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Vascular mechanisms of testosterone: The non-genomic point of view

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ABSTRACT

Testosterone (T) is the predominant endogenous androgen in the bloodstream. At the vascular level, T presents genomic and non-genomic effects, and both effects may overlap. The genomic actions assume that androgens can freely cross the plasma membrane of target cells and bind to nuclear androgen receptors, inducing gene transcription and protein synthesis. The non-genomic effects have a more rapid onset and may be related to the interaction with protein/receptor/ion channels of the plasma membrane. The key T effect at the vascular level is vasorelaxation, which is primarily due to its rapid effect. Thus, the main purpose of this review is to discuss the T non-genomic effects at the vascular level and the molecular pathways involved in its vasodilator effect observed in *in vivo* and *in vitro* studies. In this sense, the nuclear receptor activation, the influence of vascular endothelium and the activation or inhibition of ion channels (potassium and calcium channels, respectively) will be reviewed regarding all the data that corroborated or not. Moreover, this review also provides a brief update on the association of T with the risk factors for cardiovascular diseases, namely metabolic syndrome, type 2 diabetes mellitus, obesity, atherosclerosis, dyslipidaemia, and hypertension. In summary, in this paper we consider the non-genomic vascular mode of action of androgen in physiological conditions and the main risk factors for cardiovascular diseases.

1. Testosterone in the endocrine system

Testosterone (T) is the predominant endogenous androgen in the bloodstream [1]. T circulates at concentrations much higher than its solubility, since it is mostly bound to serum proteins, such as sexual hormone binding globulin (SHBG) and albumin [2], and only 2–3% of all circulating T is free [1]. The SHBG protein is not only involved in the regulation and availability of sex hormones [3], but also has the ability to influence the hormones effect, due to the presence of the SHBG receptor (SHBG-R) in the membrane [4]. Circulating T concentration reaches its maximum in 30-year-old men, after which its levels continuously decrease at a rate of 1–2% per year [5–7]. Conversely, recent

Abbreviations: $[Ca^{2+}]_i$, Ca^{2+} intracellular concentration; 11KDHT, 11-ketodihydrotestosterone; 11OHA4, 11β-hydroxyandrostenedione; 5-oxo-ETE, 5-oxoeicosatetraenoic acid; A4, androstenedione; ANP, atrial natriuretic peptides; AR, androgen receptors; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNG, cyclic nucleotide-gated; COX, cyclooxygenase; CRP, C-reactive protein; CVD, cardiovascular diseases; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; ED, endothelial disfunction; eNOS, endothelium nitric oxide synthase; ER, oestrogen receptors; GPCR, G protein-coupled receptor; GPR6A, GPCR family C group 6 member A; H_2S, hydrogen sulphide; HDL, high-density lipoprotein; HPG, hypothalamic–pituitary–gonadal; HUA, human umbilical artery; HUVEC, human umbilical vein endothelial cells; ICP_{max}/MAP, intracavernous pressure/mean arterial pressure; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channels; K_{ATP}, ATP-sensitive K^+ channels; KCl, potassium chloride; K_{IR}, inward-rectifier K^+ channels; K_V, voltage sensitive K^+-channels; LDL, low-density lipoprotein; LH, luteinizing hormone; L-type VOCC, L-type voltage operated Ca^{2+} channels; mAR, membrane androgen receptor; mTORC1, mechanistic target of rapamycin complex 1; NA, noradrenaline; nNOS, neuronal NOS; NOS, nitric oxide synthases; OXER1, Oxoeicosanoid receptor 1; PCa, prostate cancer; PDE, phosphodiesterase's; PDE5i, phosphodiesterase type 5 inhibitor; pGC, particulate guanylyl cyclase; t-PA, plasminogen activator; PAI-1, plasminogen activator inhibitor-1; PGE2, prostaglandins; PGF2α, prostaglandin F2α; PGI2, prostacyclin; PKA, protein kinase A; PKG, protein kinase G; PLCβ-IP_3, phospholipase C-inositol 1,4,5-trisphosphate; ROCC, receptor-operated Ca^{2+} channels; sGC, soluble guanylyl cyclase; SHBG, sexual hormone binding globulin; SHBG-R, sexual hormone binding globulin receptor; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channels; SMC, smooth muscle cells; SNP, sodium nitroprusside; SOCC, store-operated Ca^{2+} channels; T, testosterone; TRPC3, transient receptor potential channel 3; TRPM8, transient receptor potential melastatin 8; TRPV4, transient receptor potential vanilloid 4; TRT, testosterone replacement therapy; T-type VOCC, T-type voltage operated Ca^{2+} channels; VLDL, very low-density lipoprotein; VOCC, voltage-dependent Ca2+ channels; VSM, vascular smooth muscle; ZIP9, ZRT-and Irt-like protein

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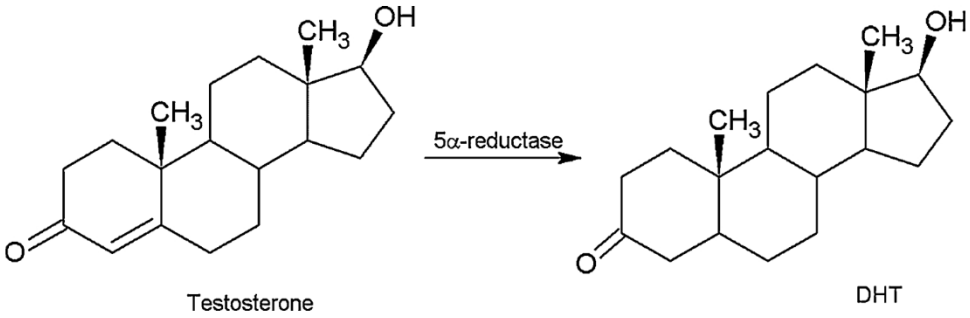

Fig. 1. Chemical structure of testosterone (T) and formation of DHT from T by the 5α-reductase enzyme.

studies have shown that SHBG levels are higher in older men, mainly after the age of 40, increasing about 1% per year [8]. Therefore, with increasing age, free T levels decline faster than total T levels [9,10]. Several studies have shown a correlation between low T levels and an increased prevalence of cardiovascular diseases (CVD) [10,11]. A seven year case control study, with 2314 men aged 40–79 years, showed that an increase of 173 ng/dL in serum T was associated with a 21% lower risk of all-cause mortality, even when correcting for age, body mass index, systolic blood pressure, cholesterol, cigarette smoking, diabetes, alcohol intake, physical activity, social class, education, and SHBG [12,13]. However, it is still controversial whether this is a causal relationship and whether a low T level is a suitable biomarker [5]. But, the use of free and/or SHBG bound T concentration as a clinical marker has gained relevance over the years [4,14].

T can be converted to dihydrotestosterone (DHT) by the 5α-reductase enzyme (Fig. 1), it can also be converted to oestradiol by aromatase (CYP19A1), a ligand of α and β oestrogen receptors (ER) [15]. Thus, both DHT and oestradiol can share not only the same binding site as T, but also the SHBG receptor [9]. DHT is considered the most active androgen, since it has more affinity for the androgen receptor (AR), however, its plasma concentration is much lower compared to T [16]. In general, adult male testes produce large amounts of T (2.5–10 mg, daily) resulting in plasma levels of 350–1000 ng/dL, whereas DHT is found at 35–75 ng/dL. However, these concentrations vary with circadian rhythm and stress. T is not a male-exclusive hormone, since women can produce other types of androgens in the ovaries and adrenal glands, and this hormone can reach plasma levels of 15–65 ng/dL [5,17–22].

The main androgenic hormones secreted by the adrenal gland are dehydroepiandrosterone (DHEA), Androstenedione (A4) and 11β-hydroxyandrostenedione (11OHA4) [23]. Furthermore, downstream metabolism of adrenal androgen precursors must be taken into account, as it has been demonstrated that C11-oxy C_{19} and C_{21} steroids such as 11OHA4, 21 dF and 21dE have prominent roles in clinical conditions [24]. While the biosynthesis of the C11-oxy C_{19} and C_{21} steroids is catalysed by the adrenal specific CYP11B isozymes, their peripheral conversion by steroidogenic enzymes leads to the production of 11-ketodihydrotestosterone (11KDHT) and 11β-hydroxydihydrotestosterone (11OHDHT) through the backdoor pathway [24–26], which may also have non-genomic effects. Although not all these metabolites exhibit androgenic activity, they contribute to the pool of circulating androgenic precursors that can be metabolized to more potent androgens in peripheral target tissues [27]. Due to the importance of adrenal steroids, the development of a steroid profile is therefore essential to accurately diagnose steroid levels, as well as an androgen excess or deficiency in adrenal-linked endocrine diseases. In this sense, Du Toit et al. [28] developed a method capable of simultaneously separating and quantifying the C_{19} and C_{21} steroids, together with their C11-oxy steroid metabolites. A very promising method, since, as well as profiling steroid metabolism and abnormal enzyme activity in patients, is an asset in the identification of new steroid markers in these diseases [28]. Additionally, the inclusion of C11-oxy C_{19} and C_{21} steroid reference ranges in routine steroid analyses should also be considered [29]. Despite these recent findings, the importance of these compounds on human pathophysiology needs further investigations.

When it comes to pregnancy, the fetoplacental unit can synthesize androgens and oestrogens, which may play an important biological role during pregnancy [30]. The concentrations of dehydroepiandrosterone (DHEA), 16α-DHEA and the corresponding sulphates are higher in the human umbilical artery (HUA) than in the human umbilical vein, suggesting that androgen production occurs mostly in the foetal compartment. At the end of gestation, DHEA sulphate production is approximately 200–220 mg/24 h, of which 90–95% are secreted by the foetus. DHEA is mainly biosynthesized by the adrenal glands and liver, specially from three precursors: pregnenolone, 17-hydroxypregnenolone and 17,21-dihydroxypregnenolone (Fig. 2). Most androgens produced by the foetus are subsequently converted into oestrogens in the placenta. However, biosynthesized T in the testes of the foetus is determinant for its sexual differentiation [31,32]. Most circulatory T is converted into various metabolites in the liver, including androsterone and etiocholanolone, which after conjugation with glucuronic and sulfuric acids are excreted in the urine [33].

Historically, the biological action of T and of its metabolite DHT has been only attributed to an effect on gene transcription and protein synthesis [34]. This classical model for steroid hormones action assumes that these hormones can freely cross the plasma membrane of target cells and bind to intracellular receptors, which for androgens are the nuclear AR [35,36]. These are members of the nuclear receptors' superfamily, functioning as genetic transcription factors. Androgens binding to AR causes receptor dimerization, and these dimers act as transcription factors that bind to specific DNA sequences (androgen response elements) and regulate target genes expression [37–41]. This type of action leads to the so-called genomic effects and accurately describes the molecular mechanisms for the response to androgens and to many other steroid hormones.

In addition to the steroid genomic actions, it has been demonstrated that these hormones can also act through non-genomic effects. These are considered of faster onset and may be related to the interaction with structures in the plasma membrane. The first evidence of these effects was reported in 1967 by Szego and Davis [42]. These authors demonstrated that physiological doses of 17β-oestradiol caused a very rapid (15 s) increase in the concentration of cyclic adenosine monophosphate (cAMP) in mice uteri after extraction of the ovaries [42].

More recently, some authors demonstrated that T genomic and non-genomic actions seem to overlap, and the hypothetical mechanism linking these genomic and non-genomic effects (vasodilation) is represented in Fig. 3. Briefly, androgens can cross the plasma membrane, enter the cytoplasm, dissociate from chaperone proteins and bind to AR. This binding leads to alterations in specific genes, inducing an increase in the hydrogen sulphide (H_2S) production, and consequently vasodilation via transient receptor potential vanilloid 4 (TRPV4) and large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) [36]. Moreover, Saldanha et al. showed that the exposure of smooth muscle cells (SMC) from HUA to androgens down-regulates L-type voltage operated Ca^{2+}

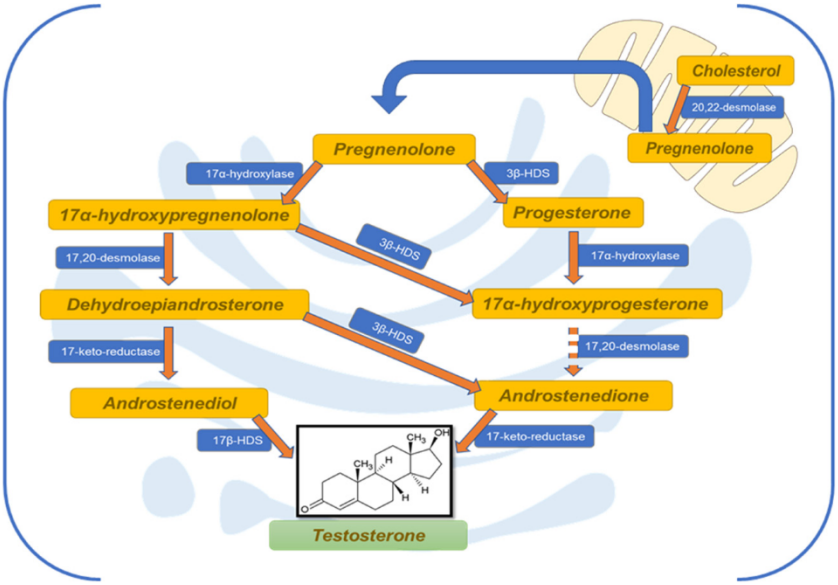

Fig. 2. Testosterone biosynthesis in humans. Testosterone is synthesized from cholesterol through a sequence of enzymatic steps, within Leydig cells located in the mature testes. Each enzymatic reaction/step is represented by an arrow and the enzyme names involved in each reaction are shown. The conversion reaction of 17α-hydroxyprogesterone to androstenedione is represented with a broken line arrow as only insignificant amounts are converted, although this reaction occurs in other species. 20,22-desmolase (P450scc, CYP11A1), 17α-hydroxylase (P450c17, CYP17), 3β-HDS (3 beta-Hydroxysteroid dehydrogenase/delta 5-4 isomerase), 17,20-desmolase (or 17,20 –lyase, P450c17, CYP17), 17-keto-reductase (17 alpha-hydroxysteroid dehydrogenase).

channels (L-type VOCC) and that T up-regulates β1 subunit of BK_{Ca} and this gene alteration also modifies the vascular tone [43].

This review will focus on T non-genomic effects at the vascular level and present the mechanisms involved in the T vasodilator effect observed in *in vivo* and *in vitro* studies. This paper also reviews the current literature about the association of T with the main risk factors for CVD, namely metabolic syndrome, type 2 diabetes mellitus, obesity, atherosclerosis, dyslipidaemia, and hypertension.

2. Vasodilator mechanism of testosterone

As previously mentioned, T and DHT can regulate numerous cellular functions through binding to AR. After crossing the plasma membrane, androgens bind to the receptor forming the hormone-receptor complex, which in turn, interacts directly with nuclear DNA, modulating gene transcription and therefore, protein synthesis. Some authors consider that the accomplishment of this process takes dozens of minutes. Therefore, from a temporal point of view, these genomic effects are considered slow, when compared to the non-genomic effects [36,44–46]. For this reason, T vasodilatory effects that occur within a short time (seconds to minutes) are considered non-genomic (or rapid) effects [44]. But, the first question to be asked is whether the non-genomic effects observed are from T or from oestradiol, since 17β-oestradiol also causes vasodilation in several vessels [9,39,41,47–51] and is converted from T by aromatase. However, several studies with aromatase inhibitors [52,53] and classical ER antagonists have demonstrated that this non-genomic effect is not due to oestradiol [54–56]. Recently, other authors also reported that T conversion to oestrogen is not responsible for the vasodilatory effects of T *ex vivo* [57,58]. Furthermore, DHT (which cannot be converted to oestradiol) has been shown to produce a vasodilatory effect like T [59–63]. Therefore, according to these studies, the non-genomic effects are due to T and not to oestradiol.

At the vascular level, previous studies have shown that T acts as a direct coronary vasodilator in a variety of species, including rabbit, dog, pig and mouse, both *in vivo* [54] and *in vitro* [52,64–66]. It has also been reported that T exhibits a vasodilatory action on thoracic aortas [52,67–69] and vessels isolated from mesenteric [53] and pulmonary [48,70] vasculature of experimental animals and, more recently, also on human pulmonary [71], mammary [55], radial [72] and umbilical arteries [43,73,74] and human corporal SMC [75].

The mechanisms responsible for the T vasodilatory effect will be

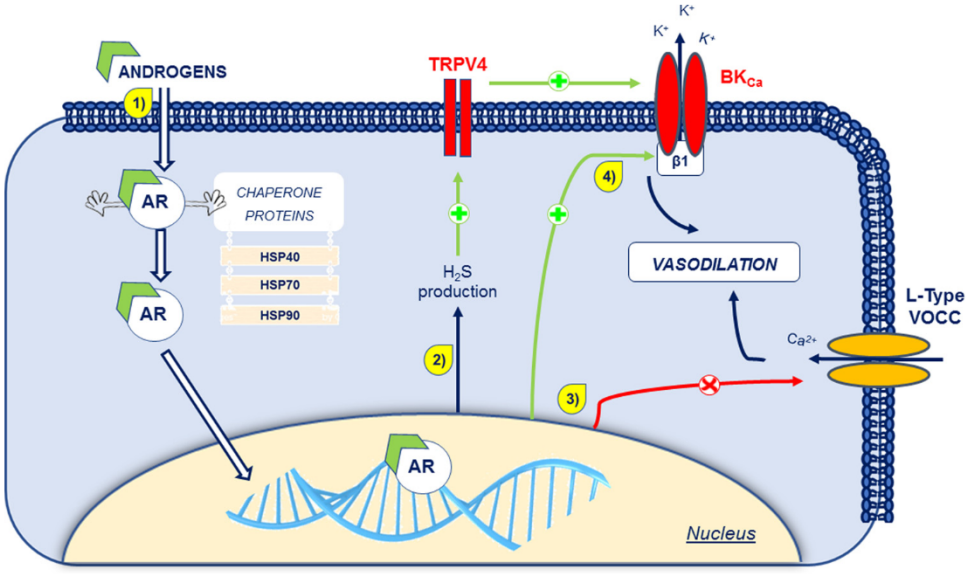

Fig. 3. Schematic representation of androgens genomic mechanisms: **1)** Androgens cross the plasma membrane, enter the cytoplasm, dissociate from chaperone proteins and bind to androgen receptors (AR); **2)** Androgens increase H_2S production, leading to vasodilation via TRPV4 and BK_{Ca}; **3)** Androgens down-regulate L-type VOCC; **4)** Androgens up-regulate β1 subunit BK_{Ca}. **LEGEND:** -Androgens; AR – androgen receptors; HSP - Heat shock proteins; H_2S - Hydrogen sulphide; TRPV4 - transient receptor potential vanilloid 4; BK_{Ca} - large-conductance Ca^{2+}-activated K^+ channels; L-Type VOCC- L-type voltage operated Ca^{2+} channels; /Green arrows – Stimulation; /Red arrows – inhibition.

described in the following sections, namely nuclear receptor activation, influence of vascular endothelium and activation or inhibition of ion channels.

2.1. Activation of androgen nuclear receptors

Initial studies on intracellular mechanisms of T vasodilation focused on the analysis of a possible AR involvement. It has been shown that the T vasodilatory effect is rapid and reversible, and is unlikely to involve nuclear receptor activation [52–54,69,70,73,76]. Furthermore, several studies using flutamide (an AR antagonist) have also shown that AR is not involved in T vasodilator effect [52–54,69,70,73]. This has been confirmed using albumin-bound androgen analogues, which cannot pass through the membrane [69], and protein synthesis inhibitors [77]. In addition, Jones et al. (2002) observed that T induced vasodilation in rats without functional AR, thereby demonstrating that this effect was not mediated by this receptor [70]. In contrast, studies in pig coronary artery [64], mouse renal afferent arterioles [76], human Sertoli cells [78] and prostate cancer cells (PCa) [79] suggested that the T vasodilatory effect was inhibited by flutamide. In general, the participation of AR in vasodilation continues to be highly controversial, with some authors suggesting the presence of this receptor on the cell membrane, as already demonstrated in caveolae of human aortic endothelial cells [80].

Research in this field has grown, and in the last five years, four new membrane AR (mAR) mediators of these nonclassical androgen actions and unrelated to nuclear AR have been discovered [46,81]. The candidates for mAR are, G protein-coupled receptor family C group 6-member A (GPR6A), Oxoeicosanoid receptor 1 (OXER1), transient receptor potential melastatin 8 (TRPM8) and zinc-regulated transporter [Zrt]- protein (ZIP9). The two first candidates are G protein-coupled receptor (GPCR), while the last two are ion channels/transporters for Ca^{2+} and zinc, respectively [46].

GPRC6A, also known as seven-(pass)-transmembrane domain receptors family C group 6-member A, is a recently identified androgen membrane receptor and it was shown that its (coupled to Gi protein) inhibited the non-genomic mechanisms [82]. Pi et al. [83] observed in the human embryonic kidney (HEK-293) cell line, that the removal of GPRC6A suppressed androgen responsive intracellular signalling. In addition, GPRC6A-null mice lacked some typical responses of exogenous androgen administration, such as restoration of seminal vesicle size. Concerning signalling, GPRC6A can activate the ERK pathway in both cell cultures and tissues [83]. However, in contrast to what has been observed for other sex steroids, the interaction between this receptor and androgens is not specific, being also activated by extracellular Ca^{2+}, cations, L-amino acids and osteocalcin [82,84,85]. Pi et al. reported that T binds directly to GPRC6. It has been found that GPRC6A directly mediates the T non-genomic effects, and through computational structural models, the authors further suggest that this happens through unrecognized endocrine networks [85]. Recently, the same research group [86], working with PCa cells, demonstrated that the human GPRC6A mediates testosterone-induced mTORC1 (mechanistic target of rapamycin complex 1) signalling activation. These activation by T occurred via ERK and Akt signalling. Moreover, the authors also reported that GPRC6A mediated testosterone-induced cell proliferation and autophagy. Nevertheless, further studies are needed to know the clinical relevance of this receptor in human pathophysiology [86].

Regarding the other GPCR, the OXER1, it is highly expressed in tissues of PCa and their major ligand is 5-oxoeicosatretraenoic acid (5-oxo-ETE) [46]. In 2017, Kalyvianaki et al. (2017) demonstrated that T competes for 5-oxo-ETE binding to OXER1 and antagonizes 5-oxo-ETE-mediated inhibition of cAMP production [87]. However, T treatment alone does not alter this intracellular signaling pathway in OXER1-transfected cells. Therefore, for this reason, it does not meet one of the criteria to be designated mAR [46,88] and further studies are needed to clarify this issue.

TRPM8, a transient receptor potential melastatin 8, is a Ca^{2+} channel that mediates androgen-induced increases in the Ca^{2+} levels and survival of PCa cells [46]. Asuthkar researches demonstrated that T directly interacts with TRPM8 to activate Ca^{2+} channel activity and increase cytosolic Ca^{2+} levels [89,90]. Nevertheless, there is no consensus on the regulation of TPMR8 activity by T in PCa cells and its physiological importance [91]. Further studies for AR binding to clarify some parameters such as steroid specificity, limited capacity, high affinity androgen binding, and ready dissociation are needed to finally confirm its role as mAR [46,88].

Moreover, Thomas group suggested the existence of another mAR, ZIP9, which is also a 7-domain transmembrane protein [81,92,93]. Interestingly, it shows high sequential similarity with the ZIP9 zinc subfamily (zinc-regulated transporter [Zrt]- and Irt-like proteins or SLC39A proteins) which carry zinc into the cytoplasm across cell membranes from the extracellular fluid or from intracellular organelles, unlike that observed for classical GPRC receptors [82]. This protein is highly specific for binding to androgens, having low affinity for other steroids (in contrast to others mAR) and are coupled to G proteins [81,82]. ZIP9 acts through several signal transduction pathways, including Gs (stimulatory Protein G) in granulosa cells, Gi (inhibitory G Protein) in cancer cells and Gq11 in spermatogenic cells [81]. However, androgens activate a G_s protein coupled to ZIP9, consistent with an increase in cAMP levels, which appears to have effects on breast cancer and PCa cell lines [81,82]. Also, ZIP9 mediates T regulation of tight junction formation in Sertoli cells and nonclassical testosterone signaling in spermatogenic cells [81]. The androgen signalling functions of ZIP9 have been confirmed in other cells, but the overall importance of these transporter in the physiology of androgens remains unclear [46].

In summary, several mechanisms have been proposed to explain these rapid cellular responses to sex steroids, such as steroid nuclear receptor translocation to the cell membrane surface, nonspecific effects of steroids on plasma membrane fluidity, direct allosteric modification of ion channels dependent of binders and GPRCs [82]. However, concerning T, only nuclear receptor translocation to the cell membrane surface has been reported; GPRC6A, OXER1, TRPM8 and ZIP9 zinc transporter were never related to the rapid androgens' vascular responses. Findings of these new mRA are quite promising and suggest that there is a complexity of mechanisms involved in (rapid) non-genomic T functions that remain unknown.

The androgen effects related to the activation of androgen nuclear receptors are summarized in Table 1 and in Fig. 4.

2.2. Effect on vascular endothelium

Over the years, several studies have been performed to analyse the association between T and endothelial function [94,95]. The main regulator of endothelial function is nitric oxide that is produced by endothelium nitric oxide synthase (eNOS) [94]. The endothelium plays a key role in the regulation and maintenance of vascular homeostasis and tone [96,97]. As an essential metabolic and endocrine organ, the endothelium can synthesize and release vasoactive mediators, of which the most significant are prostacyclin (PGI2) and nitric oxide [98]. PGI2 is synthesized by cyclooxygenase (COX) isozymes, and there are two main COX isoforms: COX-1, which is a constitutive enzyme expressed in most tissues, including vascular endothelium, and mediates basal physiological functions; and COX-2, an inducible isozyme that is stimulated by several stimuli, such as inflammatory cytokines, and that is expressed in the cardiovascular and immune systems, thus being frequently associated with pathological conditions [99,100]. This mediator causes vasorelaxation by activating specific cell-surface receptors that are G-protein-coupled to adenylyl cyclase and thereby increasing the cAMP levels [98].Nitric oxide is produced by a group of enzymes called nitric oxide synthases (NOS), which convert L-arginine into citrulline. This mediator causes vasorelaxation via activation of soluble

Table 1
Androgen effects induced by nuclear receptor modulation.

	Assays	Species	Cell/Tissue	Effects	References
Activation of androgen nuclear receptors	***In vitro***	Yorkshire pigs	Coronary artery	T vasodilatory effect was inhibited by flutamide	[64]
		C57BL/6 J mice	Renal afferent arterioles	T vasodilatory effect was inhibited by flutamide	[76]
		Wistar Rat	Sertoli cells	T activation of transmembrane influx of extracellular Ca^{2+} was inhibited by flutamide and by finasteride	[78]
		Human	Prostate cancer cells	T-induced changes in intercellular Ca^{2+}, as a result of Ca^{2+} influx through L-type channels in the plasma membrane, was inhibited by flutamide	[79]
		Human	Embryonic kidney cell line (HEK-293 cells)	Removal of GPRC6A from HEK-293 cells suppressed androgen responsive intracellular signalling	[83]
		Human	Prostate cancer cell lines	Human GPRC6A mediates testosterone-induced mTORC1 signalling activation via ERK and Akt signalling.	[86]
		Human	Prostate cancer cell lines	T antagonizes 5-oxo-ETE by binding to OXER1, that induced the inhibition of cAMP production.	[87]
		Human	Prostate cancer cell lines	T directly interacts with TRPM8 to activate Ca^{2+} channel activity and increase cytosolic Ca^{2+} levels	[89,90]
	In vivo	Mouse	Seminal vesicle	GPRC6A-null mice do not restore seminal vesicle size upon exogenous androgen administration	[83]

guanylyl cyclase (sGC) generating cyclic guanosine monophosphate (cGMP) [101].

Several studies have shown that the T vasodilatory effect is endothelium-independent [52,59,64,67,102,103]. In different types of arteries from a variety of species, indomethacin (a cyclooxygenase inhibitor) did not inhibit T-induced vasodilation [52,54,55,70,104]. Furthermore, studies have shown that the vasodilator effect of this hormone is also not inhibited by NOS or sGC inhibitors [52,59,68,105]. In bovine and human endothelial cells, it has been shown that T has no effect on NOS activity, suggesting that the T vasodilatory effect is endothelium-independent [106,107]. However, some authors have suggested that the T vasodilatory effect is partially endothelium-dependent, and that this hormone stimulates the production of different vasodilation endothelial mediators. Proteinoids (cyclooxygenase action products) and nitric oxide effects have been the subject of intense investigation. Studies have shown a decrease in the T vasodilatory effect in the presence of NOS inhibitors on rat aorta [108], dog coronary artery [54], rat mesenteric artery [53] and mouse renal afferent arterioles [76]. It was also shown a decrease in the T vasodilatory effect in the presence of indomethacin on rat aorta [109]. Other authors demonstrated that the T induced vasodilatory effect was almost null in the absence of the endothelium, in rabbits' renal arteries and male sheep coronary arteries [110,111]. However, in female sheep coronary arteries, the T vasodilator effect was only partially mediated by the vascular endothelium, since the removal of endothelium partially reduced the T effect [111]. The authors postulated that this difference could be due to other vasodilator mechanisms that occur in female sheep, other than that of endothelial factors, for example, activation of K^+ channels. Yu et al. [112] nvestigated T-effect on nitric oxide biosynthesis and its molecular mechanism using human aortic endothelial cells. The authors found that T, at physiological concentrations (1–100 nm), induced a rapid (15–30 min) increase in nitric oxide production by AR-dependent phosphorylation and activation of eNOS. Phosphorylation of eNOS was due to, at least in part, the PI3-kinase/Akt signaling activation and a direct interaction of AR with p85α [112]. Moreover, the same authors suggested that T induced a rapid assembly of a membrane signaling complex among AR, caveolin-1 and c-Src, being this latter kinase the critical upstream regulator of -Src/ PI3-kinase/Akt cascade for eNOS activation in endothelial cells [80]. Other authors demonstrated that T and DHT directly regulate human umbilical vein endothelial cells (HUVEC) *in vitro* and *in vivo*. This occurs by activation of eNOS through both nuclear and extra-nuclear mechanisms and by regulating plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) expression [113]. In the same sense, other authors demonstrated that neither T inhibition aromatization nor ER antagonism affected nitric oxide production or cellular growth, a non-genomic and genomic vascular actions of T, respectively [114]. However, flutamide completely blocked the production of nitric oxide and DNA synthesis suggesting that AR is therefore involved in mode of action of T in rat aortic cells. These results suggest that, in female rat aortic cells, T has a direct action on isolated aortic cells to increase the concentration of endothelial nitric oxide and the SMC proliferation [114]. Moreover, T at physiological concentrations inhibits PGF2α-induced Ca^{2+} fluxes, leading to vasodilation. These effects are mediated by ROCC through a non-genomic mechanism in cultured vascular SMC [115].

One the other hand, other studies have shown only a small but significant decrease in the vasodilatory effect of T in the absence of endothelium [53,69], suggesting that vasorelaxation of T involves, mainly, BK_{Ca} channel activation. In spontaneously hypertensive rats, T-induced relaxation appears to be mediated by the release of endothelium-derived substances, which can open voltage sensitive K^+-channels (K_V) and BK_{Ca} in vascular smooth muscle [68]. Moreover, a more recent study performed by Ruamyod et al. [116], using human coronary artery endothelial cells, suggested that small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and BK_{Ca} channels are responsible for the T vasodilatory effect mediated by the vascular endothelium.

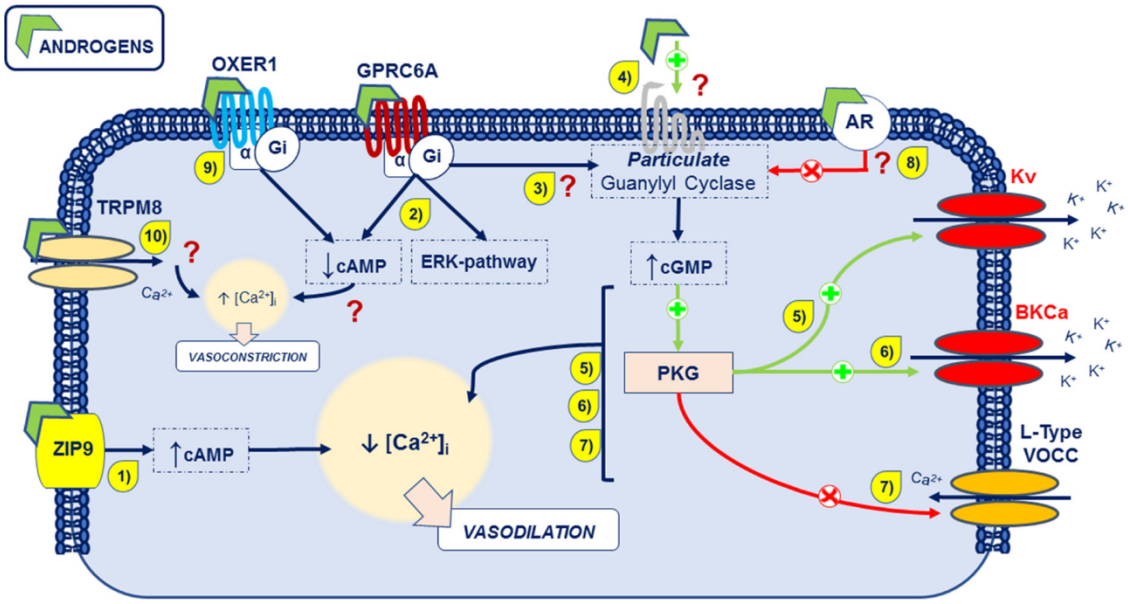

Fig. 4. Schematic representation of androgens non-genomic mechanisms: **1)** ZIP9 activation induced by androgens leads to a cAMP increase. **2)** Through binding to GPRC6A ($G_i\alpha$-activation), androgens lead to a cAMP decrease and ERK-pathway. **3)** Through binding to $G_i\alpha$, androgens are thought to activate particulate guanil cyclase, by a mechanism that is not yet known. **4)** Androgens are thought to activate protein receptors, leading to a cGMP increase by particulate guanil cyclase activation. This increase of cGMP levels leads to PKG activation. Through PKG activation, androgens activate **5)** K_v and **6)** BK_{Ca} channels and inactivate **7)** L-Type VOCC channels through mechanisms still unknown. The action of PKG on the ion channels (represented at 5, 6 and 7) induced by testosterone leads to a decrease in intracellular calcium levels and consequent vasodilation. **8)** via androgen receptors (AR), it is thought that these sex hormones inhibit particulate guanil cyclase. **9)** Through binding to OXER1 ($G_i\alpha$-activation), T lead to the inhibition of cAMP production. **10)** T directly interacts with TRPM8 and increase cytosolic Ca^{2+} levels that may lead to a vasoconstriction. **LEGEND:** - Androgens; ZIP9- Zrt- and Irt-like protein 9; cAMP - cyclic adenosine monophosphate; Ca^{2+} - calcium; GPRC6A - G protein-coupled receptor family C group 6-member A; ERK - extracellular-signal-regulated kinase; - peptide receptor; cGMP - cyclic guanosine monophosphate; PKG - protein kinase G; K_v - voltage-gated K^+ channels; BK_{Ca} - large-conductance Ca^{2+}-activated K^+ channels; K^+ - potassium; L-Type VOCC- L-type voltage operated Ca^{2+} channels; /Green arrows – Stimulation; /Red arrows – inhibition; **?** – unknown mechanism.

This process occurs through $G_{i/o}$ protein activation in the plasma membrane, which activates protein kinase A (PKA) causing hyperpolarization and consequent activation of SK_{Ca} and BK_{Ca} channels [116]. Recently, another study, in rat penile corpus cavernosum, also evidenced that low levels of T down-regulated the $SK_{Ca}3$ and IK_{Ca} (Intermediate-conductance Ca^{2+}-activated K^+ channels) channels expression. This will decrease the ration of the maximum intracavernous pressure/mean arterial pressure (ICP_{max}/MAP), leading to an inhibition of P-eNOS/eNOS and reduction of eNOS bioactivity [117].

Another important factor, considered as determinant for the role of vascular endothelium, is the T concentration. In vessels without endothelium, T induced vasodilation at concentrations higher than 10 μmol/L, but at lower physiological concentrations, nitric oxide appears to be involved in the vasodilatory effect of this hormone. For example, in human male pulmonary arteries, a biphasic response was observed at physiological and supra-physiological concentrations: only vessels with intact endothelium developed T-induced vasodilation within the physiological range (10^{-9} mol/L), while endothelium-denuded vessels required a concentration as high as 3×10^{-5} mol/L to show a dilatory response [71].

Another study was conducted to investigate the sex-specific differences in adrenergic vasoconstriction and vasorelaxation. The authors showed that oestrogen increases vascular β1- and β3-adrenoceptor expression in female rats. They also demonstrated that coactivation of endothelial β1- and β3-adrenoreceptors, when contracted with norepinephrine, induced a higher nitric oxide release in vessels, promoting a higher vasorelaxation in females than in male rats [118]. Moreover, the same authors showed that endothelium denuded mammary arteries of women had a 2.5-fold increase in vasoconstriction toward norepinephrine. In men arteries, endothelium denuded mammary arteries had no change in the vasoconstriction induced by norepinephrine [118].

Several studies have also investigated the effect of T in endothelial function. Cui et al. (2019) observed that T supplementation in the castrated rats increased testosterone serum levels, endothelial function and erectile function through activation of sphingosine-1-phosphate receptor 1 (S1P1)/Akt/FOXO3a pathway. This data may be relevant to elucidate how the androgen deficiency mechanism induces endothelial dysfunction [119]. Moreover, other studies suggest that the combination of testosterone replacement therapy (TRT) with phosphodiesterase type 5 (PDE5i) inhibitors may be a promising alternative to endothelial dysfunction treatment [95,120]. In addition, Usselman et al. demonstrated that androgens lead to a microvascular endothelial dysfunction in women with polycystic ovary syndrome through complete inhibition of ET-1-induced nitric oxide production [121].

In summary, several studies have shown an effect of T on the endothelium, it seems that the T vasodilatory effect may also be due to its direct action on vascular smooth muscle. Recent studies have shown that the mechanism by which T causes endothelium-dependent vasodilation seems to be related with receptor activation (coupled to $G_{i/o}$ protein), which activates PKA causing hyperpolarization and consequent activation of SK_{Ca} and BK_{Ca} channels.

The androgen effects related to the vascular endothelium are summarized in Table 2 and in Fig. 4.

2.3. Effects on ion channels

The control and regulation of the vascular tone and the vascular SMC membrane potential are mainly determined by Ca^{2+} and K^+ channels, of which the later are the dominant ion conductance [122,123]. Membrane potential controls the open-state probability of voltage-dependent Ca^{2+} channels (VOCC) and the influx of Ca^{2+} through these channels, the intracellular Ca^{2+} concentration and the vascular SMC contraction [122,123]. The release of Ca^{2+} from internal

Table 2
Androgen non-genomic effects in vascular endothelium.

	Assays	Species	Cell/Tissue	Effects	References
Effect on vascular endothelium	***In vitro***	Sprague-Dawley rats	Thoracic aortae	Pre-treatment of (+ENDO) aortae with both T and L-NAME (N omega-nitro-L-arginine methyl ester) reversed in part the attenuating effects of T alone	[108]
		Male Wistar rats	Mesenteric artery	A nitric oxide synthase (NOS) inhibitor L-NAME or removal of the endothelium significantly inhibited maximal relaxations to T	[53]
		Male C57BL/6 J mice	Renal afferent arterioles	In the presence of nitric oxide inhibition, L-NAME, T-induced dilation was reduced	[76]
		Wistar-Kyoto rats	Thoracic aortae	T vasodilatory effect decreased in the presence of indomethacin (a cyclooxygenase inhibitor)	[109]
		New Zealand white rabbits	Renal artery	Mechanical rubbing of the endothelium almost abolished relaxations elicited by T in noradrenaline precontracted renal arteries	[110]
		Male sheep	Coronary artery	T-induced vasodilatory effect was almost null in the absence of endothelium. However, in female sheep the T vasorelaxant effect was only partially mediated by vascular endothelium	[111]
		Humans	Thoracic aortae endothelial cells	T, at physiological concentrations (1-100 nm), induces a rapid increase in nitric oxide production by AR-dependent phosphorylation and activation of eNOS.	[112]
		Human	Thoracic aortae endothelial cells	T can activate the c-Src/PI3-kinase/Akt cascade with consequent endothelial NOS (eNOS) activation in vascular endothelial cells	[80]
		Human	Human umbilical vein endothelial cells (HUVEC)	T and DHT directly regulate human endothelial cells *in vitro* and *in vivo* by activating eNOS through both nuclear and extra-nuclear mechanisms and by regulating t-PA and PAI-1 expression	[113]
		Wistar Rats	Aortic endothelial and smooth muscle cells	Inhibition of T aromatization not affect nitric oxide production or cellular growth. Flutamide (an AR antagonist) completely blocks the production of nitric oxide and DNA synthesis	[114]
		Sprague-Dawley rats	Thoracic aorta	T at physiological concentrations inhibits PGF2α-induced Ca^{2+} fluxes, via ROCC	[115]
		Sprague-Dawley rats	Thoracic aortae	T-induced vasorelaxation was slightly but significantly greater in the aortae with endothelium than in aortae without endothelium; this effect of the endothelium was significant at 5–150 mM T	[69]
		Male Wistar rats	Superior mesenteric artery	T-induced vasorelaxation was partly sensitive to L-NAME and the removal of the endothelium decreased the T vasorelaxation effect	[53]
		Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)	Thoracic aortae	T-induced relaxation seems to be due to the release of endothelium-derived substances, which can open voltage sensitive K^+-channels (K_V) and large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca})	[68]
		Human	Commercial human coronary artery endothelial cells (HCAECs)	T vasodilatory effect mediated by vascular endothelium is due to the activation of small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and BK_{Ca} channels. This process occurs through $G_{i/o}$ protein activation in the plasma membrane, which activates protein kinase A (PKA) causing hyperpolarization and consequent activation of SK_{Ca} and BK_{Ca} channels	[116]
		Sprague-Dawley rats	Corpus cavernousus	Low levels of T down-regulated the $SK_{Ca}3$ and IK_{Ca} channels expression, which lead to inhibition of P-eNOS/eNOS and reduction of eNOS	[117].
		Human	Male pulmonary arteries	A biphasic response was observed at physiological and supra-physiological concentrations: only vessels with intact endothelium developed T-induced vasodilation within physiological range (10^{-9} mol/L), while endothelium-denuded vessels required a concentration as high as 3×10^{-5} mol/L to show a dilatory response	[71]
		Wistar Rats	Aorta and mesenteric arteries	Coactivation of endothelial β1- and β3-adrenoreceptors, when contracted with norepinephrine, induced a higher nitric oxide release in vessels, promoting a higher vasorelaxation in females than in male rats	[118]
		Human	Mammary arteries.	Endothelium denuded mammary arteries of women had a 2.5-fold increase in vasoconstriction toward norepinephrine. In men arteries, endothelium denuded mammary arteries had no change in the vasoconstriction induced by norepinephrine	[118]
		Sprague-Dawley rats	Corpus cavernousus	T supplementation in the castrated rats increased testosterone serum levels, endothelial function and erectile function through activation of sphingosine-1-phosphate receptor 1 (S1P1)/Akt/FOXO3a pathway.	[119]
		Bovine	Aortic endothelial cells	Complete inhibition of ET-1-induced nitric oxide production in androgen-pretreated ECs	[121]
	In vivo	Dogs	Coronary artery	Pre-treatment with L-NAME to block nitric oxide biosynthesis decreased T-induced increase in cross-sectional area, average coronary peak flow velocity, and coronary blood flow	[54]
		Human	Forearm arteries	Androgens lead to a microvascular endothelial dysfunction in women with polycystic ovary syndrome through complete inhibition of ET-1-induced nitric oxide production	[121]

stores and the Ca^{2+} sensitivity of the contractile machinery are also important in the modulation of the membrane potential, and the Ca^{2+} and K^+ channels participate in all aspects of the regulation of the vascular smooth muscle (VSM) contraction [124]. The main classes of VOCC expressed in the vascular SMC and involved in tone regulation are the L-type VOCC and T-type voltage operated Ca^{2+} channels (T-type VOCC) [125,126]. The main classes of K^+ channels expressed in the vascular SMC are the K_V, BK_{Ca}, ATP-sensitive K^+ channels (K_{ATP}) and inward-rectifier K^+ channels (K_{IR}) [51,122,123].

The vasodilator effect of T can also be due to its action on ion channels, as demonstrated by several authors over the last years [43,73,127–129]. These studies were performed in different blood vessels of several animal species, including human, and showed that various K^+ and Ca^{2+} channels are implicated in the T effects. However, the question of how T regulates ion channels remains answered.

To answer this question, several studies have been performed and different signalling pathways for the T actions have been proposed. These studies support the hypothesis that T effects are due to the activation of K^+ channels and/or the inhibition of voltage-dependent Ca^{2+} channels. However, there is no consensus as to whether these T effects are direct or due to the activation of a signalling pathway. This section will review the studies of the T effects on ion channels that are summarized in Table 3 and in Fig. 4.

2.3.1. Activation of potassium channels

Numerous studies with different types of arteries and in several animal species (including humans) have demonstrated a modulating T effect on K^+ channels. In 1995, Yue et al. performed the first study using rabbit aorta and coronary arteries contracted with prostaglandin F2α (PGF2α) and potassium chloride (KCl). These authors demonstrated that T relaxes the arteries contracted by depolarization (KCl, 30 mM) and this effect was due to the activation of K_V and BK_{Ca} channels, and not to K_{ATP} activation or to VOCC inhibition [52]. However, the participation of K_{ATP} is not clear since other authors have suggested that the relaxing T effect depends on these channels' activation. Chou et al. (1996) observed that the T vasodilatory action in dog coronary microcirculation was significantly reduced after infusion of glibenclamide (K_{ATP} blocker), indicating that K_{ATP} may be involved in the T vasorelaxant effect [54]. Further studies performed by Honda et al. (1999) with aorta of non-hypertensive and hypertensive rats, showed that the T relaxing effect was reduced after K_{ATP} channels inhibition [68], and in hypertensive rats the T effect was also inhibited by blocking BK_{Ca} and K_V channels. The results of this study are interesting, as they demonstrated that during hypertension there may be a modification of K^+ channels function and/or expression [147,148]. On the other hand, Ding and Stallone (2001) observed that the T effect on noradrenaline (NA)- and KCl-contracted rat thoracic aorta is exclusively due to K_V activation, without the involvement of K_{ATP} and BK_{Ca} channels [69]. This conclusion is in agreement with Won et al., using rabbit coronary arteries [130], and with Ohya et al. in castrated rats. They showed that changing T levels controls the functional expression of K_v channels and induced a down-regulation of these channels in rat vas deferens SMC [131].

Other studies have demonstrated the participation of BK_{Ca} channels in the T vasodilator mechanism in rat mesenteric and aorta arteries, but not K_V and K_{ATP} channels [53,56]. Deenadayalu et al. have shown through *Patch-clamp* experiments with SMC of porcine coronary arteries, that T-induced vasodilation is due to activation of BK_{Ca} channels [59]. More recently, the same authors suggested that this effect was due to a cGMP increase, which involves nitric oxide production (derived from neuronal NOS - nNOS) with subsequent activation of cGMP-dependent phosphorylation via protein kinase G (PKG) [132]. Furthermore, this study also showed that a highly specific antagonist of BK_{Ca} channels (iberiotoxin) inhibited T-induced dilation, suggesting that BK_{Ca} channels are the primary target of T action in porcine coronary artery smooth muscle [132]. Hristov et al. also reported that, under physiological conditions, T nanomolar concentrations activates directly BK_{Ca} channels in detrusor SMC. The authors demonstrated that this pathway was independent from genomic AR and through it, T decreases the urinary bladder smooth muscle excitability [133]. Recently, another study evidenced the participation of other K^+ channels in T vasodilation, demonstrating that low levels of T down-regulated the $SK_{Ca}3$ and IK_{Ca} channels expression [117]. Also, Ramírez-Rosas et al. discovered that T relaxes canine basilar artery, in a lesser extent, by activation of $K_{IR,}$ K_V and BK_{Ca}. These authors refer that this effect does not involve genomic mechanisms, production of cAMP/cGMP nor the conversion of testosterone to 17β-oestradiol [58].

Studies on the T vasodilatory effect have also been performed in human arteries, namely in mammary artery [55], radial artery [72], HUA [73] and human corporal SMC [75]. In human radial arteries, T also causes vasodilation, which is reduced when K_{ATP} is inhibited [72]. The effect on these channels has also been demonstrated by *Patch-clamp* techniques in human corporal SMC, when whole-cell inward K^+ currents of K_{ATP} channels were also increased in the presence of T [75]. In mammary arteries, the T vasodilatory effect appears to be due to BK_{Ca} activation [55], and this effect was also demonstrated in HUA [43,74] and human corporal SMC [75]. In these studies performed by Cairrao et al. the effect on these channels was demonstrated by *Patch-clamp* techniques, where T activated the K^+ currents [74]. Regarding the HUA, it was shown that T also activated the K_V channels, in addition to BK_{Ca}, where K^+ channels activation was shown to be due to PKG activation [74], which occurred due to an intracellular increase of cGMP [134]. This increase of cGMP was solely due to particulate guanylyl cyclase (pGC) activation, that induced a similar vasodilation to the one observed for T in HUA. In this artery, using cyclic nucleotide-gated (CNG) channels as cGMP biosensors, it was also demonstrated the existence of an intracellular cGMP spatiotemporal distribution depending on the activation of two differently located cyclases: 1) when pGC is activated by atrial natriuretic peptides (ANP), cGMP rises near the membrane; 2) when sGC is activated by nitric oxide donors (sodium nitroprusside, SNP), cGMP increases in the cytosol and also near the membrane. Moreover, phosphodiesterases (PDE) play a key role in this compartmentalization, because different PDE subtypes (PDE3 and PDE5) regulate particulate and cytosolic cGMP pools. The authors concluded that PDE5 appears to control the particulate cGMP pool but not the soluble pool, and that the latter is under the exclusive control of PDE3 [149]. This study indicated that, in humans, T vasodilation may be related with the pool near the membrane (controlled by PDE5) and not with the pool induced by NO.

In summary, T-induced vasodilation occurs by modulation of different K^+ channels. Smooth muscle tone is controlled by an ion flow, where the activation of K^+ channels represents the main mechanism for relaxation [124,150]. Activation of K^+ channels causes a membrane hyperpolarization that closes Ca^{2+} channels and leads to vasorelaxation. Taken together, the mentioned studies point to a T-induced K^+ channels activation, with BK_{Ca} e K_v the main channels involved in this process in several species, mainly in human.

2.3.2. Inhibition of calcium channels

The studies mentioned above suggested that T causes the opening of K^+ channels. This effect results in a decrease of the membrane potential, and consequent closure of voltage-dependent Ca^{2+} channels. In this sense, the results observed in some of those studies [43,52,56,69,73] are consistent with the hypothesis that T may lead to an inhibition of Ca^{2+} channels. Other authors have already demonstrated or suggested that this hormone directly inhibits Ca^{2+} channels [67,135]. In fact, currently, it is relatively consensual that the T relaxing effect is also due to the blocking of Ca^{2+} channels, as this effect has already been demonstrated in several arteries, including pig coronary [64,102], rat pulmonary [70] and rat aorta [60,135,145] and also in A7r5 cells (a rat aorta cell line) [143].

The first study to propose that T has an antagonistic effect on the

Table 3
Androgen non-genomic effects on ion channels.

	Assays	Species	Cell/Tissue	Effects	References
Potassium Channels Activation	***In vitro***	New Zealand White rabbits	Aorta and coronary artery	T vasodilatory effect was due to activation of K_V and BK_{Ca} channels, and not to activation of ATP-sensitive K^+-channels (K_{ATP}) or to inhibition VOCC	[52]
		Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats	Thoracic aortae	In WKY rats, T relaxing effect is reduced after K_{ATP} channels inhibition, but in SHR rats, T effect is also inhibited by blockade of BK_{Ca} and K_V channels	[68]
		Sprague-Dawley rats	Thoracic aortae	T vasodilatory effect is exclusively due to K_V activation, without involvement of K_{ATP} and BK_{Ca} channels	[69]
		Rabbit	Coronary artery	T vasodilatory effect is due to K_V activation	[130]
		Male Wistar rats	vas deferens SMC	Changing T levels controls the functional expression of Kv channels and induced a down-regulation these channels in rat vas deferens SMC	[131]
		Male Wistar rats	Superior mesenteric artery	T vasodilator effect was due to BK_{Ca} channels activation, but not K_V and K_{ATP} channels	[53]
		Wistar rats	Thoracic aortae	T vasodilator effect was due to BK_{Ca} channels activation, but not K_V and K_{ATP} channels	[56]
		Pigs	Coronary artery	T vasodilation involved potassium efflux and direct evidence from patch-clamp studies confirmed that T opened BK_{Ca} channels	[59]
		Pigs	Coronary arteries and single myocytes	T relaxation was mediated by the PI3 kinase-Akt/NO/cGMP/PKG signalling cascade that induced the BK_{Ca} channels activation and the repolarization of VSM cells	[132]
		Guinea pigs	detrusor smooth muscle	T nanomolar concentrations activates directly BK_{Ca} channels in detrusor SMC, decreasing the urinary bladder smooth muscle excitability	[133]
		Sprague-Dawley rats	Corpus cavernousus	Low levels of T down-regulated the $SK_{Ca}3$ and IK_{Ca} channels expression	[117]
		dog	canine basilar artery	T induces relaxation, in a lesser extent, by activation of K_{IR}, K_V and BK_{Ca}	[58]
		Human	Mammary artery	T vasodilatory effect appeared to be due to BK_{Ca} activation	[55]
		Human	Radial artery	T vasodilation was reduced when K_{ATP} was inhibited	[72]
		Human	Umbilical artery	T vasodilatory effect was due to BK_{Ca} and K_V activation	[73]
		Human	Umbilical artery	T vasodilatory effect was due to BK_{Ca} and K_V activation	[43]
		Human	Corporal SMC	T vasodilatory effect appeared to be due to BK_{Ca} and K_{ATP} activation	[75]
		Human	Umbilical artery	T vasodilatory effect was mediated by PKG/BKCa and K_V activation signalling cascade	[74]
		Human	Umbilical artery	T vasodilatory effect was mediated by PDE5/particulate cGMP/PKG/BK_{Ca} and K_V activation signalling cascade	[134]
	In vivo	Dogs	Coronary artery	T vasodilatory action in coronary microcirculation was significantly reduced after infusion of glibenclamide (K_{ATP} blocker)	[54]

(*continued on next page*)

Table 3 (*continued*)

	Assays	Species	Cell/Tissue	Effects	References
Calcium Channels inhibition	***In vitro***	Wistar rats	Thoracic aortae	T, 5β-DHT and 5α-DHT induced a vasodilation effect on noradrenaline (NA)-contracted aortae due to direct or indirect inhibition of voltage-independent Ca^{2+} channels, namely, receptor-operated Ca^{2+} channels (ROCC)	[67]
		Wistar rats	Thoracic aortae	5β-DHT vasodilatory effect occurred through inhibition of ROCC and VOCC, being this effect more pronounced in VOCC	[135]
		Guinea Pigs	Airway smooth muscle	T-induced relaxation was due to the blockade of the L-type VOCC at nanomolar and store-operated Ca^{2+} channels (SOCC) at micromolar concentrations, and prostaglandins (PGE2) were also involved in this phenomenon	[136]
		Guinea pigs	airway smooth muscle	T blocks L-type VOCC, constitutively active TRPC3 channel, and probably the PGE2 biosynthesis of smooth muscle of guinea pigs	[137]
		Guinea Pigs	Tracheal smooth muscle	T at physiological concentrations (nanomolar, nM) induced a decrease in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) through the PLCβ-IP3 signalling pathway	[138]
		Yorkshire pigs	Coronary artery	T vasodilation may be due to inhibition of Ca^{2+} entry through other types of Ca^{2+} channels than VOCC or suppression of other contractile mechanisms, and did not affect the Ca^{2+} release from intracellular deposits	[102]
		Sprague-Dawley rats	Thoracic aortae	T vasodilation may be due to inhibition of Ca^{2+} entry through Ca^{2+} channels or suppression of other contractile mechanisms, and did not affect the Ca^{2+} release from intracellular deposits	[139]
		Yorkshire pigs	Coronary artery	T vasodilation may be due to inhibition of calcium entry through other types of Ca^{2+} channels than VOCC or suppression of other contractile mechanisms, and did not affect the Ca^{2+} release from intracellular deposits	[64]
		Sprague-Dawley rat	Aortic VSMCs	T was able to inhibit the increase of intracellular calcium induced by high KCl concentrations through an interaction between T and VOCC present on membranes	[140]
		dog	canine basilar artery	T relaxes the canine basilar artery by blockade of L-VOCC	[58]
		Wistar rats	Pulmonary artery	T induced pulmonary dilation via a mechanism that involved VOCC and ROCC inhibition	[70]
		Wistar rats	Coronary artery	T acted as a Ca^{2+} antagonist against extracellular Ca^{2+} entry and also reduced action against intracellular Ca^{2+} release	[66]
		Wistar rats	Thoracic aortae	T induced dilation via a mechanism that involved VOCC and SOCC inhibition	[105]
		Human	Umbilical artery	T vasorelaxing effect may be mediated by a nitric oxide-independent pathway; and/or a decrease in external Ca^{2+} influx by VOCC inhibition, but not by activating potassium channels	[128]
		Adult pigs	Prostatic small arteries	T vasodilation was suggested to be produced via blockade of extracellular Ca^{2+} entry through L-type VOCC and ROCC	[141]
		Rats	A7r5 cells line, derived thoracic aorta	Higher concentrations of T inhibited L-type VOCC and T-type VOCC	[142]
		Human	HEK 293 cells stably transfected with human α1C and α1H	Low T-concentrations inhibited L-type VOCC and higher supraphysiological concentrations inhibited T-type VOCC	[142]
		Rats	A7r5 cells line, derived thoracic aorta	T and 5β-DHT, at low concentrations inhibited L-type VOCC and did not inhibit the T-type VOCC	[143]
		Human	HEK 293 cells stably transfected with human α1C	T inhibitory effect on L-type channels was selective and direct	[144]
		Wistar rats	Thoracic Aortic rings and myocytes	5β-DHT vasorelaxation may be due to its selective blockade on VOCC by acting as a pure Ca^{2+} antagonist from nM to μM concentrations. T at nM concentrations is a powerful Ca^{2+} antagonist, but at μM concentrations its Ca^{2+} antagonist property stops	[60]
		Rats	A7r5 cells line, derived thoracic aorta	T inhibited L-type VOCC in A7r5 cells but not K^+ current	[145]
		Human	Umbilical artery	T relaxation of KCl- and 5-HT-contracted human umbilical arteries may be due to inhibition of VOCC or ROCC	[128]
		Human	Umbilical artery	A minor vasorelaxing effect induced by T may be mediated by L-type VOCC	[43]
	In vivo	Spontaneously hypertensive rats	Carotid artery	The blockade of L-VOCC is involved in T antihypertensive effect on hypertensive rats	[146]

Ca^{2+} channels was performed in 1996 by Perusquia et al. In this study, the authors concluded that T, 5β-DHT and 5α-DHT have a vasodilatory effect on NA-contracted aorta due to direct or indirect inhibition of voltage-independent Ca^{2+} channels (specifically, receptor-operated Ca^{2+} channels (ROCC)) [67]. Further studies have suggested that 5β-DHT vasodilatory effect on NA- and KCl (60 mM)-contracted rat thoracic aorta occurred through the inhibition of ROCC and VOCC, with this effect being more pronounced in VOCC [135]. More recently, the same authors [136] demonstrated that, in smooth muscle of Guinea pigs, T-induced relaxation was due to the blockade of the L-type VOCC at nanomolar concentrations and store-operated Ca^{2+} channels (SOCC) at micromolar concentrations, and that prostaglandins (PGE2) are also involved in this phenomenon [136]. Two years later, the same research group demonstrated that T diminishes airway smooth muscle tone and intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in guinea pig. These effects seem to occur by blocking of L-type VOCC and a constitutively active Transient Receptor Potential Channel 3 (TRPC3) channel, and probably by PGE2 biosynthesis [137]. In the same year, the authors also demonstrated that the blockade of L-type VOCC was involved in T antihypertensive effect on hypertensive rats, and once again, that this effect was non-genomic [146]. More recently, the team demonstrated, that T at physiological concentrations (nanomolar, nmol/L) induced a decrease in the $[Ca^{2+}]_i$ on rat thoracic aorta through the phospholipase C-inositol 1,4,5-trisphosphate (PLCβ-IP_3) signalling pathway [138]. In the same study, T had no effect on histamine-induced contraction, supporting the hypothesis that this androgen (at nmol/L concentrations) does not interfere with the function of L-type VOCC, as they play a minor role in the histamine response and the major pathway seems to be the IP_3 signalling cascade [138].

In 1999, using porcine coronary arteries, Crews et al. demonstrated the T antagonistic effects on Ca^{2+} channels and the absence of this effect when the contractions were due to Ca^{2+} release by intracellular deposits [102]. Similar results were observed in rat thoracic aorta [139]. These studies suggest that the T vasodilatory effects are restricted to the inhibition of Ca^{2+} entry through ROCC and/or VOCC. It was also suggested that T reduces Ca^{2+} entry from the extracellular space in KCl- or PGF2α-contracted porcine coronary arteries and does not affect the Ca^{2+} release from intracellular deposits [64]. More recently, Hu et al. (2016) demonstrated, for the first time, that T is able to inhibit the increase of $[Ca^{2+}]_i$ induced by high KCl concentrations. This inhibition occurred through an interaction between T and the VOCC present in the membrane of rat aorta SMC [140]. Ramírez-Rosas et al. also reported that T relaxes the canine basilar artery by a blockade of L-type VOCC. The authors refer that this effect does not involve genomic mechanisms, production of cAMP/cGMP nor the conversion of testosterone to 17β-estradiol [58].

Several studies performed by the Hugh Jones group demonstrated the T vasodilatory effect on coronary, pulmonary and rat aorta arteries contracted by different agents (KCl, prostaglandin F2alpha, BAY K8644), suggesting that T has an antagonistic effect on VOCC, ROCC and /or SOCC [66,70,105]. Further studies demonstrated the T effect on VOCC and/or ROCC in small porcine arteries and in HUA, showing that the vasodilation is produced by the blockade of extracellular Ca^{2+} entry through these channels [128,141].

These T-elicited effects on Ca^{2+} channels were also observed in *Patch-clamp* experiments, in which T inhibits L-type VOCC [60,142–145] and T-type VOCC [142] in rat aorta A7r5 cells, fresh mouse aortic cells and in HEK 293 cells transfected with the α_{1C} subunit (Cav 1.2). Specifically, Scragg et al. (2004) observed a potent inhibitory effect of low T-concentrations (1 nmol/L) on HEK 293 cells transfected with α_{1C} subunit. Concerning A7r5 cells, the effect on native T-type and L-type was only observed at higher concentrations of T [142]. Hall et al. (2006) reported that T and 5β-DHT, at low concentrations (3.1 and 6.1 nmol/L, respectively), inhibit L-type VOCC but not T-type VOCC in A7r5 cells [143]. Later, Scragg et al. demonstrated that the inhibitory effect of T on L-type channels of HEK 293 cells transfected with the α_{1C} subunit is selective and direct [144]. Using mouse aortic cells, Montano et al. showed that T may be antagonist, when in low concentrations (nmol/L) or agonist, at high concentrations (μmol/L), of the L-type VOCC. In this study it was also observed that high concentrations of T levels increase cAMP levels, suggesting that this increase leads to the activation of the PKA, inducing the activation of the L-type VOCC [60]. More recently, Alvarez et al. [145] showed that T inhibits L-type VOCC in A7r5 cells but not the K^+ current, and that the effect is not mediated by the activation of K^+ channels. In addition, the authors showed that cholesterol has similar effects to those induced by T, in rat aorta and in A7r5 cells, suggesting a mutual action mechanism for both steroids that could be also shared by other steroids [145].

As indicated previously, the effect of this hormone was also studied in human arteries. Firstly, Fausett et al. observed that T did not significantly relax depolarization-contracted HUA [49]. Subsequently, Perusquia et al., under similar experimental conditions, suggested that this hormone caused relaxation of KCl- and serotonin-contracted HUA due to the inhibition of VOCC or ROCC [128]. More recently, Saldanha et al. demonstrated that nifedipine (a specific Ca^{2+} channels inhibitor) may induce a small influence on T vasodilator effect, once the combined effect of nifedipine and T depended on the contractile agent used [43].

In summary, T-induced vasodilation occurs by modulation of different Ca^{2+} channels. At vascular level, Ca^{2+} channels are closely related with K^+ channels, where a membrane hyperpolarization by K^+ channels activation closes the Ca^{2+} channels and leads to vasorelaxation. Overall, the mentioned studies, point to a T-induced inhibition of Ca^{2+} channels, VOCC and ROCC, the main channels involved in this process.

3. Is testosterone a risk factor for CVD?

Testosterone was initially considered to be harmful to the cardiovascular system, as men have a high prevalence of CVD and the cardiovascular morbidity and mortality is over 2-fold greater in men compared to women [151]. However, several clinical and epidemiological studies have challenged this idea. In fact, it was observed that men with CVD had low levels of T [10,152–155]. In addition, the prevalence of CVD increases in aging men, when T production declines. These observations suggest that this hormone has beneficial effects on the cardiovascular system and that testosterone replacement therapy (TRT) may become a therapeutic reality for some of these pathologies. In contrast, more recent studies have shown that TRT may be associated with an increased incidence of adverse cardiovascular events and these studies have created a great controversy regarding the cardiovascular benefits of TRT [10,11,156]. This led the FDA to issue a warning statement about the potential CVD risk factors of TRT. However, no study published to date on TRT has been adequately designed to assess and correlate this therapy with CVD risk factors, so its cardiovascular safety remains uncertain [11].

In this sense, it is urgent to answer the question: is testosterone associated with an increase or decrease in the risk factors for CVD? Several clinical and epidemiological studies observed that men with type 2 diabetes mellitus, obesity, metabolic syndrome, atherosclerosis, dyslipidaemia, and higher blood pressure had lower levels of T.

Concerning metabolic syndrome and type 2 diabetes mellitus, most studies showed that low levels of T are associated with an increased risk for metabolic syndrome [157–160] and type 2 diabetes mellitus [158,161,162]. TRT seems to be beneficial to these disorders in hypogonadal males [163], improving the fasting glucose, glycated haemoglobin, cholesterol levels and obesity. Studies performed by Corona et al. demonstrated that TRT improves the central obesity and metabolic control as well as body fat mass in these patients. Specifically, a reduction of waist circumference, fasting glucose, and insulin resistance were reported, and also an increase in high-density lipoprotein (HDL) levels [157,158]. Cai et al. also suggested that TRT could improve

glycaemic control and decrease triglyceride levels of type 2 diabetes mellitus in hypogonadal men [163]. Moreover, the prescription of TRT to correct low T levels was associated with a decrease in the incidence of some CVD [10], and with improvement in mortality of men with type 2 diabetes and hypogonadism. These findings are suggestive that T plays an important role in human health. However, it remains unclear whether their actions are involved in protecting cardiovascular health and/or stabilizing/improving CVD already established [3].

Furthermore, another disorder, obesity, was associated with low levels of T, and the hypogonadism-obesity cycle may be an explanation for this CVD risk factor [164–166]. Moreover, these low T levels may also contribute to an increase in obesity [167,168], that may be inversely related by a bidirectional mechanism [169]. Besides, several studies also showed that TRT improves obesity, reducing visceral and body fat mass, BMI and waist circumference [166]. In summary, the T/obesity/CVD relation is multifactorial and very complex, and so far, remains poorly understood [169].

Regarding atherosclerosis, an inverse association between T levels and carotid Intima–media thickness, plaque score, endothelial dysfunction and higher levels of high-sensitivity C-reactive protein has been showed [170–175]. Makinen et al. (2005) suggested that TRT decelerate the atherosclerosis progression and protects from its clinical sequelae (such as coronary heart disease, ischemic stroke, and peripheral vascular disease) [175]. So, individuals with normal levels of T seem to have an anti-atherosclerotic pattern. Recently, a study performed by Snyder et al. (2018) to determine the efficacy of increasing T levels in older men with low T, demonstrated that T treatment increases the coronary artery noncalcified plaque volume. Although T was not associated with more cardiovascular adverse events than the control, the authors refer that more trial studies are necessary to determine whether T increases CVD risk [176].

Concerning dyslipidaemia, most of the studies showed that there is a correlation between low T levels and high levels of low-density lipoprotein (LDL), total cholesterol, triglycerides e very low-density lipoprotein (VLDL) [167,177]. Dyslipidaemia is the main cause of atherosclerosis [14], with T playing a key role. Several studies have demonstrated that TRT induces a more favourable lipid profile, contributing to a decrease in the occurrence of CVD, concluding that androgens seem to provide a protective effect against the development and/or progression of atherosclerosis [14,178]. Recently, Mohler et al. [179] attempted to determine TRT effect on cardiovascular biomarkers in older men with low T levels. The biomarkers studied included lipids and markers of glucose metabolism, fibrinolysis, inflammation, and myocardial damage. The authors found that 1-year T treatment was only associated with small cholesterol and insulin reductions, and was not associated with glucose and inflammation markers, fibrinolysis or troponin. The authors point out that the clinical importance of their results is unclear, and therefore further clinical studies are needed [179]. Only a few studies in hypogonadal men demonstrated an association between T and blood pressure, demonstrating beneficial effects of T in the diastolic and systolic blood pressure as well as the resting heart rate [180,181]. Furthermore, an excess or insufficient androgen production during pregnancy may trigger the development of preeclampsia or gestational hypertension [62]. Moreover, Keya et al. also suggested that T may have an important role in preeclampsia pathogenesis, as they found that women with preeclampsia had higher T-free levels than normotensive women [182]. Consequently, these studies indicate that the administration of androgens in hypertensive disorders of pregnancy is a near possibility, however more clinical studies are needed.

In summary, T levels decrease with advancing age. Low T levels have been associated with increased incidence of various CVD [10] and therefore it appears to be a marker of these diseases. Nevertheless, a pathogenic link was not proved. TRT has been shown to improve the CVD risk factors [183]. In contrast, TRT was also associated with an increased incidence of adverse CV events, which has raised major questions about the safety of this therapy [10]. Further studies are needed to assess the TRT safety in order to guide their prescription [156,184].

4. Conclusions and future directions

Testosterone is an important sex hormone that triggers several genomic and non-genomic pathways, leading to improvement of several CVD risk factors and quality of life in men. At the vascular level, the main non-genomic effect of T is vasorelaxation. The underlining mechanisms at this process involve the activation or inhibition of ion channels, with the vascular endothelium also playing an important role, depending on species and gender. In this sense, the knowledge of the molecular pathways involved in the T beneficial effect in the cardiovascular system and the respective clinical outcomes may lead to the identification of new therapeutic agents to minimize the adverse effects or risks associated with TRT. However, the question persists: is TRT important for aging men, hypogonadal men or all men with low levels of T? Further studies are needed to elucidate the molecular and clinical mechanisms of androgens at the vascular level and thus, to contribute to the prevention and treatment of CVD.

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Declaration Competing Interest

None

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