Testosterone therapy and cardiovascular diseases

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Abstract

Since it was first synthesised in 1935, testosterone (T) has been viewed as the mythical Fountain of Youth, promising rejuvenation, restoring sexual appetites, growing stronger muscles, and quicker thinking. T is endowed with direct effects on myocardial and vascular structure and function, as well as on risk factors for cardiovascular (CV) disease. Indeed, low serum T levels are a risk factor for diabetes, metabolic syndrome, inflammation, and dyslipidaemia. Moreover, many studies have shown that T deficiency *per se* is an independent risk factor of CV and all-cause mortality. On this background and due to direct-to-patient marketing by drug companies, we have witnessed to the widespread use of T replacement therapy (TT) without clear indications particularly in late-life onset hypogonadism. The current review will dwell upon current evidence and controversies surrounding the role of T in the pathophysiology of CV diseases, the link between circulating T levels and CV risk, and the use of replacing T as a possible adjuvant treatment in specific CV disorders. Specifically, recent findings suggest that heart failure and type 2 diabetes mellitus represent two potential targets of T therapy once that a state of hypogonadism is diagnosed. However, only if ongoing studies solve the CV safety issue the T orchid may eventually 'bloom'.

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Key words: Heart; Testosterone; Hormones; Pathophysiology; Prognosis**.**

1. Introduction

Since it was first synthesised in 1935, testosterone (T) has been viewed as the mythical *Fountain of Youth*, promising rejuvenation, restoring sexual appetites, growing stronger muscles, and quicker thinking. T is endowed with direct effects on vascular and myocardial structure and function, as well as on risk factors for cardiovascular (CV) disease (D). Reduced T levels are linked to premature coronary artery disease (CAD), unfavourable effects on CVD risk factors, including type 2 diabetes (T2D) and metabolic syndrome (MS), and with increased risk of CV mortality. Although serum T concentrations decrease with age, several epidemiological studies showed that this association remains even following correction for age. The key symptoms suggesting T deficiency (TD) mostly involve the sexual life, with reduced libido and erectile dysfunction, but also include fatigue (due to anaemia and muscle loss), back pain (osteoporosis), weight gain, gynecomastia, and vasomotor symptoms. Testosterone replacement therapy (TT) is currently employed to treat men with hypogonadism, but it also has specific CV effects.

This review will dwell upon current evidence and controversies surrounding the role of T in the pathophysiology of CVD, and its potential use in the treatment of these disorders. Notably, we preferred the term biochemical TD (BTD) and syndromic TD (STD) to indicate low T states associated or not with clinical symptoms or signs, and TT rather than TRT or TST in accordance with the current thought of the leaders in the field.

2. The Hypothalamus-Pituitary-Testes Axis

The T release from the Leydig cells in the testis is highly regulated by a complex feedback loop (Figure 1). The gonadotropin-releasing hormone (GnRH), secreted from the hypothalamus, governs the hypothalamus-pituitary-testes (HPG) axis[1] by stimulating the anterior pituitary to produce the luteinizing hormone (LH)[1]. Then, LH binds to its receptors on Leydig cells and stimulates T synthesis[1].

The release of GnRH into the pituitary portal circulation, and LH from the adenohypophysis, is under the negative feedback regulation of T and oestradiol (E2) derived from T aromatisation. Circulating T is bound predominantly to sex hormone-binding globulin (SHBG) and albumin, and to a lesser extent to corticosteroid-binding globulin and orosomucoid[2]; free T is only 1-4%[2]. According to the free hormone hypothesis, unbound T is believed to be the biologically active form[2]. T is peripherally converted to oestradiol, *via* the action of the aromatase enzyme, and to dihydrotestosterone (DHT), *via* 5α-reductase [3]. T and DHT bind to the androgen receptor (AR) in several tissues, with DHT having a greater affinity than testosterone; however, DHT is considered clinically relevant only for tissues where the 5α-reductase is highly expressed, mainly the genitalia (during development) and the adult prostate, skin, and liver[4, 5]. Most human studies exploring the effect of androgens on CV function have focused only of the most abundant testosterone, and despite some evidence support a role for DHT levels in sexual symptoms, should not be routinely included in the primary diagnosis of hypogonadism[4, 5]. The activation of AR by T or DHT is inversely related to the number of polyQs, a common polymorphism of the receptor, responsible for an attenuation of signal transduction[6]. In experimental and pre-clinical studies, an additional non-canonical (non-genomic action) AR-independent action has been reported, to explain some of the ultrarapid effects of T[3]. Finally, an effect of the SHBG-androgen complex, through the megalin system has been recently postulated[3].

In summary, the complex of mechanisms underlying the synthesis, delivery, pre- and post-receptorial regulation of the androgenic pathway should be taken into account when reviewing the effect of T on the CV system.

3. Mechanism(s) of action of testosterone on the CV system from bench to bedside

A schematic representation of the T effects on various targets of the CV system is depicted in Table 1. For simplicity, the sets of evidence have been cauterized into four groups (i.e., effects on vascular structure, vascular function, myocardial structure, and myocardial function). In light of the interplay with the CV function, a subchapter on the effect of T on the immune system has also been included among putative mechanisms.

Vascular structure

T exerts a modulating action on apoptosis and proliferation of vascular smooth muscle cells (VSMC), through an AR-dependent regulation of growth arrest-specific gene 6 (GAS6) transactivation[7], a pro-survival molecule that, *via* a PI3K/Akt pathway, decreases VSMC apoptosis and reduces VSMC inorganic phosphate (Pi)-induced calcification[7] (Figure 2A). Conversely, T increases extrinsic apoptosis of VSMC *via* AR activation, mitochondrial-ROS generation, and procaspase-8 and -3 activation[8]. However, contrasting results on AR-induced VSMC calcification have been reported[9] (Figure 2A).

T is protecting against VSM senescence[10]. GAS6/Axl pathway plays a pivotal role in T-mediated improvement of angiotensin II (Ang II)-induced VSMC senescence and collagen overexpression[10]. The T-induced reduction of collagen synthesis can be attributed to a reduced expression and activity of matrix metalloproteinase-2 (MMP-2)[10] (Figure 2A). Indeed, T induces migration of VSMC *via* NADPH oxidase-derived reactive oxygen species (ROS) production and *via* a c-Src-dependent pathway by both genomic and non-genomic mechanisms[11]. Specifically, T induces the expression of NADPH subunits Nox1, Nox4, and p47phox protein[11] and activates NAPH oxidase rapidly through

increased phosphorylation of c-Src[11]. Furthermore, T has been demonstrated to induce VSMC proliferation, increasing the expression of the human prostate overexpressed protein 1 (PTOV1) gene[12] (Figure 2A).

In animal studies, T inhibits fatty streak formation[13]. The enhanced expression of vascular cell adhesion protein 1 (VCAM1), which promotes leukocytes attachment to endothelial surfaces, is a key step in the initial development of atheroma[14]. T downregulates the expression of VCAM1 induced by tumour necrosis factor (TNF) in human endothelial cells[15]. The T aromatisation could mediate this protective effect to oestradiol[15] (Figure 2B).

A relevant role in T actions on vascular structure, in particular in atherogenesis, could also be held by T immune-modulating effects. T has anti-inflammatory effects, suppressing serum pro-inflammatory cytokines expression, such as TNF- α and interleukin (IL)- 1β, and IL-6, and promoting anti- inflammatory cytokine IL-10 expression[16] and reduces the inflammatory response induced by lipopolysaccharide and TNFα in endothelial cells[17] (Figure 2B).

Vascular function

In the 50s several reports described a relieving effect of T injection on angina; forty years later, controlled studies demonstrated a T vasodilating action[18, 19]. The rapid- onset vasodilation induced by T suggests a non-genomic mechanism[18, 19]. Preclinical studies, confirmed by animal models, described an *endothelium-independent* mechanism is involved in T-induced coronary vasodilation[20]. More specifically, T was found to modulate the activity of potassium and calcium channels[20], and activate guanylate cyclase, leading to a cGMP increase and protein kinase G (PKG) activation[21]. In turn, PKG stimulates the opening of large-conductance calcium-activated potassium ion channels and voltage-sensitive potassium ion channels[22] (Figure 2A). Intracellular calcium handling was thus considered responsible for the rapid, endothelial-independent, myorelaxation induced by T. Additionally, an *endothelium-dependent* mechanism involving protein kinase A

activation, leading to hyperpolarization and activation of small and large-conductance calcium-activated potassium ion channels, has been described[23] (Figure 2B).

It is conceivable that the long-term genomic effect of T on L-type calcium ion channels is a compensatory mechanism for the non-genomic T-induced inactivation of L-type calcium ion channel[24] (Figure 2C). Long-term genomic effects mediated by the AR are involved in endothelium-dependent action of T on vascular cells[25]. T induces vasodilation *via* transient-receptor-potential-cation channel subfamily V member 4 (TRPV4) and large-conductance calcium-activated potassium ion channels activation through an increase in hydrogen sulphide production[26] (Figure 2B). Furthermore, T upregulates endothelial nitric oxide synthase (eNOS), leading to increased nitric oxide (NO) production and increases eNOS activity *via* rapid AR-dependent activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/eNOS pathway[27] (Figure 2B). Recently, T and DHT have been shown to affect phosphatidylinositol-3-kinase (PI3-K)/Akt signalling (Figure 2B). This pathway has been found to be important in regulating proliferation, adhesiveness and reparative potential of human endothelial progenitor cells (EPCs)[28, 29]. In mice, EPCs pre-treated with androgens, augmented blood flow recovery and angiogenesis. In men with CAD, circulating testosterone was positively associated with the number of circulating EPCs[30] and the extent of coronary collateralization[29]. The net balance of the above-described mechanisms suggests T favour vasorelaxation through calcium-handling in smooth muscle cells and promotes neovascularization and collateralization in coronary arteries by enhancing EPCs activity.

Myocardial structure

T can induce cardiac hypertrophy *via* several molecular pathways, including the mammalian target of rapamycin complex 1 (mTORC1)/ S6 kinase 1 (S6K1) axis, glycogen synthase kinase-3β (GSK-3β)/nuclear factor of activated T-cells (NFAT) signalling, Ca2+/calmodulin-dependent protein kinase II (CaMKII), and myocyte-enhancer factor 2 (MEF2) activation[31] (Figure 2C).

In animal studies, maladaptive remodelling induced by the renin-angiotensin-aldosterone system (RAAS) activation has been associated with T administration[32]. However, in mouse models, angiotensin II-induced cardiac remodelling is counteracted by T *via* AR pathway through activation of the Akt pathway and up-regulation of cardiac mitochondria transcription factor A (Tfam)[33] (Figure 2C).

In heart failure (HF) rats, T treatment diminishes the imbalance between IL-10 and TNF- α, suppressing ventricular remodelling and improving cardiac function[34]. In addition, there are other mechanisms involved in T-induced suppressed ventricular remodelling, leading to an improvement in left ventricular (LV) function by reducing atrial natriuretic peptide, brain natriuretic peptide, MMP-2, sarcoendoplasmic reticulum Ca2+-ATPase 2a, and increased expression of glycogen synthase kinase 3β and tissue inhibitor of MMP-2[35]. T treatment also significantly reduces caspase-3 expression leading to reduced cardiomyocyte apoptosis[35] (Figure 2C).

Some evidence shows that T has a beneficial effect on myocyte survival. Indeed, T is important for both immediate and delayed cardioprotection of ischemic preconditioning[36], *via* increased synthesis of heat shock protein 70 (HSP-70)[36]. Finally, T induces ATP-sensitive potassium channels in the myocytes mitochondrial inner membrane conferring cytoprotection[37] (Figure 2C).

The T effects on cardiac structure have been reported detrimental in case of experimental MI or acute pressure-overload triggering pathological hypertrophy[38]. In mice, testosterone deficiency seems to attenuate adverse remodelling during pressure overload or post-MI. This evidence was based on studies showing that inhibiting the 5-alpha reductase, the enzyme involved in conversion of testosterone into the more potent DHT, help counteracting interstitial fibrosis, by reducing cardiomyocyte hypertrophy and increased capillary density induced by ischaemia[38], possibly attenuating Akt signalling myocytes.[39] However, finasteride and dutasteride are potent in lowering DHT levels in the prostate and in the skin, where the enzyme is highly expressed, but circulating total testosterone increases in treated subjects, suggesting that an anti-androgen effect on remodelling requires more compelling evidence.

Myocardial function

T exerts an effect on cardiac contractility and relaxation. In cardiomyocytes isolated from rats, T reverses castration induced cardiomyocyte hypocontractility[40]. In animal cardiac myocytes, T acts as a positive inotropic agent *via* AR[41]. In orchiectomised animals treated with T, the increased contractile velocity is related to an increased expression of faster myosin heavy chain α in place of the slower myosin heavy chain β[41]. Enhanced functional expression of L-type calcium-channel and Na/Ca exchanger[42] are also related to the increased contractile velocity. Furthermore, positive inotropic response and myocardial relaxation to stimulation of α1-adrenergic receptor and β1-adrenergic receptor are elicited by T physiological levels through AR-dependent mechanism[43]. Other mechanisms by which T can determine increased cardiac contractility and prompter relaxation include enhanced calcium release *via* the ryanodine receptor and increased calcium clearance by sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and sarcolemmal Na/Ca exchanger[43] (Figure 2C).

In animal models, T administration shortens the action potential duration, thus facilitating cardiomyocyte repolarization. In detail, T increases the slowly activating delayed rectifier potassium currents (IKs) and inhibits the inward depolarizing L-type calcium current (ICaL) *via* a non-transcriptional AR-mediated pathway involving c-Src, PI3K, Akt, and NOS3[44]. AR and PI3K are also involved in stimulating the rapidly activating delayed rectifier potassium current (IKr) induced by T [45]. Moreover, T increases the ultra- rapid potassium current (IKur)[46]. However, contrary to acute administration, chronic T administration increases ICaL current through a genomic pathway[47]. (Figure 2C).

In rat cardiomyocytes, long-term T administration increases, whereas acute administration decreases, T-type calcium currents, contributing to spontaneous pacemaker activity[48]. These effects appear to be mediated respectively by a genomic and a nongenomic mechanism, respectively[48]. Besides pacemaker activity, T-type calcium currents are also involved in the maintenance of vascular tone, modulation of cell growth, regulation of atrial natriuretic peptide secretion, and CV remodelling[48].

Studies on androgen deprivation reveal no major effect on systolic function (e.g., ejection fraction), but a mild impairment in isovolumic relaxation time, indicative of diastolic dysfunction[49]. In cardiac fibroblasts, testosterone has been reported affecting inositol trisphosphate (IP3) receptor, Ca2+/calmodulin-dependent protein kinase II (CaMKII) expression and ERK signalling pathways leading to an inhibition of Angiotensin II-stimulated collagen synthesis[50, 51]. When testosterone is combined with resistance exercise was found to increase capillary density, improving collagen deposition in the interstitial space.[52] The latter observations are consistent with impaired contraction dynamics in hypogonadism, due to altered myocardial stiffness involving the extracellular matrix[53].

Immune function and atherogenesis

Several immunology studies demonstrated that T exerts an anti-inflammatory effects that has been claimed to account for the lower susceptibilities to antibody-mediated autoimmunity, allergy, and anaphylaxis and mitigated response to immunization and infection of males vs. females[54]. T is crucial, starting from pre-natal life, to re-programming mast-cell release of histamine[55] and, in adulthood, regulates monocytes and neutrophil maturation to control immune response[56].

The so called anti-inflammatory action of T, affecting the innate as well as the adaptive immune system, at developmental and functional levels has been advocated to prevent vascular damage when this is sustained by uncontrolled inflammation[57]. Androgens have been claimed to suppress proinflammatory leukotrienes formation (for which biosynthesis inhibitors are under clinical investigation as CVD treatments)[58].

While immunology studies all converge toward a T anti-inflammatory role, the trials in hypogonadal subjects reveal conflicting results, with some showing T reducing TNFα and IL-1β, while increasing IL-10[16, 59], and others showing no differences in IL-6 or C reactive proteins[60, 61]. Similarly, the trials investigating the effects of T on the atherosclerotic plaque failed to demonstrate any benefit of T over placebo[62]. With regard to T and lipid profile, results are inconsistent[61, 63].

In summary, experimental studies suggest T may exert an immune-modulatory effect, mitigating excessive responsiveness and autoimmunity; such an effect in the short term does not seems to affect large vessel disease. However, as recently shown in COVID-19, in the presence of non-gonadal illnesses, men with higher number of poliQ (reduced androgen activity) seems at higher risk to develop hyperinflammation[64], suggesting that T level and action should always be evaluated in the context of the very frequent AR variants (poliQ or CAG repeats). Conversely, shorter polyQ in the AR have been associated with an increased risk of prostate cancer, raising an important warning[64]. How AR polymorphism may explain some of the controversies on the association between T and CVD remains unaddressed and such uncertainty may limit the generalizability of clinical findings thereafter reported.

4. Testosterone and CV risk: the never-ending story

The issues of whether BTD increases risk of CVD and whether TT affects CV outcomes are both still pending despite decades of intense research[65, 66]. There is a body of evidence linking low T circulating levels to CV events, CV mortality, and all-cause mortality in middle-aged and older men[67-80], although contradictory findings are equally reported[81-87]. Table 2 summarises the key studies investigating the association of T levels with major adverse CV events and mortality[67-87]. Many explanations have been proposed to reconcile such apparently opposite findings. First of all, one of the dogmas of epidemiology research states that association does not imply causation, being not possible to control, in epidemiological models, all the unknown possible confounders. Indeed, population studies cannot exclude reverse causality and some studies suggested that association between T and CV outcomes might not be causal but resulting from reverse causation or residual confounding[84]. More in general, the prognostic significance *per se* of T decline in the frame of chronic diseases has been questioned since it is well known that sex steroid levels decrease in both acute and subacute illnesses, and therefore BTD might be the consequence of the morbidity rather than the

cause. In other words, T reduction should be viewed as an adaptive response, being T a biomarker of good health. Finally, there are several methodological limitations that make comparisons across studies very difficult. Indeed, most studies did not consider diurnal T variations, since only reported single T measures. In addition, the methodology employed to measure circulating T has been questioned, since most studies employed immunoassays which are not as precise as gold-standard techniques particularly at low concentrations. Further, different cut-offs to define BTD have been used. Finally, one must consider that population studies enrolled patients with BTD and not STD, whose prevalence is much lower.

The TT controversy

While the use of TT in congenital hypogonadism or young is well described, TT in age-related TD has been a matter of controversy. The prevalence of BTD in the United States is approximately 20% in men older than 60, reaching 50% in those older than 80 years, while STD (defined as at least 3 sexual symptoms with a total T level <11.1 nmol/L), which represents the current indication for TT, is much less frequent[88]. As a prototype, in the European Male Aging Study (EMAS) population STD was 5.1% in men 70–79 years old[88]. Despite this, direct-to-patient marketing by drug companies led to a dramatic increase of T product sales, particularly in US and Canada. From 2000 through 2011 T sales increased 12-fold globally, rising from $150 million in 2000 to $1.8 billion in 2011, despite the percentage of STD was substantially constant[89]. In fact, up to a third of men who are placed on TT do not meet the criteria to be diagnosed as STD. Such disheartening statistics paralleled by safety concerns raised by RCT and retrospective trials led to an FDA warning concerning potential increased CVD risk of TT. Therefore, despite several encouraging findings of T use in age-related TD and as adjunctive therapy in a broad variety of CVD briefly reviewed in the following paragraphs, any further indication to TT except STD waits for CVD safety trials.

There are numerous studies showing CV benefits of TT. Their pathophysiological background is provided, as outlined before, by the demonstration that TT reduces fat mass, increases lean mass,

improves glycaemic controls and exercise capacity, and reduces insulin resistance and waist circumference (Table 1). On the other hand, there are several studies dwelling upon the main alleged mechanisms for the association between TT and CVD. The first involves the dose-dependent stimulatory effect of T on erythropoiesis, which is more pronounced in older men and may cause aggravation of vascular disease due to polycythemia[66]. The second is the increase in aromatization of T to oestradiol in older men, which may lead to dyslipidaemia and promote atherosclerosis[66]. Finally, excess T is associated with gynecomastia, PSA elevation, and worsening sleep apnea[66]. Considering these important controversies, adequately powered trials are awaited to provide a definitive answer to the TT purported beneficial actions and to the CV safety issues [66].

Many retrospective analyses evaluated TT and risk of CV events. Prior to summarizing their results, that certainly provide interesting information on CV effects of TT, it is important to acknowledge their major limitations. The principal pitfall is obviously inherent to the retrospective nature of the study design, that hampers definitive conclusions regarding CV safety. Another major limitation is the lack of information regarding the adequacy of TT, since most studies lack information about T levels during treatment. To further complicate the issue, it has been suggested that also the type of T formulation might influence CV outcomes, since those patients receiving intramuscular injections attain supraphysiological T levels, with consequent higher CV risk than those receiving transdermal T gel

As a result, there is conflicting evidence in literature, ranging from increased to reduced risk, to lack of association thereof. With regard to studies showing increased risk, two main studies received major media attention and contributed to the Food and Drug Administration (FDA) warning about potential increase of CVD events in T users[89,90]; Vigen R et al. performed an observational retrospective cohort study of men with low testosterone levels (BTD) who underwent coronary angiography in the Veterans Affairs (VA) system, concluding that the use of T therapy was associated with increased risk of adverse cardiovascular outcomes [89]. A few months later, Finkle WD and co-workers published the results of a cohort study about the risk of acute non-fatal MI following an

initial TT prescription in a large health-care database, concluding that the risk of MI following initiation of TT prescription was substantially increased[90]. These two studies, despite several criticisms[91], were not disproved since their results strongly pointed to an increase in CV risk for patients undergoing TT. Etminan M et al.[92] performed a case- control study within a cohort of the IMS LifeLink Health Plan Claims Database, reporting a statistically significant association between first- time TT exposure and MI, although the absolute risk was low. Further, Martinez C and co-workers[93] showed that starting T treatment was associated with a transient (limited to the first phase of treatment) increased the risk of venous thromboembolism in 370 general practices in UK primary care, with some differences with regard to the presence or not of hypogonadism.

Other studies reported no association between TT and CV outcomes. Specifically, a case-control study of 30,572 men 40 years and older who were enrolled in one of the nation's largest commercial insurance programs[94], showed that TT was not associated with an increased risk of VTE. A large cohort analysis found no significant association between exogenous T therapy and incidents of idiopathic or overall venous thrombotic events in hypogonadal men[95]. The Registry of Hypogonadism in Men (RHYME) was designed as a multi-national, longitudinal disease registry of men diagnosed with hypogonadism at 25 clinical sites in six European countries[96]. TT use did not predict new-onset CV events. Another retrospective cohort study of the Veteran Affairs database[97] did not find a significant association between TT and risk of DVT/PE in adult men with TD at low to moderate baseline risk of DVT/PE.

Contrary to negative or neutral results, at least other 10 retrospective analyses reported beneficial effects of TT on a broad variety of CV outcomes, ranging from stroke, MI, mortality, all-cause death, major adverse cardiovascular events (MACE), and atrial fibrillation [78, 94, 98-105]. Since all evidence came from observational studies, which only imply correlation and not causation, more robust evidence of putative beneficial CV effects was expected from randomized controlled trials[106-108]. Unfortunately, none of them was adequately powered to answer definitively to the controversy surrounding TT and CV system. Considerable media attention received the TOM trial, that was

stopped prematurely because of a significantly higher rate of adverse CV in the T group[106]. In this population of older men with limitations in mobility and BTD, TT was associated with an increased risk of CV adverse events. Subjects were randomly assigned to receive placebo or T gel, to be applied daily for 6 months[106]. The T's Effects on Atherosclerosis Progression in Aging Men (TEAAM) trial explored the association between TT and progression of atherosclerosis in elderly men with BTD or low-normal T levels, employing a placebo-controlled, double-blind, parallel-group randomized trial design[107]. No difference in the progression of subclinical atherosclerosis between the men assigned to receive TT or placebo was found in terms of intima media thickness of the carotid artery and coronary artery calcium after 3 years. Interestingly, T did improve overall sexual function or health-related quality of life[107]. The T Trials (TTrials) included 7 placebo-controlled, double-blind trials in 788 men with a mean age of 72 years to determine the efficacy of increasing the T levels of older men with STD[108]. In general TT was not associated with more CV or prostate adverse events than placebo. TT increased sexual activity, sexual desire, and erectile function, the distance walked, haemoglobin, volumetric bone mineral density, and the estimated strength of the spine and hip[108].

Several meta-analyses were subsequently published pooling data from controlled trials, mostly reporting CV safety of TT, although no definitive conclusion could be drawn because of inhomogeneity of patient population, lack of adequate statistical power, and many low-medium quality studies involved[66].

On this basis, in 2018 the TRAVERSE trial was implemented, aimed at evaluating the effect of TT on MACE and efficacy measures in hypogonadal men (NCT03518034). The adequate statistical power of the trial enrolling 6,000 men will hopefully provide next year (2022) a definitive answer to the outstanding issue of CV safety of TT.

Side effects

International scientific societies guidelines (i.e., the Endocrine Society from United States of America, the European Society of Endocrinology, and the European Academy of Andrology) outline that trials in young men with STD report a low frequency of serious adverse effects, most commonly including erythrocytosis, acne, oiliness of skin, and breast tenderness[109, 110]. T administration increases haemoglobin and haematocrit (erythrocytosis – the most frequent adverse effect), particularly in older man when compared to younger. Therefore, physicians should monitor these values and, if altered, cease therapy until haematocrit has returned in the normal range, restarting TT at a lower dosage[109]. Regarding cardiovascular effects, international guidelines state that there is no conclusive evidence that TT is associated with increased CV risk[109, 110]. Despite the FDA revised an alert, the European Medicines Agency (EMA) concluded that there is no consistent evidence of an increased risk of coronary heart diseases associated with TT. TT may induce secondary hypertension[111], fluid retention and oedema, and such untoward effects need to be monitored, particularly in HF patients. However, RCT of TT in HF did not show an increased risk of pulmonary oedema or other adverse effects. Venous thromboembolism (VTE) has been also associated with TT. Specifically, it has been hypothesised an increase in the risk of VTE within the first 6 months of TT in the presence of thrombophilia[93]; despite case control and epidemiological studies having not confirmed such findings[109], the FDA has required manufactures to include a warning about VTE risk. Finally, the relationship between T administration and risk of prostate cancer remains poorly understood, with no strong evidence available in literature[109]. Despite some inconsistencies, meta-analyses of prospective epidemiologic studies found no significant association between T levels and prostate cancer[109]. Further, TT increases the risk of detecting subclinical, otherwise indolent, prostate cancer because of closer surveillance and T-induced PSA levels. To date there is no compelling evidence that TT induces a “de-novo” prostate cancer. As a precaution, considering the role of T in the biology of prostate cancer, guidelines recommend avoiding T supplementation in men with a

diagnosis of prostate cancer and require assessing for the prostate cancer risk before the initiation of a T treatment, discussing with the patients the risk-benefit ratio[109, 110].

T excess

Although the present review is focused on TD and TT, it is worthy to mention possible adverse cardiovascular effects (e.g., myocardial infarction, cardiac failure, cardiac tamponade, strokes, thrombosis) promoted by T excess, when supraphysiological dosage are used (i.e., sport doping and transgender males). Four main mechanisms have been identified as potential players: accelerated atherogenesis, thrombosis, vasospasm, direct cardiotoxicity[112] .

Sport doping represents a human model of androgen excess. A recent cross-sectional study involving 86 illicit anabolic-androgenic steroids (AAS) users and 54 non-using male weightlifters showed a statistically significant reduction in ejection fraction and in diastolic function, with a higher coronary artery plaque volume, closely associated with the lifetime use[113]. Another study showed, in 37 AAS users, higher aortic stiffness and mean systolic blood pressure than controls, with a reduction of plasma MR-pro ANP associated with increased levels of aldosterone and noradrenaline[114]. However, as recently reviewed by Handelsman[115], available evidence is mainly anecdotal, and the lack of population-based studies, makes unclear the pathophysiology of such cardiovascular effects in particular their direct relation to androgen abuse. [115, 116].

Concerning gender affirming hormone therapy, a recent metanalysis showed that androgen therapy increases LDL-C and TG levels and decreases HDL-C level[117]. However, these changes have not been associated with higher morbidity or mortality[117, 118] and the effect of these findings on major cardiovascular events (e.g., myocardial infarction and stroke) during long-term therapy remains elusive[117, 118].

5. Effect of TT in specific cardiovascular diseases

Heart Failure: not ready for prime time

Worldwide, the burden of HF increased to an estimated 23 million people, thus becoming a major public health concern with substantial morbidity and mortality. Despite recent therapeutic breakthroughs, HF survival is still poor and lags behind other serious conditions, and the quest for novel approaches is highly needed. In this regard, the concept has emerged that HF progression is not only secondary to the hyperactivation of maladaptive pathways, but also to a reduction of the anabolic drive that leads to anabolic/catabolic imbalance. In particular, circulating levels of IGF-1, DHEA-S, and total T are strongly and independently related to both clinical status and exercise performance, and, more importantly, to survival[119].

Specifically, TD has been described in approximately 1/3 of male with HF, ranging from 26 to 37% [119-126]. The larger prospective study on CHF and hormone abnormalities, the Trattamento Ormonale nello Scompenso CArdiaco – Hormone replacement treatment in HF (T.O.S.CA.) Registry, reported a prevalence of BTD of 42%[127, 128]. The question arises as to whether TD is a mere biochemical marker of the underlying disease or plays a relevant role in CHF progression. Previous studies have shown a strong relation between BTD and EF, haemodynamic, and exercise capacity [129-131]. Notably, it has been shown that BTD is an independent marker of mortality in men with CHF. In addition to the landmark study of Jankowska and co-workers that explored the impact on mortality of the main anabolic systems, Santos et al. also showed the relationship between BTD and hospital readmission for HF and mortality[119, 132]. However, other studies failed to demonstrate an independent prognostic role of BTD in men with CHF[133, 134].

In addition to the occurrence of BTD in HF and its role in disease progression, several pathophysiological considerations provided a solid background to implement trials of TT in HF[135]. First, the restoration of the anabolic drive *per se* could balance the increase of the catabolic forces taking place in HF. Second, the well know T mediated vasodilating properties may be beneficial at

various levels, by decreasing both afterload and preload through reduced systemic vascular resistance and pulmonary vessel pressure, respectively, and increasing blood flow to ischemic areas through coronary vasodilation. Third, T may enhance carotid sinus baroreceptor sensitivity. Fourth, T may partially correct chronic anaemia of CHF. Fifth, T action on circulating levels of TNF-alfa may reduce the chronic low inflammation state of CHF. Sixth, T improves insulin sensitivity and reduces fasting glucose and body fat percentage, thus improving the disturbed metabolic profile of CHF. Overall, the multifaceted actions of TT in CHF appears to act mainly on peripheral rather than central mechanisms, in particular on skeletal muscle myopathy, muscle size, strength and oxidative capacity[135]. Abnormalities in skeletal muscle structure, function, and cell viability are linked to each other and contribute to the abnormal exercise response, enhanced fatigability and progressive symptom complex that can be reversed by TT. The multiple putative beneficial effects of TT in CHF are summarized in Figure 3. However, even if none of the trials for TT showed safety concerns, with no major events reported and no-significant changes in PSA assays, it is important to specify that there are also possible negative effects, mostly related to a possible salt-water retention, and the choice of a possible TT needs to be evaluated on a case-by-case basis and careful monitored.

The first uncontrolled open-label trial in HF demonstrated that oxymetholone reduced LV diameter and mass as well as BNP circulating levels [136]. Seminal human studies of TT in CHF came from the Cardiology Department of Sheffield, UK. The first, performed on 12 stable male patients[137], monitoring central hemodynamic with a pulmonary flotation catheter, showed reduced systemic vascular resistance followed by increased cardiac output after the administration of a single T dose on two consecutive days[137]. Notably, effects were more evident in patients with a lower baseline circulating T level. Malkin et al. reported T effects on cardiac electrophysiology with T reducing QT dispersion in HF[138]. The following year, a pilot double-blind, placebo-controlled trial, enrolling 20 male CHF patients[139] showed a significant increase in the incremental shuttle walk test, and in quality-of-life score indexes in TT group, while skeletal muscle bulk did not change significantly. A larger randomized, double-blind, placebo control study, involving 76 CHF patients of TT at physiological

doses over a 12-months follow-up period[138], showed that symptoms improved by at least one functional class on T in the active group. TT was overall safe, and no significant changes were found in both groups as to handgrip strength, skeletal muscle bulk by cross-sectional computed tomography, or in tumour necrosis factor levels. Subsequent studies focused on TT in elderly and women[140, 141], showing that TT improved exercise capacity, muscle strength, insulin, and baroreflex sensitivity in the elderly[140], and improved functional capacity, insulin resistance, and muscle strength in 36 women with advanced stable CHF[141]. A subsequent revaluation of the two trials by Schwartz et al. shed new lights on the effects of T on QT interval [142]. Specifically, T shortened Q- T and Q- Tc intervals without heart rate changes in both sexes, supporting a direct effect of T to shorten Q- T intervals in the absence of HR changes or hypogonadal status. Stout and colleagues evaluated the feasibility and efficacy of TT on top of a program of cardiac rehabilitation [143]. Forty-one male patients with CHF and low T levels were randomly allocated to T or placebo group in an exercise program lasting 12 weeks. Exercise improved peak oxygen uptake, Beck Depression Inventory, leg strength, and quality of life indexes in the T-treated group, but not in the placebo group. Dos Santos and colleagues further explored this topic selecting 39 male subjects with HF and BTD[144]. Patients were randomised to three groups (i.e., exercise training – ET alone, intramuscular T alone, and training plus T). Muscle sympathetic nerve activity was reduced in patients who performed ET; on the other hand, no differences were observed in patients receiving T. No differences regarding forearm blood flow were detected. Regarding lean mass, ET patients showed an increase, while T patients a decrease. Finally, biopsies showed an increase in the response of cross-sectional area of type I and type II fibres in patients receiving T and performing ET when compared to the patients only receiving T. Taken together, the authors showed a greater effect on muscle sympathetic nerve activity, muscle wasting, and functional capacity when ET and T were combined. Mirdamadi et al randomised fifty male patients suffering from CHF to receive either an intramuscular long-acting T injection once every four weeks or saline for 12 weeks [145]. While echocardiographic parameters did not differ between the study groups, patients receiving T showed a significant increased trend in 6-walk mean distance.

Finally, a prospective, randomized, double-blind, placebo-controlled, and parallel-group trial comparing TT with placebo in CHF males[146] demonstrated no significant changes of clinical status, functional capacity, EF, and NT-proBNP levels over a 12-month period in the TT group.

Three meta-analyses were performed dwelling upon TT in CHF. Toma and colleagues included four trials with 198 patients[147]. As a result, a significant improvement in exercise capacity was observed in TT patients when compared to placebo, with a mean increase in the 6-minute walk test (+54.0 m), incremental shuttle walk test (+46.7 m), and peak oxygen consumption (2.70 mL/kg/min). No significant adverse CV events were reported. Notably, this improvement was superior to the effect of other pharmacological CHF treatments (e.g., ACE inhibitors and beta blockers). More recently, Wang at al analysed data from eight eligible trials[148] and concluded that TT improved significantly exercise capacity, muscle strength, and electrocardiogram indicators; however, no significant changes in ejection fraction, systolic or diastolic blood pressure, N-terminal pro-brain natriuretic peptide or inflammatory biomarkers were observed. TT appeared to be safe, with any obvious adverse reactions. However, a recent updated meta-analysis reported opposing results[149], concluding that TT, when maintained at a physiological level, is not related to an improvement in cardiac function, exercise capacity, quality of life, or prognosis in HF[149]. Specifically, eight studies for a total of 170 patients enrolled in the T group and 162 in the placebo group were included. Interestingly, T supplementation increased systolic blood pressure in CHF patients while no effects were reported with regard to diastolic BP or heart rate.

Gap in evidence and future directions

TT in CHF will remain an interesting working hypothesis until a robust phase III with a large population of both sexes will be implemented. Data collected so far point to a beneficial effect of TT in CHF, particularly in subjects with low basal T. Insofar as no significant changes were detected in cardiac architecture and function even in positive trials, T actions appear mainly peripheral, probably mediated by its anabolic effects on skeletal muscle and peripheral vasodilation[135]. Notably, none of these trials, although of limited duration, raised significant safety concerns. Moreover, it maybe conceivable that if a replacement rather than supraphysiological androgen replacement therapy is implemented, side effects might be negligible. The most relevant issue relates to the ideal recipient of TT. Although most studies were performed regardless of basal T levels, T therapy is more effective in CHF patients with low T[135]. In this concern, multiple hormone deficiency syndrome might be the target of future investigations[127, 128] as recently demonstrated in a pilot trial of combined GH and T replacement treatment[150].

In the future, investigations should analytically assess different dosages and administration routes, enrol wider populations – including female patients too- and try to explore other correlates; finally, future studies should be aimed to collect clinical outcome and prognosis data. This novel information hopeful may help in providing novel insights into the precise mechanisms and possible clinical benefits of TT in HF.

<u>Ischemic Heart Disease: The T conundrum</u>

The T conundrum in myocardial ischemia is still unsolved: on the one hand, male gender is one of the strongest independent risk factors for coronary artery disease (CAD)- leading to the idea that T may exert detrimental action on cardiac ischemia; on the other hand, CAD increases with age paralleled by a marked fall in circulating T. A great deal of animal and epidemiological studies has led to the assumption that it is low T rather than male sex to be associated with CAD, and that T might beneficially modulate the atherosclerotic process[65].

The concept of T cardioprotection in ischemic heart disease (IHD) dates back to the 40's when Lesser reported beneficial actions of T propionate on clinical status first in a preliminary small trial of 14 patients with angina pectoris and subsequently in a larger study of 100 patients, 91 of them showing benefits[151, 152]. Since then, many studies have been published demonstrating an improvement of anginal indexes including ST segment depression, angina episodes, and total ischemic burden. Specifically, in the first RCT on the topic, Jaffe et al.[153] showed a large post-exercise ST reduction at 4 and 8 weeks of T therapy in 50 men. Wu et al.[154] included 62 elderly men in a 2.5 month trial demonstrating significant differences between T- and placebo-treated groups. In the former, angina pectoris was relieved, and sings of myocardial ischemia in ECG and 24-hour Holter recordings were improved and paralleled by serum T level increase. English et al.[155] in another RCT showed that low-dose TT in men with chronic stable angina reduced exercise-induced myocardial ischemia and improved QOL indexes. Malkin et al.[156] demonstrated that TT in hypogonadal men delays time to ischemia, improves mood, and is associated with potentially beneficial reductions of total cholesterol and serum TNF-alfa in a single blind placebo controlled crossover study in 10 men with ischemic heart disease and hypogonadism. Mathur and colleagues[157], in a small RCT showed that T increased time to ischemia and haemoglobin, and reduced body mass index and triglycerides. No side effects were reported nor changes in mood and symptom scores. In a larger RCT, Conroldi et al[158] randomised 87 elderly diabetic male subjects with proven CAD to a 12-weeks treatment with either T or placebo. Compared to placebo, TT significantly reduced the number of anginal attacks/weeks by 34%, silent ischemic episodes by 26% ($p < 0.05$), and the total ischemic burden by 21% on ambulatory ECG monitoring. After 12 weeks total cholesterol, plasma triglycerides, and HOMA index were significantly reduced in the T group as compared to placebo group.

The idea of the T cardioprotection was reinforced by parallel studies investigating the acute effects of TT on cardiac ischemia. Acute or short-term T administration improved exercise-induced ischemia in two independent studies[18, 19]. This finding may be secondary to a rapid and direct vasodilatory action on the coronary arteries. Contrasting data reported neither a beneficial nor a

deleterious effect on the onset and magnitude of stress-induced myocardial ischemia in men with stable CAD[159]. Effects of longer-term T treatment on myocardial perfusion and vascular function were investigated in 21 men with CHD and low T[160]. Although no difference was found in global myocardial perfusion after T, myocardium supplied by unobstructed coronary arteries showed increased perfusion. However, the results of the Cardiovascular Trial -implemented within the frame of the TTrials, a coordinated set of RCT trials aimed at evaluating the efficacy of T in older men with syndromic TD- dampened enthusiasm surrounding T use in CVD, since it reported an increase of noncalcified plaque volume in the active treatment group, measured as surrogate outcome, after 1 year of TT[62].

The main putative mechanisms by which T may improve myocardial ischemia include a) augmented coronary blood flow secondary to direct vasodilatory actions; b) overall anti-atherogenic action, supported by numerous animal studies, whereby castration accelerates aortic plaque build-up in models of atherosclerosis [13, 161, 162]. TT in these animals significantly diminished plaque formation, indicating a direct role for T in the aetiology of atherogenesis; c) enhanced myocardial function; and d) increased haemoglobin concentrations.

Taken together, although initial results appeared intriguing, TT use in IHD entered a dead end, considering that, independent of the study limitations of the Cardiovascular Trial, any decrease in the coronary artery lumen should be viewed as deleterious. Thus, until novel evidence will be available, TT was discouraged in patients with IHD.

TT: a novel drug to prevent diabetes?

A bi-directional link exists between DM and CVD; on the one hand, CVD are the major T2D complications; on the other hand, T2D is the strongest CV risk. In this context, a robust association subsists between BTD and T2D: a) BTD is associated with increased risk of incident diabetes[163] and an association between BTD and insulin resistance, metabolic syndrome, and T2DM has been described[164]. Intriguingly, in a natural human model of androgen deprivation (i.e. Klinefelter Syndrome), an impaired metabolic risk profile has been demonstrated, characterised by an increased prevalence of MS, T2D, and CVD risk[165]; b) men with DM and prediabetes often have BTD[166]; c) recent mendelian randomization studies suggest that higher T concentrations are casually related to lower T2D risk[167]; and d) T improves glycaemic control in T2D; indeed, interventional studies show an improvement in insulin resistance and glycaemic control in men with T2DM taking TT[168-170]. In the European TIMES2 study, T gel reduced measures of insulin resistance by up to 16% after one year in men with MS with or without T2DM[168]. The BLAST study reported a decrease in HbA1c levels in diabetic men assigned to T undecanoate for 24 weeks, greater in men with poorly controlled T2DM [171]. Finally, in a registry study aimed at evaluating whether T in men with hypogonadism and prediabetes prevents progression to T2D, long-term TT completely prevented prediabetes progression to T2D in men with hypogonadism and improved glycemia, lipids, and AMS score[172]. Mortality as well as incidence of nonfatal MI were both lower in the T-group compared to the untreated group[172].

A landmark study dwelling upon this topic was published a few weeks ago[173]. The T4DM study, a randomised placebo-controlled, 2-year trial of intramuscular T in 1007 men overweight or obese, with modestly low or low-normal T concentrations with prediabetes or recently diagnosed T2D, reported a 41% reduction in the proportion of participants with T2D after 2 years beyond the effects of a lifestyle program. Moreover, body composition improved, as well as sexual function, although modestly.

Notwithstanding the encouraging results, considerable gap in evidence prevents T use in clinical practice and need to be accounted for, including the lack of long-term surveillance and safety

issues. Despite no significant differences among cardiovascular side effects, the T4DM study reported a high frequency of haematocrit increase as well as higher PSA and serious prespecified adverse events in the T group compared with placebo[173]. However, before to indicate a TT as a novel drug to prevent T2D, it should be tested in an adequately powered CV outcome trial; also in this context, results from the TRAVERSE study will hopefully provide further information on this important issue.

Strength and limitations

Reviewing current literature about T and CVD leaves several controversies still pending, mostly because of the weaknesses of the study design of the majority of clinical studies available (e.g., observational studies, which do not prove causation given their intrinsic nature) and the lack of robust trials (e.g., randomised double blind placebo-controlled studies) enough powered to provide definitive answers. Further, considering the impressive wideness of the field, our review could not cover all the aspects of the intricate relationship between T and CV.

On the other hand, the major strengths include an up-to-date and deep review of most published studies in the field, and the coverage of several hot topics, with a particular attention to translational data; furthermore, the current review provides readers with a snapshot about the potential use of T replacement treatment in specific CV diseases. Hopefully, it may be viewed as a thought stimulating article in a field that has been widely explored in the past originating significant controversies.

Conclusions

Reviewing the rich literature dwelling upon TT and CV system leaves a feeling of incompleteness and unexpressed potential, like an orchid that never blooms. One the one hand, the exploitation of T properties might in theory portend to widespread use of a TT in a broad CV disorders; on the other hand, safety issues strongly limit T utilisation in clinical practice.

Conflict of Interest: none declared

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Table 1. Effects of testosterone treatment on cardiovascular risk factors and target organs and possible CV side effects.

CAD: coronary artery disease; CHF: chronic heart failure; T2D: type 2 diabetes mellitus; LVEDP: left ventricular end-diastolic pressure; LVEF: left ventricular ejection fraction; QTc: QT interval corrected for heart rate; STAT3:cardioprotective signal transducer and activator of transcription; cIMT: carotid intima media thickness, SVR: systemic vascular resistance; BRS: baroreceptor cardiac reflex sensitivity; LV: left ventricular; NYHA: New York Heart Association; 6MWT: six minute walking test; HDL‐C: high‐density lipoprotein‐cholesterol; HbA1c: glycated hemoglobin; TNF‐α: tumor necrosis factor‐α; IL‐10: interleukin‐10; tPA: tissue plasminogen activator; PAI‐1: plasminogen activator inhibitor‐1

Target organs	Variables	Diseases	T Effects
Heart	Myocardial perfusion	CAD	Increase in perfusion of coronary territory[157]
	Q-T interval	CAD, CHF	Decrease in QT and QTc duration[143] Decrease in Qt dispersion[139]
	Exercise induced ischemia	CAD	Increase in time to 1 mm ST depression[157]
	Immediate and delayed cardioprotection of ischemic preconditioning	Pre-clinical models	Increased synthesis of heat shock protein 70 (HSP-70)[28] ATP-sensitive potassium channels in the myocytes mitochondrial inner membrane[37]
Haemodynamics	Decrease in SVR	CHF	Reduced preload[138] Reduced after-load[138]
	Increase in cardiac output	CHF	
	Baroreceptor cardiac reflex sensitivity	CHF	Increase in carotid baroreceptor sensitivity[141]
Endothelial function	Coronary artery flow	CAD	Increase in blood flow and coronary diameter[18,19]
	Peripheral blood	CAD	Increase in peripheral blood flow[18,19]
Inflammation	TNF-alfa, IL- 1β, and IL-6	CAD, CHF	Inconsistent results. Decrease or stable levels[16,17, 60,61]
	Il-10	CAD	Increase[16]
	tPA/PAI-1/fibrinogen	CAD	No significant changes[16]
Atherosclerosis	cIMT and coronary artery calcium score	Healthy men	No changes in cIMT and coronary artery calcium scores[61]
Metabolism	Body composition	CAD, T2D	Increase in lean mass and decrease in fat mass; decrease in waste circonference[145,158]
	Fasting glucose	T2D, HF	Reduced in HF, stable or reduced in T2D[169-171].
	HbA1c	T2D	No demonstrated effects[169-171]
	Insulin resistance	T2D, CHF	Improvement[169-171]
	Glucose tolerance	T2D, HF	Improvement[169-171]
	Cholesterol	CAD, T2D	Reduction of total cholesterol and LDL levels[61]
	HDL	CAD, T2D	Different responses, seems to decrease levels, without affect general function[61]
	Triglycerides	CAD, T2D	No effects[61]
Clinical performance	NYHA class	CHF	Improvement[141,142]

	6MWT	CHF	Increase max walking distance[140-142]
	VO_2	HF	Increase VO2 max[141,142]
CV Side Effects			
Hypertension		Healthy subjects with testosterone abuse Pre-clinical models	genomic and non-genomic mechanisms of testosterone action on vascular smooth muscle cells in arterial hypertension[11,111]
Salt retention		Healthy, HF	Possible salt retention with worsening of oedema[109]
VTE		Healthy	increase in the risk of VTE within the first 6 months of TT in the presence of thrombophilia[93]

Table 2. Key observational studies dwelling upon the association between Testosterone levels and outcomes

First author yr Study name	Population Follow-up duration Study design	Primary Outcome Method of T assay	Principal Findings	Definition of low T
Ärnlöv J [81] *2006* *Framingham Study*	2084 M 10 yr Prospective cohort study	Incident CVD diseases (coronary heart disease, cerebrovascular disease, congestive heart failure, peripheral vascular diseases) Radioimmunoassay	Serum levels of T or DHEA-S were not associated with CVD risk	Lowest T quartile: < 11.5 nmol/l (330 mg/dl)
Shores MM[85] *2006*	858 M 4.30 yr Retrospective cohort study	All-cause mortality Immunoassay	Low T levels were associated with increased mortality	T <8.7 nmol/L) (<250 ng/dL) or fT <30 pmol/L (<0.75 ng/dl)
Jankowska EA[119] *2006*	208 HF M 3 yr Prospective cohort study	All-cause mortality Immunoassay	Reduced serum levels of T were related to increased risk of death	Age adjusted 10th percentile healthy population: <45 yr 11.1 nmol/L (320 ng/dL), 45-55 yr 10.41nmol/L (300ng/dL), 55-65 yr 9.37 ng/dL (270 ng/dL) >65 yr 9.02 ng/dL (260 ng/dL)
Araujo AB[82] *2007* *Massachusetts Male Aging Study*	1686 M 15.3 yr Population-based cohort study	All-cause or cause-specific mortality Radioimmunoassay	fT levels were positively associated with IHD mortality and inversely associated with respiratory disease mortality. DHEAS was positively associated with IHD mortality T was not associated with mortality from any cause in multivariate models.	Lowest T quintile: < 12.8 nmol/L (< 370 ng/dL) Lowest fT quintile: < 0.28 nmol/L (8.0 ng/dL)
Khaw KT[68] *2007* *(European Prospective Investigation Into Cancer in Norfolk) EPIC-NORFOLK*	2314 M 7 yr Nested case-control study	All-cause mortality Chemiluminescent immunoassays	Low T levels were associated with increased mortality, with lower risk in the highest compared with the lowest quartile	excluded men with CVD and cancer diseases at baseline
Tivesten A[70] *2008* *MrOS Sweden cohort*	3014 M 4.5 yr Multicentre prospective study	All-cause mortality. Gas chromatography-mass spectrometry (GC-MS)	Low levels of both T and oestradiol were associated with mortality. Subjects with low levels of both nearly doubled risk of mortality.	Lowest T quartile <11.6 nmol/L (<334 ng/dL)
Laughlin GA[69] *2008* Rancho Bernardo Study	794 M 11.8 yr Prospective population study	All-cause mortality Radioimmunoassay	Men whose T levels were in the lowest quartile more likely to die. Low T predicted increased risk of cardiovascular and respiratory disease mortality but was not significantly related to cancer death	Lowest T quartile <8.36 nmol/l (<241 ng/dl)

Vikan T[72] *2009* The fourth Tromsø Study	1568 M 11.2 for all cause 10.8 for CVD and IHD Population-based prospective cohort study.	First-ever MI (fatal or nonfatal), all-cause, CVD, and IHD mortality. Chemiluminescent immunoassays	The lowest fT quartile compared with the highest quartiles was associated with poor outcome, whereas T was not associated	Lowest T quartile <9.7 nmol/L (279.54 ng/dL) or lowest fT quartile <158 pmol/L (4.6 ng/dL)
Yeap BB[71] *2009* *Health in Men Study (HIMS)*	3443 M 3.5 yr Prospective observational study	Incident stroke and TIA Chemiluminescent immunoassays	Lower T and fT predicted increased incidence of stroke or TIA SHBG and LH were not	T< 11.7 nmol/L (<337 ng/dL) fT<222 pmol/L (6.4 ng/dL)
Malkin CJ[74] *2010*	930 M IHD 7 yr. Longitudinal study	All-cause mortality and vascular mortality ELISA	TD is common and impacts significantly negatively on survival.	T< 8.1nmol/L (233 ng/dL) or Bio-T < 2.6 nmol/L (74ng/dL)
Haring R[75] *2010* *Study of Health in Pomerania*	1954 M 7.2 yr Prospective population-based study	All-cause mortality and cause-specific mortality Chemiluminescent immunoassays	Low T levels predicted increased risk of CVD death and cancer, but not from respiratory diseases or other causes.	T< 8.7 nmol/L (250 ng/dL)
Menke A[73] *2010* *The Third National Health and Nutrition Examination Survey Mortality Study (NHANES III)*	1114 M 16 yr A stratified, multistage probability survey	All- cause and cause specific mortality Electrochemiluminescence immunoassays	Low fT, and Bio-T were associated with an increased risk of all-cause and cardiovascular mortality at 9 years (phase 1) but not at 18 years (phase 2). T levels were not associated	T < 4.5 mmol/L (129 ng/L) or fT <312 pmol/L (8.9 ng/dL) Data analysed as a decrease in concentration equal to the difference between the 90th and 10th percentiles
Hyde Z[76] *2012* Health in Men Study (HIMS)	3637 M 5.1yr Population-based cohort study	Cause-specific mortality Chemiluminescent immunoassays	Low fT was associated with increased all-cause mortality and CVD mortality. T was not associated with outcomes	T < 15 nmol/L (432 ng/dL) fT< 280 pmol/L (8 ng/dL)
Haring G[75] *2012* *Study of Health in Pomerania (SHIP)*	1182 M 10 yr Population based cohort study Mendelian randomization study	CV mortality Chemiluminescent enzyme immunoassay	No evidence for causal associations of T levels with cardiometabolic risk factors and mortality	T< 6.9 nmol/L (200 ng/dL)
Muraleedharan V[78] *2013*	581 type 2 DM M 5.8 (1.3)	All-cause mortality Chemiluminescent assay and a solid phase enzyme immunoassay	Mortality was increased in the low T group	T< 10.4nmol/l (300ng/dl)

Soisson V[77] *2013* *Three city study (3C-S)*	495 M 4 yr Post-hoc analysis of the multicentre prospective cohort study (Case-cohort study)	Ischemic artery diseases (coronary artery disease + stroke) Immunoassay	A J-shaped association between plasma T and IHD risk	Optimal range of plasma testosterone may confer cardiovascular protection
Magnani JW [79] *2014* *Framingham Study*	1251 M 10 yr Prospective observational study	The 10-year risk of atrial fibrillation Radioimmunoassay	T and oestradiol are associated with incident AF	T<10.4nmol/l (300 ng/dl)
Yeap BB[80] *2014* *Health In Men Study (HIMS) wawe 2*	3690 M 6.6 yr. Prospective population-based cohort study	First hospital admission or death due to MI or stroke were Liquid chromatography-tandem mass spectrometry (LC-MS)	T, DHT, and E2 were not associated with incident MI; with a lower incidence of stroke	T < 9.82 nmol/l (283 mg/dl)
Chan YX [86] *2016* *Busselton Health Study (BHS)*	1804 M 14.9 yr Population based cohort study	time to death from any cause, time to death from CVD and time to first fatal or non-fatal CVD even Liquid chromatography-mass spectrometry	T was not associated with mortality, CVD death or CVD events	T<12.8 nmol/l (368 ng/dL)
Srinath R[87] 2016 Atherosclerosis Risk in Communities *(ARIC) study*	1558 M 14.1 yr Population based cohort prospective study	no association of T with incident stroke was found Liquid chromatography mass spectrometry	No association between T and incident clinical stroke or ischemic brain changes	T≤11 nmol/L (317.7 ng/dL)
Cittadini A[128] 2021 *The Trattamento Ormonale Scompenso CArdiac/hormone treatment heart failure (T.O.S.CA.) Registry*	480 HF M and F 3 yr Prospective multicentre observational study	All-cause mortality, CV-hospitalization, composite Immunoassay	TD was associated with poor outcomes	T<10.4 nmol/L (300 ng/dL) M T <0.87 nmol/L (25 ng/dL) F

T: Total testosterone; fT: free testosterone, DHT: dihydrotestosterone; M: males; yr: years; HF: heart failure; IHD: ischemic heart disease; CV: cardiovascular; CVD: cardiovascular diseases; TD: testosterone deficiency; SHBG: sexual hormone binding globulin.

Figure Legend:

Figure 1. The Hypothalamus-Pituitary-Testes Axis.

Abbreviations: GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; ABG: androgen binding globulin; SHBG: Sex Hormone Binding Globulin.

Figure 2 Summary of experimental evidence on the molecular effects of testosterone in: A endothelial cell, B cardiomyocyte, and C vascular smooth muscle cell. Depicted targets are related to murine or human in-vivo or in-vitro studies using testosterone or DHT.

Abbreviations: TRPV4 transient-receptor-potential-cation channel subfamily V member 4, BK_{Ca} large conductance calcium-activated potassium channels, SK_{Ca} small conductance calcium-activated potassium channels, H2S hydrogen sulfide, PKA protein kinase A, AR androgen receptor, eNOS endothelial nitric oxide synthase, PI3K phosphoinositide 3-kinase, Akt protein kinase B, VCAM-1 vascular cell adhesion protein 1, IKs delayed rectifier potassium current, IKr rapidly activating delayed rectifier potassium current, ICaL L-type calcium current, NCX sodium-calcium exchanger, Ca_v T-type calcium current, SERCA sarco-endoplasmic reticulum calcium ATPase, Tfam cardiac mitochondria transcription factor A, GSK-3β glycogen synthase kinase-3β, TIMP-2 tissue inhibitor of matrix metalloproteinase-2, ANP atrial natriuretic peptide, BNP brain natriuretic peptide, NFAT nuclear factor of activated T-cells, mTORC1 mammalian target of rapamycin complex 1, S6K1 S6 kinase 1, CaMKII Ca2+/calmodulin-dependent protein kinase II, MEF2 myocyte-enhancer factor 2, $mitK_{ATP}$ mitochondrial ATP-sensitive potassium channels, HSP-70 heat shock protein 70, K_{ATP} ATP-sensitive potassium channels, cGMP cyclic guanosine monophosphate, K_v voltage-sensitive potassium ion channels, SOC store-operated calcium channels, PKG protein kinase G, PTOV1 human prostate overexpressed protein 1, ROS reactive oxygen species, NADPH nitrate reductase, GAS6 growth arrest-specific gene 6, MMP-2 matrix metalloproteinase-2

Figure 3 and graphical abstract. Effects of testosterone treatment in heart failure

Heart failure, a possible novel application field of testosterone replacement therapy, represents a perfect example depicting the multitargeted action of testosterone on several cardiovascular risk factors and metabolic impairment, leading to a positive net effect.

Abbreviations: HF: heart failure; TT: testosterone treatment; NYHA: New York Heart Association; MLWHFQ: Minnesota Living with Heart Failure Questionnaire.

Figure 1

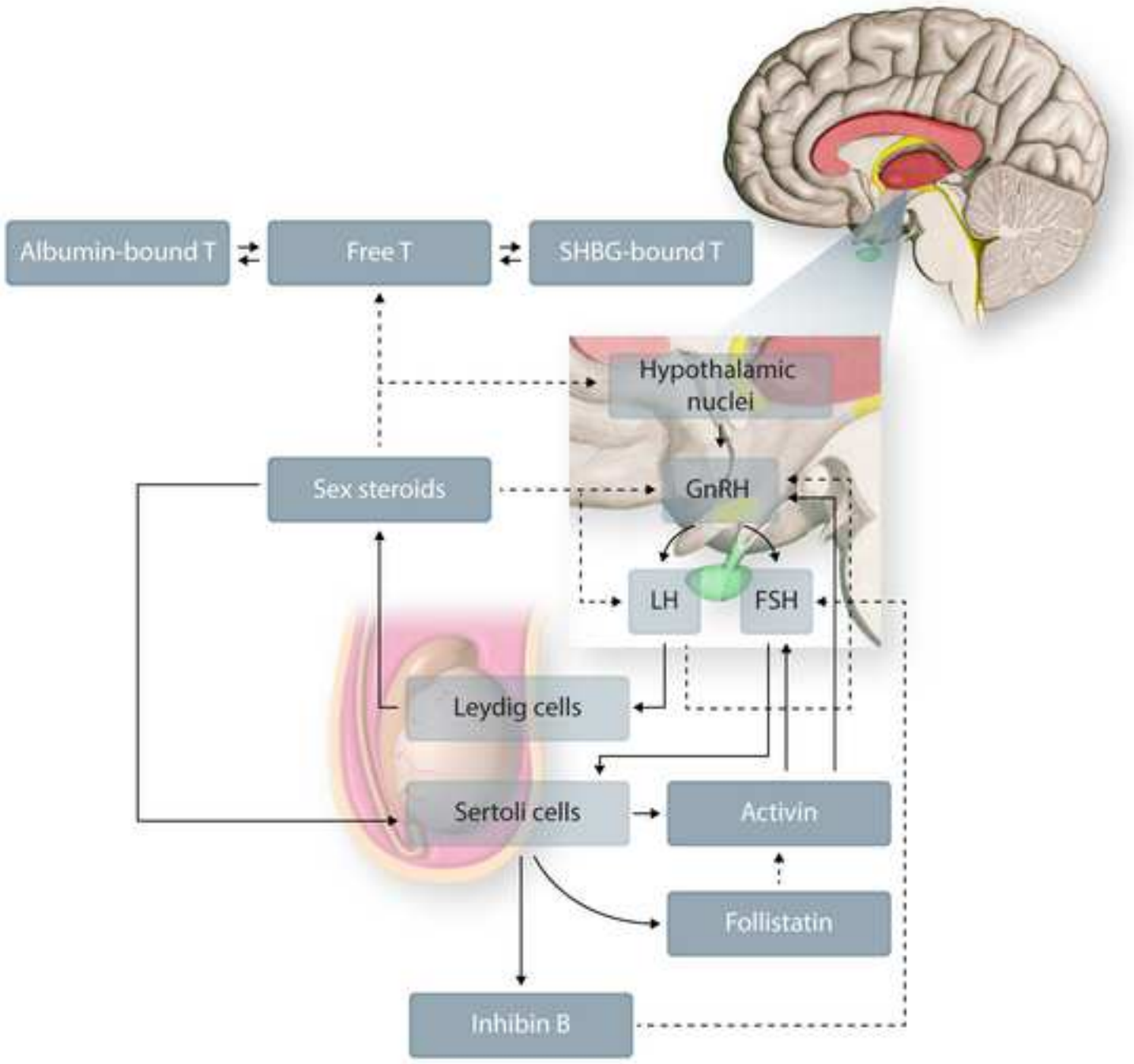

Figure 2

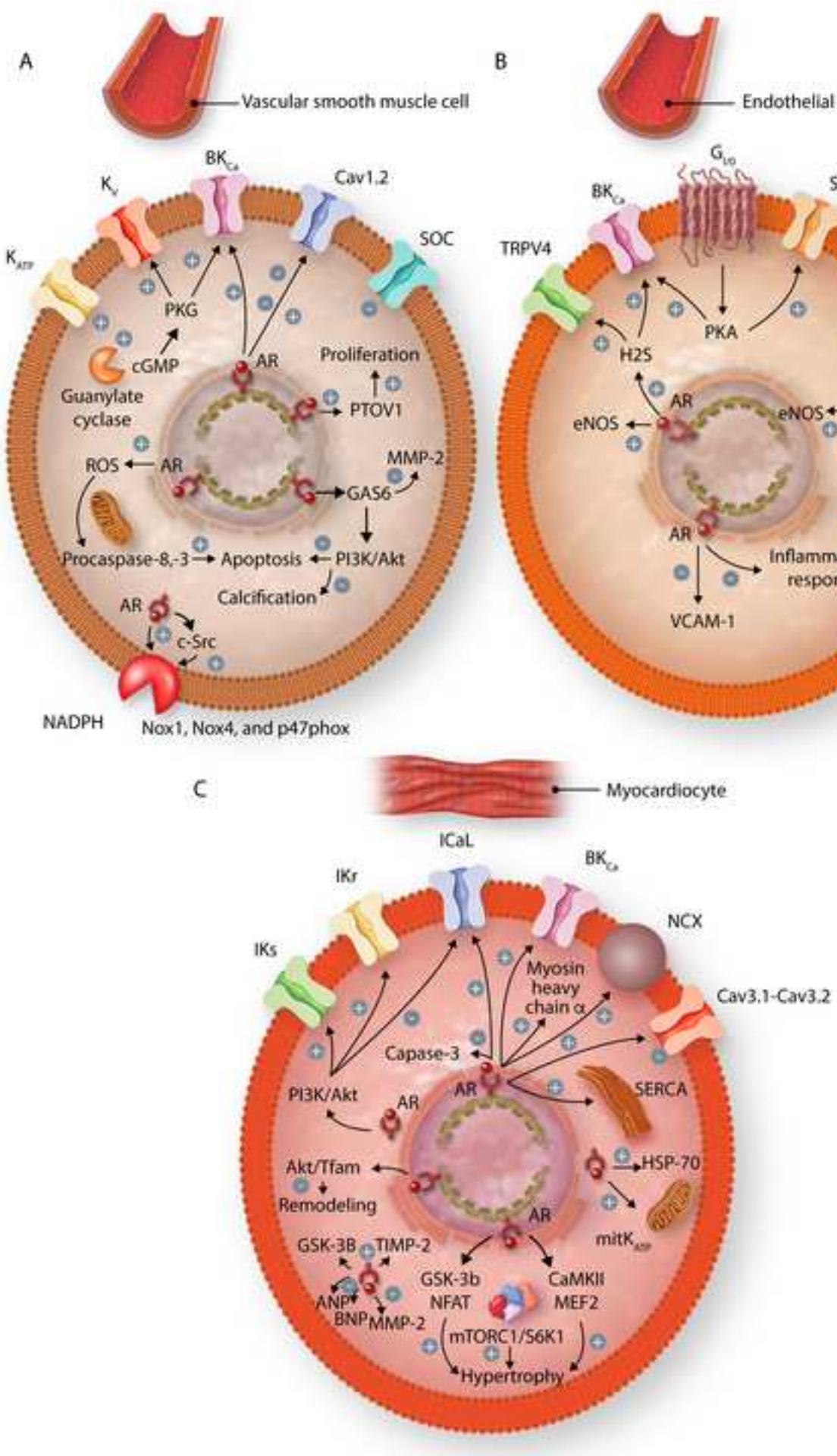

Figure 3