript, DSB and DJH performed experiments and revised/edited the manuscript, GW and SF revised/edited the manuscript, LKH analysed data and wrote the manuscript.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

All authors have been given opportunity to comment on the content of this manuscript and have approved submission.

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Appendix A. Supplementary data

Supplementary data (French résumé) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.diabet.2014.03.006.

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