

Testosterone and type 2 diabetes prevention: translational lessons in the T4DM study

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Abstract

Testosterone acting via the androgen receptor, and via aromatisation to oestradiol, an activator of the oestrogen receptor, plays key roles in adipose tissue, bone, and skeletal muscle biology. This is reflected in epidemiological studies associating obesity and disordered glucose metabolism with lower serum testosterone concentrations and an increased risk of type 2 diabetes (T2D) in men. Testosterone also modulates erythrocytosis and vascular endothelial and smooth muscle cell function, with potential impacts on haematocrit and the cardiovascular system. The Testosterone for the Prevention of Type 2 Diabetes (T4DM) study enrolled men aged 50 years and over with a waist circumference of 95cm or over, impaired glucose tolerance or newly diagnosed T2D, and a serum testosterone concentration (as measured by chemiluminescence immunoassay) <14.0 nmol/L. The study reported that 2 years treatment with testosterone undecanoate 1000mg, administered 3-monthly intramuscularly, on the background of a lifestyle program, reduced the likelihood of T2D diagnosis by 40% compared to placebo. This effect was accompanied by a decrease in fasting serum glucose, and associated with favourable changes in body composition, hand grip strength, bone mineral density and skeletal microarchitecture, but not in HbA1c, a red blood cell-dependent measure of glycaemic control. There was no signal for cardiovascular adverse events. With the objective of informing translational science and future directions, this commentary discusses mechanistic studies underpinning the rationale for T4DM, and translational implications of the key outcomes relating to glycaemia, and body composition, together with effects on erythrocytosis, and cardiovascular risk and slow recovery of the hypothalamo-pituitary-testicular axis.

1. Introduction

1.1 Testosterone, obesity, and male ageing

A decrease in serum testosterone concentration is not inevitable in ageing men (Handelsman, et al. 2015; Marriott, et al. 2022; Sartorius, et al. 2012; Shi, et al. 2013). The apparent decrease in testosterone seen in unselected middle aged and older men reflects obesity and the accumulation of chronic conditions (Shi et al. 2013) for example cardiovascular disease (Regadera, et al. 1985) and depression (Finkelstein, et al. 2013; Shi et al. 2013), medication use (most commonly opioids), health-related behaviours (e.g., excessive alcohol consumption) and psychosocial factors (Wittert and Grossmann 2022), rather than chronological ageing in itself. However, beyond the age of 70 years, declining testosterone concentrations in unselected men with co-morbidities are accompanied by increases in LH, suggesting possibly impaired Leydig cell function (Yeap, et al. 2018). The obesity phenotype associated with a decrease in serum testosterone concentration in middle to older aged men is associated with a reduction serum SHBG with mid-normal serum LH and FSH and is characterised by an accumulation of visceral adipose tissue associated with insulin resistance, aberrant glucose metabolism, and dyslipidaemia as features of the metabolic syndrome (Umapathysivam, et al. 2022). The proportionate fall in serum testosterone and SHBG concentrations under these circumstances is inherently reversible with weight loss (Grossmann 2018) and does not demonstrate androgen deficiency. Although weight loss will both increase serum testosterone and prevent T2D, it is unclear whether this effect is mediated directly by the increase in testosterone, or by beneficial effects of testosterone on body composition.

1.2 Mechanistic aspects of testosterone action

Testosterone is both a hormone, acting directly at the androgen receptor (AR), as well as a prohormone, undergoing 5-alpha reduction to DHT, a more potent AR analogue and being aromatised to oestradiol, which acts via oestrogen receptors (ER). These sex steroid receptors are ligand dependent transcription factors, and AR and ERs, upon binding of their cognate hormone are shuttled to the nucleus where they bind on specific DNA sequences (response elements), in gene promotor regions to regulate gene transcription in concert with transcriptional cofactors. In addition, there may be expression of cell membrane bound sex

steroid receptors which may mediate rapid (non-classical) actions of testosterone and oestradiol (Handelsman 2000).

1.3 Testosterone effects on body composition: basic and translational considerations

There is a positive relationship between circulating testosterone concentration with muscle mass and strength and an inverse relationship with fat mass (Bhasin, et al. 2001; Finkelstein et al. 2013). Testosterone treatment decreases fat mass and increases lean body mass (skeletal muscle and bone) and muscle strength in both healthy and hypogonadal men irrespective of age (Bhasin et al. 2001).

In skeletal muscle, testosterone, via the AR, induces hypertrophy of both type I and type II muscle fibres, increases muscle satellite cells, myogenic differentiation of muscle progenitor cells and muscle protein synthesis and inhibits adipogenic differentiation of pluripotent stem cells (Herbst and Bhasin 2004). Testosterone, following aromatisation to oestradiol increases growth hormone secretion from the pituitary (Eakman, et al. 1996) and expression of Insulin Like Growth Factor I (IGF-I) in skeletal muscle (Ferrando, et al. 2002). However, the trophic effects of testosterone on skeletal muscle do not require GH and IGF-I (Serra, et al. 2011).

In males, oestradiol (E2) is predominantly synthesised by aromatisation of testosterone in a tissue specific manner (Russell et al 2019). In females, but not males, E2 has a protective effect on functional skeletal muscle mass (McMillin, et al. 2022; Pellegrino, et al. 2022). The effect of testosterone to decrease fat mass is mediated at least in part by aromatisation to E2. Men or male mice with genetic inactivation of ER-alpha or aromatase have increased fat mass (Simpson, et al. 2005) and, in healthy men, increasing serum testosterone by administration of aromatase inhibitors does not lead to a reduction in fat mass (Burnett-Bowie, et al. 2009). Any beneficial effect of E2 on fat mass appears to require the presence of testosterone because in men made androgen deficient, supplemental E2 leads to an increase in fat mass (Russell, et al. 2022).

In males E2 promotes insulin sensitivity and glucose metabolism in skeletal muscle via an E2 receptor alpha mediated mechanism (Inada, et al. 2016). E2 may also improve skeletal muscle glucose metabolism indirectly by increasing overnight pulsatile growth hormone (GH) secretion from the pituitary while inhibiting the hepatic IGF-1 response to GH (Russell et al 2019). In middle aged and older males, higher fat mass (particularly visceral fat accumulation) increases risk of T2D, an effect reversed with reduction in fat mass and there is an inverse

relationship between skeletal muscle mass and strength and risk of T2D (Atlantis, et al. 2009; Qadir, et al. 2021, Li, et al. 2016).

Applying both epidemiological and mechanistic perspectives implicating testosterone in adipose and skeletal muscle biology, and diabetes risk, we designed a randomized controlled trial (RCT), “The Testosterone for the Prevention of Type 2 Diabetes (T4DM) study”. T4DM sought to determine whether treatment with testosterone, on the background of a lifestyle program, would prevent progression of prediabetes to T2D or reverse newly diagnosed T2D in men aged 50 and over with visceral obesity as reflected by a waist circumference of 95cm or greater and mild lowering of circulating testosterone, but excluded those with pathological hypogonadism.

All men in T4DM were enrolled in a lifestyle program as this is the standard of care for T2D prevention and management of T2D. The lifestyle intervention delivered by WW (formerly Weight Watchers) was selected because it is standardised, widely accessible nationally and evidenced-based for the cost-effective prevention of T2D in men (Fuller, et al. 2013).

2. Design of T4DM.

2.1 The serum testosterone cut-off for inclusion

To reflect the typical at-risk population, we excluded men with pathological hypogonadism, where the benefit and safety of testosterone is widely accepted. Ultimately, an upper limit of serum testosterone concentration of 14nmol/L, as measured by a chemiluminescent immunoassay was adopted as a screening compromise between treatment thresholds of serum testosterone advocated elsewhere with our observations that the association between serum testosterone concentration and T2D risk in men with obesity and the metabolic syndrome, persists up to about 16 nmol/L (Atlantis, et al. 2016), implying that higher testosterone concentrations are associated with protection from T2D. After enrolment in the study serum testosterone was measured at baseline by LCMS resulting in serum concentrations from 4 to 30 nmol/L, a finding confirming the unreliability of the testosterone immunoassays (Rosner, et al. 2007; Sikaris, et al. 2005). The absence of a relationship between baseline concentration of testosterone regardless of the method of measurement and the primary outcome of the study highlights the pharmacological effect of testosterone treatment in this study.

2.2 Waist circumference to assess obesity.

The details of the design of the study are published (Wittert, et al. 2019). Waist circumference (WC) rather than body mass index (BMI) was used as the anthropometric criterion for enrolment because an increase, reflecting abdominal obesity, is associated with T2D risk even if BMI is normal (Janiszewski, et al. 2007). Abdominal obesity, where fat accumulates around the viscera and in the liver, and infiltrates skeletal muscles mechanistically underpins the metabolic syndrome, which is characterised by insulin resistance, dyslipidaemia (typically raised triglycerides and reduced high-density lipoprotein concentrations), abnormal glucose metabolism, and hypertension. The Metabolic Syndrome is associated with a reduction in serum testosterone, but normal LH and FSH concentrations most likely reflecting a reduction in SHBG (Brand, et al. 2014). SHBG is synthesised in the liver and tightly coupled to *de novo* lipogenesis. When *de novo* lipogenesis increases SHBG synthesis decreases (Selva, et al. 2007). All testosterone assays measure total testosterone which predominantly reflects SHBG bound testosterone and therefore if SHBG decreases this will be reflected in a decrease in total testosterone concentration (Gleicher, et al. 2020; Kim, et al. 2020; Zhou, et al. 2020). The extent to which, and under what circumstances, there are direct defects of excess adipose tissue on the regulation of the hypothalamic pituitary testicular axis is not completely resolved but there is some evidence for mediation by hypothalamic leptin resistance, central effects of inflammatory cytokines, and inhibitory effects of increased oxidised low-density lipoprotein, adenosine and dysregulated iron metabolism on Leydig cell function in the testis (Umapathysivam, et al. 2022).

Beyond the age of 60 years skeletal muscle mass decreases and visceral lipid filled adipose tissue increases. Unless there is a very substantial increase in fat mass, BMI tends to remain stable and may even decrease therefore not reflecting the increase in viscerally located adipose tissue. Furthermore, with advancing age the risks (including T2D) associated with higher BMI flatten. Waist circumference which is simple to perform and reproducible when done correctly (Ross, et al. 2020) provides an approximation of visceral fat and on average increases with increasing age; values 95cm or above predict increased risk for T2D in middle-aged and older Caucasian men (Siren, et al. 2012).

2.3. Outcome measures

The primary outcome of T4DM was based on an oral glucose tolerance test (OGTT). Although inherently variable, the OGTT has better sensitivity and specificity for diagnosing T2D compared with fasting glucose or with HbA1c, which at its standard cut-point has high specificity but low sensitivity (Jesudason, et al. 2003). The OGTT was used to define the primary outcome in the Diabetes Prevention Program (DPP) (Knowler, et al. 2002) and remains generally accepted as the gold standard diagnostic test for T2D (Collaboration 2015). Secondary outcome measures relevant to this paper included fasting serum glucose, HbA1c, body weight, waist circumference, body composition (fat mass, skeletal muscle mass, and bone density) by Dual-Xray Absorptiometry (DXA), and muscle strength by peak non-dominant handgrip. Haematocrit was measured and data relating to cardiovascular events collected at each clinic visit. Other outcome and safety measures have previously been described in detail (Wittert, et al. 2019).

3. Glycaemic status: Main findings and questions arising

3.1 Testosterone and prevention/remission of type 2 diabetes

The T4DM study achieved its recruitment target, enrolling 1007 men, across 6 centres in Australia. All were enrolled in the WW lifestyle program and randomly allocated to be treated with either 3 monthly intramuscular TU or placebo for 2 years, making it, the largest and longest RCT of testosterone treatment at the time it was done. Outcome data available for primary analysis at two years comprised 443 and 413 testosterone and placebo treated men respectively. The primary and secondary outcomes are published (Wittert et al. 2021). Key findings and some implications relevant to translational science and future directions are detailed below.

In the testosterone compared to placebo-treated group the proportion of participants with T2D at two years, based on a two-hour glucose ≤ 11.1 mmol/L on the OGTT, was reduced by 40% (55/443, 12.4% vs 87/413, 21%). There was a 0.75 mmol/L greater decrease from baseline in the two-hour OGTT glucose concentration and a 0.17 mmol/L greater decrease in fasting glucose in the testosterone as compared to the placebo group.

Blood glucose normalised (2-hour glucose < 7.8 mmol/L) in 51.9% of testosterone and in 43.3% of placebo treated men respectively. While the difference is significant, the translational importance of over 40% of these high-risk men even if randomised to placebo treatment

achieving normal glucose tolerance two years after the initiation of a relatively low intensity lifestyle intervention, of proven cost-effectiveness, is a crucial finding.

3.2 Glucose response to oral challenge vs HbA_{1c}: Impact of red blood cell dynamics

In the T4DM study, the benefits of injectable testosterone undecanoate (TU) for glycaemia were shown in the 2hr GTT but were not accompanied by any change in HbA_{1c} (Wittert, et al. 2021). Previous small studies had previously reported little or no consistent benefit of TU treatment for HbA_{1c} in studies of men without (Svartberg, et al. 2008) or with type 2 diabetes (Gianatti, et al. 2014; Hackett, et al. 2014; Ramachandran, et al. 2020).

HbA_{1c} is a function of the average blood glucose to which haemoglobin in the red blood cell (RBC) is exposed, and the lifespan of that RBC (Cohen, et al. 2008). Improved glycaemia would be expected to produce a lower HbA_{1c} so the unchanged HbA_{1c} may reflect modest or minimal chronic glycaemic changes from testosterone treatment. However, the discordance between testosterone effects on acute (fasting glucose and 2-hour OGTT) vs chronic (HbA_{1c}) glycemia may be due to testosterone's erythropoietic effects influencing RBC lifespan; if testosterone increases RBC lifespan this could increase HbA_{1c} masking a glycaemic benefit of testosterone treatment. However, RBC lifespan does not differ significantly between males and females (Zhang, et al. 2018) making changes in RBC lifespan due to ambient testosterone concentrations unlikely. Other possible explanations for the discrepancy is that testosterone treatment may abrogate the effects of endogenous inhibitors of deglycation such as fructosamine-3-kinase (Delpierre, et al. 2002; Szwegold, et al. 2001) or alternative deglycation mechanisms (Szwegold and Beisswenger 2003) operative in erythrocytes; however, their testosterone sensitivity has not been established. If testosterone inhibits deglycation enzymes, then HbA_{1c} will increase. This area warrants further investigation.

3.3. Pharmacological not physiological effect of testosterone

For purposes of screening, and entry into the study serum testosterone concentration was measured by platform chemiluminescent immunoassay (CLIA) (Wittert et al. 2019). Blood was subsequently sampled fasting in the morning prior to the first injection of TU or matched placebo and prior to each subsequent TU injection, serum testosterone concentration was measured by LC MS/MS in batched samples at study end. Based on the CLIA data testosterone concentrations at screening were <8 nmol/L, 8-11 nmol/L, and >11-14 nmol/L in

approximately 20%, 40% and, 40% of participants, respectively. By contrast, baseline analysis by LCMS showed that 67% of participants had serum testosterone concentrations >11 nmol/L and 41% >14nmol/L. However, there were no significant relationships between serum testosterone concentration measured either by screening (CLIA) or baseline (LC-MS/MS) and glycaemic outcome variables. This suggests that pharmacological studies of testosterone effects on glycaemia in men without pathological hypogonadism should not employ baseline entry thresholds and should avoid the use of unreliable testosterone immunoassays.

4. Testosterone effects on body composition

4.1 Changes in fat and lean mass: translational considerations

In placebo treated participants fat mass, skeletal muscle mass, and non-dominant hand grip strength decreased. In testosterone treated participants there was a greater decrease in fat mass and increases in skeletal muscle mass non-dominant hand grip strength (Wittert. et al. 2021). The magnitude of changes in lean and fat mass and hand grip strength are summarised in the table 1 below.

	Placebo	Testosterone	Between group difference	P
Fat mass	-1.9kg	-4.6kg	2.7	p<0.0001
Skeletal Muscle Mass	-1.3kg	+0.4kg	1.7kg	p<0.0001
Non-dominant hand grip strength	-1.3kg	+1.7kg	2.19kg	p<0.0001

Because fat mass is positively, and skeletal muscle mass (Qadir, et al. 2021) and grip strength (Li, et al. 2016) are inversely, associated with risk of T2D we have sought to determine the extent to which changes in total fat mass (kg), abdominal fat mass (%), lean mass (kg), and non-dominant handgrip (kg) mediate the effect of testosterone treatment on glycaemia. Preliminary data (Wittert, et al. 2022) show that some, but not all, of the testosterone effect (when coupled with lifestyle changes) is mediated via a decrease in fat mass and to some extent also by the increase in oestradiol concentration in accordance with the known effects of oestradiol on glucose metabolism in skeletal muscle documented in section 1.3. The

observation that there is limited effect mediation by skeletal muscle mass and strength does not exclude non-trophic mechanisms other than E2, for example increased growth hormone secretion (Eakman, et al. 1996), adiponectin production, and GLUT-4 expression and translocation (Antinozzi, et al. 2017; Inada, et al. 2016) that may mediate the beneficial effects of testosterone.

Lifestyle modification programs are effective to reduce weight in obesity when adherence is high, but even under optimal circumstances, engagement is variable and adherence wanes with time (Fabricatore, et al. 2009). Because sex steroid receptors are expressed in the brain, a motivational benefit of testosterone to increase adherence to the lifestyle program was proposed at the outset as a hypothetical mechanism (Wittert et al. 2019). However, the T4DM study provided no support for this motivational hypothesis as engagement in the lifestyle program during the study was not sensitive to testosterone treatment while both groups achieved 70% sufficient physical activity at 2 years. Further we observed no mental or physical quality of life benefits and no improvement in quality of sleep (Wittert, et al. 2021), findings that are consistent with data from the smaller and shorter T trials (Snyder, et al. 2016)

4.2 Bone Mineral Density and Ultrastructure: mechanistic considerations

Ageing is causally associated with decreasing bone mineral density (BMD) a process accelerated by diet-induced weight loss.

In men, testosterone both acting directly via the AR, as well as via its conversion by aromatization to E2 to act on the ER, is required for the acquisition of peak bone mass attained at the completion of growth, as well as for the maintenance of bone mass during adulthood. Men with untreated congenital hypogonadism (e.g., congenital hypogonadotropic hypogonadism, or those with Klinefelter Syndrome) fail to achieve peak bone mass during puberty, and men with acquired pathological hypogonadism experience accelerated bone loss during adulthood (Vanderschueren, et al. 2014). Small and ethically constrained, uncontrolled studies report that testosterone treatment of men with pathological hypogonadism reduces bone turnover markers and increases bone density (Katznelson, et al. 1996; Snyder, et al. 2000). The role of sex steroids in skeletal health in unselected older men without pathological hypogonadism is less clear. Population based studies report that circulating sex steroids are inversely associated with accelerated bone loss, both if assessed by conventional dual energy absorptiometry (DXA) measuring areal bone mineral density

(aBMD), and by high resolution- peripheral quantitative computed tomography (HR-pqCT) measuring volumetric BMD (vBMD) and parameters of bone microarchitecture (David, et al. 2022). Moreover, circulating sex steroids are inversely associated with fracture risk (David et al. 2022; Vanderschueren et al. 2014). However, such observational studies cannot ascribe causality.

4.3. Testosterone effects on bone structure and strength

The effects of testosterone treatment on skeletal outcomes in men without pathological hypogonadism have only come into focus more recently. An early meta-analysis of RCTs enrolling 264 men aged 60-76 years with a baseline serum testosterone of 10-15 nmol/L receiving transdermal or intramuscular testosterone (short acting esters) reported that testosterone treatment, compared to placebo, increased aBMD at the lumbar spine by 3.7%, without significant effect on the femur (Isidori, et al. 2005). A more recent large placebo-controlled study of men aged 60 years or older (n=211) with a baseline testosterone of <9.54 nmol/L showed that daily transdermal testosterone treatment over one year increased vBMD (by quantitative CT (QCT)) predominantly at the trabecular spine (+6.8%) with lesser effects at peripheral spine (+2.9%) (Snyder, et al. 2017). Studies using higher resolution HR-pqQCT (82 μ m vs ~500 μ m with standard QCT) allow assessment cortical and trabecular bone microarchitecture which predicts fracture risk independent of aBMD and the FRAX score, a validated aBMD-independent predictor of fracture risk incorporating clinical risk factors for fracture (Samelson, et al. 2019). However, RCTs assessing the effects of testosterone treatment on bone microarchitecture defined by HR-pqQCT in men were lacking. Therefore, we conducted Testosterone for Bone (T4Bone), a planned sub-study of Testosterone for Diabetes Mellitus (T4DM). In T4Bone over 24 months, testosterone treatment, compared to placebo, increased total vBMD both at the tibia and radius (Ng Tang Fui, et al. 2021). vBMD increased predominantly in cortical bone at both sites with potent treatment effect sizes ranging from 2.9% to 3.1%. However, effects on trabecular microarchitecture were minor. Testosterone also significantly increased aBMD at the lumbar spine (+3.3%) and the total hip (+1.9%) (Ng Tang Fui et al. 2021). The predominant effects of testosterone treatment on cortical bone were consistent with observational HR-pqQCT studies in older men with lower sex steroid concentrations displaying deficits predominantly in cortical, rather than trabecular bone (Argoud, et al. 2014). Moreover, men commencing androgen deprivation therapy for

prostate cancer, which leads to severe sex steroid deficiency experience predominant loss of cortical, rather than trabecular bone (Hamilton, et al. 2010).

4.4. Testosterone effects on cortical vs trabecular bone

None of the RCTs evaluating the effects of testosterone treatment in older men has been powered for fracture outcomes. Of note however the findings of T4DM that testosterone induced increases in cortical bone, which comprises 80% of the human skeleton, suggests that it might. Preclinical studies report that the loss of cortical bone compromises bone strength to a greater extent than the loss of trabecular bone (Vanderschueren et al. 2014), and in observational HR-pqQCT studies in men, deficits in cortical parameters were more consistently associated with fractures than trabecular parameters (Fink, et al. 2018; Szulc, et al. 2011). Moreover, the effect sizes, ranging from 1.3%-3.1% for total and cortical vBMD in T4DM compare favorably with those reported for conventional osteoporotic drug treatments. In postmenopausal women with low BMD, antiresorptive drug therapy (using bisphosphonates or denosumab which have proven anti-fracture efficacy) over 12-24 months produced effect sizes on HR-pqQCT indices ranging from 0.3-3.8% (Burghardt, et al. 2010). Of note men participating T4Bone had a mean baseline serum testosterone of 13.6 nmol/L; there was no evident relationship of baseline serum testosterone or oestradiol concentrations and bone microarchitecture.

4.5. Mechanistic aspects of testosterone action on bone

Testosterone treatment in T4DM was pharmacological and not designed to explore the mechanisms by which testosterone treatment increased vBMD. It remains unclear firstly how much testosterone effect is direct, i.e., mediated by sex steroids receptors in bone as opposed to effects other tissues such as muscle or fat, and secondly, how much of the testosterone effect is mediated via the AR rather than via aromatization to oestradiol and the ER.

Regarding the first question, both AR and ER are expressed in bone cells, and targeted deletions of both the AR as well as the ERs in bone cells (osteoclasts, osteoblasts and osteocytes) have reported skeletal deficits in mice (Vanderschueren et al. 2014) and congruent findings are reported in men with comparable genetic inactivation. Therefore, the actions of sex steroid on skeletal health are likely to be direct, supported by observations in T4Bone that testosterone treatment reduced bone remodeling. However, in addition, testosterone also increases muscle mass (Wittert, et al. 2021), and it is possible that increased

muscle mass, although modest, promotes skeletal health either by exerting loading on the skeleton and/or by secretion of bone-anabolic myokines. In addition, increased muscle mass may reduce falls risk, a common proximal cause of fragility fractures. Moreover, in T4DM testosterone reduced fat mass and waist circumference and fat mass (especially visceral fat), all of which are negatively associated with bone mass (Gilsanz, et al. 2009). The mechanisms remain unclear but may be via secretion of osteo-catabolic adipo-cytokines and/or associated insulin resistance (Vanderschueren et al. 2014). These metabolically favorable changes in body composition are, at least in part, responsible for the reduced T2D risk observed in T4DM. Insulin resistance and T2D have been associated with increased fracture risk, although precise mechanisms are unclear (Khosla, et al. 2021). Therefore, it is possible that testosterone treatment may improve skeletal health, at least in part indirectly via effects on muscle and fat including possibly glucose metabolism. However future mechanistic and clinical studies are necessary to test these hypotheses.

Androgens promote periosteal apposition to increase bone size in men, explaining why men generally have wider long bones than women. However regarding the second question, in older men, some observational studies suggest that skeletal outcomes (reduced bone density, impaired microarchitecture and increased fracture risk) are more closely related to reductions in circulating oestradiol, rather than in testosterone (Russell and Grossmann 2019). Experimental studies among men receiving GnRH analog therapy to suppress gonadal steroids with testosterone addback with or without an aromatase inhibitor (to suppress aromatization of the administered testosterone to oestradiol) have suggested indirectly that oestradiol is important for maintaining bone architecture (Finkelstein, et al. 2016). However, these studies have inferred oestradiol effects by its drug-induced absence, rather than assessing its effect directly. To directly assess the effects of oestradiol on skeletal health in men, a more recent RCT enrolled older men receiving androgen deprivation therapy for prostate cancer which reduces both circulating testosterone and oestradiol to castrate levels (Russell, et al. 2022). In this RCT, oestradiol treatment, compared to placebo improved bone microarchitecture and bone density, thus providing direct evidence that oestradiol can improve skeletal health in men in the absence of testosterone (Russell et al. 2022). From a clinical perspective this supports the role of testosterone treatment with its full spectrum of androgen actions (rather than non-aromatisable DHT or synthetic androgens) as the prime mode of testosterone replacement in men with organic hypogonadism. Of note, the findings from T4Bone do not

endorse testosterone treatment to improve skeletal health in men without pathological hypogonadism. This is because whether testosterone treatment improve patient important health outcomes (such as fractures) remains unknown, and the long-term risks of testosterone treatment are insufficiently explored.

5. Questions about safety: erythrocytosis and cardiovascular effects

5.1 Erythrocytosis: mechanistic considerations

Testosterone has a direct and dose-related effect on bone marrow to increase erythropoiesis (Coviello, et al. 2008). Mechanisms of testosterone induced erythropoiesis include iron mobilisation and incorporation into red blood cells, increased haemoglobin synthesis and red blood cell production (Bachman, et al. 2014; Hennigar, et al. 2020). Increased iron flux is mediated by changes in erythropoietin, hepcidin and ferroportin (Roth, et al. 2019). The primary stimulus to renal production of the cytokine, erythropoietin, is hypoxia (Bachman, et al. 2014). Hepcidin inhibits absorption of iron in the gut by binding to the iron transporter ferroportin and resulting in its degradation thus preventing iron transport across the basolateral membrane of the enterocytes (Atanasiu, et al. 2007). Testosterone induces a sustained suppression of hepcidin (Guo, et al. 2013).

Murine data suggest that erythropoietin (EPO) upregulation and hepcidin suppression operate both jointly and independently (Guo, et al. 2020; Guo et al. 2013). Although one clinical trial observed no significant increase in serum EPO during testosterone treatment (Maggio, et al. 2013), testosterone produces a decrease in the threshold for erythropoietin release (Bachman. et al 2014).

Oestradiol increases haemopoietic stem cell proliferation and survival in vitro (Calado, et al. 2009) and high endogenous oestradiol following IVF hyperstimulation is reported to inhibit hepcidin in women (Lehtihet, et al. 2016). However, testosterone alone is sufficient to maintain red cell mass and to increase net erythropoiesis with supra physiological doses (Rochira, et al. 2009).

5.2 Erythrocytosis: as an adverse event in clinical trials

Erythrocytosis (haematocrit >0.54) is one of the most common adverse effects in clinical trials of testosterone therapy. Advancing age is associated with a higher risk that cannot be

explained by differential effects on erythropoietin or soluble transferrin receptor (Coviello 2008). Erythrocytosis was a particularly prominent adverse event in T4DM and occurred in 22% (106) of men treated with testosterone but only 1% (6) of those on placebo. Once the effect of dehydration was eliminated by a second non-fasting test, only 23 men were withdrawn from treatment because of the finding of elevated haematocrit on two occasions. Nevertheless this rate was substantially higher than generally reported (Warren and Grossmann 2022) possibly because we did not exclude men with obstructive sleep apnoea (OSA). Severe OSA leads to a modest increase in haematocrit (Rha, et al. 2022) and the best characterised risk factor for androgen-induced erythrocytosis is elevated baseline haemoglobin/haematocrit (Idan, et al. 2010) or trough serum testosterone in testosterone treated men (Ip, et al. 2010). The prevalence of moderate or severe obstructive sleep apnoea (OSA) in a randomly selected cohort of community dwelling men aged 40 years and over is approximately 25%, with half having severe OSA (Adams, et al. 2016). The prevalence is higher with increasing age, obesity, and is over 50% in the presence of T2D (Andayeshgar, et al. 2022). At enrolment, 14% of men were using CPAP and this remained relatively constant for the study duration (unpublished data). Therefore, it is possible that the relatively high prevalence of increased erythrocytosis in T4DM is attributable to the combined effects of testosterone and overnight hypoxia.

Another intriguing possibility is an interaction between testosterone and somatic mutations, which occur with increasing frequency over the ageing process, in genes regulating erythrocytosis. For example, somatic mutations of the Janus kinase 2 (*JAK2*) gene and *SH2B* adaptor protein 3 (*SH2B3*) gene cause most cases of primary acquired erythrocytosis (Kristan, et al. 2021). Somatic mutations indicative of clonal haematopoiesis in individuals with primary erythrocytosis have also been found in 2 homologous genes *BCOR* and *BCORL1* which play key roles in haematopoiesis (Wouters, et al. 2020). Moreover, erythrocytosis with clonal haematopoiesis has an independent association with cardiovascular morbidity and mortality (Wouters et al. 2020). This is an area of active investigation we are currently pursuing.

5.3. Implications of a high haematocrit

The relationship between high haematocrit (49 - 70%) and cardiovascular risk remains unresolved with some suggesting an increase in risk (Gagnon, et al. 1994; Kunas, et al. 2009), and others not (Brown, et al. 2001; Puddu, et al. 2002). The data from these studies cannot

necessarily be used to infer the risk of an increase in haematocrit induced by treatment with testosterone. In recent study, 5,842 men who developed polycythaemia (haematocrit $\geq 52\%$) after commencing treatment with testosterone had a higher risk of Major Adverse Cardiovascular Events/ Venous Thromboembolism (MACE/VTE) (number of outcomes: 301, 5.15%) at one year than 5,842 matched men who did not develop polycythaemia (number of outcomes 226, 3.87%) (Ory, et al. 2022).

In men treated with testosterone, aggressive management of cardiometabolic co-morbidities and risk factors including OSA decreases the risk of erythrocytosis and improves overall symptoms (Farber, et al. 2020).

5.4. Cardiovascular effects: mechanistic studies

There is ongoing debate over the cardiovascular effects of testosterone. Mechanistic studies have been conducted using *in vitro* and animal models of atherogenesis. In an experimental study using aortic ring segments cultured *in vitro* after endothelial denudation, testosterone treatment inhibited neointimal plaque development, and effect associated with increased expression of androgen receptor mRNA in treated segments (Hanke, et al. 2001). This concept was supported by an *in vivo* study in male miniature pigs, where neointima formation in response to induced coronary artery injury was increased in castrated animals compared to intact controls, and effect abrogated by testosterone treatment (Tharp, et al. 2009). Testosterone treatment induces apoptosis in vascular smooth muscle cells, another mechanism by which atherosclerotic plaque growth or stability may be affected (Lopes, et al. 2014). Of note, testosterone augmented angiogenesis, a process central to cardiovascular repair/regeneration, in male but not female endothelial cells (Sieveking, et al. 2010). Androgen receptor gene knockdown abrogated these effects in male endothelial cells, whereas androgen receptor overexpression in female endothelial cells conferred androgen responsiveness for angiogenesis. Testosterone treatment also increased cholesterol efflux in a monocyte-macrophage cell line (Kilby, et al. 2021). Thus, these experimental data suggest an effect of testosterone to modulate mechanisms relevant to atherogenesis.

In castrated male rabbits fed a cholesterol-rich diet, aortic atherosclerosis was highest in placebo-treated rabbits and lowest in testosterone-treated rabbits, as well as in untreated, sham-operated controls (Alexandersen, et al. 1999). In cholesterol-fed ovariectomised female and castrated male rabbits, oestrogen in female rabbits and testosterone in male rabbits

reduced aortic plaque formation (Alexandersen, et al. 1999; Bruck, et al. 1997). Similar results were reported in male miniature pigs fed a high-fat and high-cholesterol diet where castration resulted in increased atherosclerotic plaque and intima-media thickness (Deng, et al. 2021). In castrated atherosclerosis-prone mice the anti-atherosclerosis effect of testosterone treatment was dependent of aromatization to oestradiol (Nathan, et al. 2001). Testosterone treatment reduced atherosclerotic lesion area to a greater extent in castrated wild type compared to AR knockout mice (Bourghardt, et al. 2010). Testosterone treatment also attenuated fatty streak formation in mice with a non-functioning androgen receptor, suggesting effects independent of the classical androgen receptor (Nettleship, et al. 2007). Taken together these studies are consistent with an effect of testosterone, either directly or via aromatization to oestradiol, to regulate atherosclerotic plaque formation. However, testosterone treatment increased arterial calcification in apolipoprotein E knockout mice, with a lesser effect of the non-aromatisable androgen dihydrotestosterone (McRobb, et al. 2009). Reconciling these experimental findings with the earlier onset and greater severity of atherosclerosis in men than in women remains an ongoing challenge.

5.5. Cardiovascular effects: clinical studies

The associations of endogenous testosterone concentrations with incidence of cardiovascular events in prospective cohort studies, and the risk of cardiovascular adverse events in interventional trials of testosterone therapy have been previously reviewed (see Yeap, et al. 2022). Several recent studies of note are highlighted as follows. In a large prospective observational cohort study of 210,700 men aged 40-69 years with 8,790 cardiovascular events occurring over 9 years follow-up, baseline testosterone concentrations were not associated with incidence of myocardial infarction, stroke, heart failure or major cardiovascular adverse events (Yeap, et al. 2022). An association of lower testosterone concentrations with higher risk of stroke, but not risk of myocardial infarction, has been reported in men aged ≥ 70 years (Yeap, et al. 2014). In a clinical study of overweight men, testosterone treatment did not improve vascular function assessed using flow-mediated dilation, nor did testosterone add to the benefits of exercise on vascular health (Chasland, et al. 2021). A sub-study from the Testosterone Trials (T-Trials) of 138 men reported an increase in coronary atheroma measured as non-calcified plaque volume using coronary computed tomography angiography (CCTA) in testosterone-treated men over 12 months, insufficient duration to gauge

cardiovascular consequences (Budoff, et al. 2017). The authors concluded that larger and longer studies were needed to understand the clinical implications of this finding.

A RCT of six months transdermal testosterone or placebo gel treatment to increase lower limb strength and physical function in 209 men aged ≥ 65 years with mobility limitations was stopped prematurely due to excess adverse cardiovascular events in testosterone-treated men (Basaria, et al. 2010). However, another trial in a similar population of intermediate-frail or frail men aged ≥ 65 years randomized 274 men to testosterone gel or placebo treatment for six months, finding an improvement in lean mass, knee extension strength and physical function, with no signal for adverse cardiovascular events (Srinivas-Shankar, et al. 2010). A registry study of older men treated with testosterone for up to 10 years reported improvements in cardiometabolic risk factors, however the limitations of such uncontrolled studies need to be considered (Yassin, et al 2016).

5.6. Cardiovascular adverse events in T4DM

In T4DM, cardiovascular adverse events were comparably distributed between testosterone and placebo-treated men; 13/503 men in the placebo arm, and 7/504 in the testosterone arm experienced an ischemic heart disease adverse event over the two-year trial duration. When major adverse cardiovascular events (ischemic heart disease, cerebrovascular disease and cardiovascular deaths) were considered, there were 17/503 events in the placebo arm and 12/504 in the testosterone arm (Wittert et al. 2021). This is in keeping with the T-Trials where 788 men were randomly allocated testosterone or placebo for a year, and 7 men in each arm of the study died from cardiovascular causes or had a myocardial infarction or stroke (Snyder, et al. 2016). However, none of these trials was long enough to definitively assess adverse cardiovascular events. A recent systematic review and meta-analysis of randomised controlled trials including individual patient data, mostly of 12-months duration found no evidence of excess cardiovascular or cerebrovascular adverse events in testosterone-treated men (Hudson, et al. 2022). These and the 2-year T4DM data provide some assurance regarding short term risk however much longer-term follow-up over decades is required for a full safety evaluation. Additional information will be forthcoming when the *“Testosterone Replacement therapy for Assessment of long-term Vascular Events and efficacy ResponSE in hypogonadal men”* (TRAVERSE) study, a Federal Drug Agency-mandated five-year

cardiovascular safety trial of testosterone in middle- to older-aged men with multiple risk factors or pre-existing cardiovascular disease is completed (Bhasin, et al. 2022).

6. Recovery of the hypothalamo-pituitary-testicular axis

Another potentially detrimental outcome arising from prolonged injectable testosterone treatment even at standard testosterone replacement doses is that it creates sustained suppression of the hypothalamo-pituitary-testicular (HPT) axis. The slow but eventually complete recovery takes up to 12 months after cessation of testosterone treatment (Handelsman, et al. 2022) is comparable with the time course of recovery from androgen abuse-induced HPT axis suppression (Shankara-Narayana, et al. 2020). The congruence of these findings suggests the recovery from androgen-induced HPT axis suppression is primarily related to duration since ceasing androgen intake, rather than the doses or patterns of androgens used. Prolonged, slow recovery of the HPT axis can cause symptomatic androgen deficiency withdrawal symptoms during recovery and potential androgen dependence. These pose a practical limitation on the wider application of such injectable testosterone treatment, especially in men without pathologic hypogonadism. Whether shorter treatment duration might facilitate faster HPT axis recovery remains an interesting question; however, the minimal duration of injectable testosterone treatment to maintain similar improvements in acute (if not chronic) glycemia shown in T4DM remains unknown and careful investigation would be warranted to determine the benefits and risks of shorter duration of testosterone treatment on iatrogenic HPT axis suppression and recovery. The intermediary molecular mechanism of the HPT axis suppression by exogenous testosterone, and whether it can be modified independent of systemic androgen effects, warrants further investigation.

7. Conclusions

The primary finding was that over and above the effects of a lifestyle intervention, men treated with testosterone had a 40% lower risk of T2D diagnosis (by OGTT) after two years relative to placebo treated men. Further, at 2 years, 40% of men in the placebo group, and 50% of men in the testosterone group normalised glycemia. Yet HbA1c was not reduced so the magnitude of the chronic glycaemic changes requires further long-term evaluation before wider application of pharmacological testosterone treatment to prevent T2DM is justified.

Testosterone treatment appears to have primarily mediated its effect by inducing metabolically favourable changes in body composition. There were no motivational or quality of life benefits from testosterone treatment.

T4DM illustrates how concepts gained from basic and mechanistic studies are translatable via the platform of a placebo controlled RCT leading to new information and ongoing studies to answer questions generated. These include the importance of central adiposity, and the role of testosterone and oestradiol in regulation of adipose and skeletal muscle biology. Similarly, the observed improvements in skeletal microarchitecture and bone mineral density are grounded in the role of androgen and oestrogen receptors in bone biology. Whether the benefits on bone are durable and clinically important require further investigation.

While mechanistic studies have explored the potential role of testosterone in mechanisms relevant to atherogenesis, in T4DM there was no signal of either cardiovascular risk or benefit. The risk of an unacceptably high haematocrit may be preventable by screening for and treating sleep apnoea, but other mechanisms remain to be investigated. Suppression of the endogenous HPT axis may last for up to 12 months and therefore ill-conceived or uncontrolled trials of testosterone treatment may do more harm than good.

From a translational perspective, building on the results of T4DM, data from trials that are adequately powered to examine durability of treatment effect and offset, prevention of diabetes related complications, fragility fractures, cardiovascular events, and mortality would be important. Further studies are also needed to better define mechanisms and magnitude of testosterone effects for glycaemic and skeletal benefit.

The most important, and readily translatable, outcome of T4DM is that, in line with prior observations, T2D is preventable. Lifestyle changes are a logical first step. The health of men is likely to benefit most, not from the measurement of serum testosterone and prescription of testosterone treatment, but from careful risk assessment and an overall holistic approach to care.

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Table 1

	Placebo	Testosterone	Between group difference	P
Fat mass	-1.9kg	-4.6kg	2.7	p<0.0001
Skeletal Muscle Mass	-1.3kg	+0.4kg	1.7kg	p<0.0001
Non-dominant hand grip strength	-1.3kg	+1.7kg	2.19kg	p<0.0001