Testosterone and phosphodiesterase type-5 inhibitors: new strategy for preventing endothelial damage in internal and sexual medicine?

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Abstract: Normal vascular endothelium is essential for the synthesis and release of substances affecting vascular tone (e.g. nitric oxide; NO), cell adhesion (e.g. endothelins, interleukins), and the homeostasis of clotting and fibrinolysis (e.g. plasminogen inhibitors, von Willebrand factor). The degeneration of endothelial integrity promotes adverse events (AEs) leading to increased atherogenesis and to the development of vascular systemic and penile end-organ disease. Testosterone (T) is an important player in the regulation of vascular tone through non-genomic actions exerted via blockade of extracellular-calcium entry or activation of potassium channels; also, adequate T concentrations are paramount for the regulation of phosphodiesterase type-5 (PDE5) expression and finally, for the actions exerted by hydrogen sulphide, a gas involved in the alternative pathway controlling vasodilator responses in penile tissue. It is known that an age-related decline of serum T is reported in approximately 20 to 30% of men whereas T deficiency is reported in up to 50% of men with metabolic syndrome or diabetes. A number of laboratory and human studies have shown the combination of T and other treatments for erectile dysfunction (ED), such as PDE5 inhibitors, to be more beneficial in patients with ED and hypogonadism, who fail monotherapy for sexual disturbances.

The aim of this review is to show evidence on the role of T and PDE5 inhibitors, alone or in combination, as potential boosters of endothelial function in internal medicine diseases associated with reduced T or NO bioavailability, i.e. metabolic syndrome, obesity, diabetes, coronary artery disease, hyperhomocysteinemia, that share common risk factors with ED. Furthermore, the possibility of such a strategy to prevent endothelial dysfunction in men at increased cardiovascular risk is discussed.

Keywords: endothelial dysfunction, erectile dysfunction, phosphodiesterase type-5 inhibitors, testosterone, cardiovascular disease, hydrogen sulphide, adenosine diphosphate

Introduction

The endothelium is the single layer of cells that line the luminal surface of blood vessels. Over the past few years, it has become increasingly apparent that it is far more than just a structural lining and has a range of important physiological functions. The vascular endothelium is an active, dynamic tissue that controls many important functions on the vasculature, including regulation of vascular tone, local hemostasis, proliferative process, and maintenance of blood circulation, fluidity, coagulation, and inflammatory responses. Through these multiple functions, the endothelium is primarily responsible for enabling the arterial system to deliver sufficient tissue perfusion [Haller, 1997; Vane *et al.* 1990].

Endothelial dysfunction (EDys) has gained increasing notoriety as a key player in the pathogenesis of atherosclerosis [Ross, 1990]. As atherosclerosis is the most common cause of vasculogenic erectile dysfunction (ED) in older men, the recognition of ED as a warning sign of silent vascular disease has led to the concept that

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a man with ED and no cardiac symptoms is a cardiac (or vascular) patient until proven otherwise [Solomon *et al.* 2003]. Vasculogenic ED results from impairment of endothelial-dependent or -independent smooth muscle relaxation (functional vascular ED, initial stages), occlusion of the cavernosal arteries by atherosclerosis (structural vascular ED, late stages), or a combination of these. The association between ED and clinical atherosclerosis has been documented [Guay, 2007]. Furthermore, there is a high incidence of cardiovascular disease (CVD) in men with ED, and data suggest that ED may be an early manifestation of EDys in the presence or absence of cardiovascular risk factors (CRFs) [Gazzaruso *et al.* 2008]. The presence of traditional CRFs, such as aging, smoking, hypertension, dyslipidemia, diabetes and obesity, and some less-traditional risk factors, including inflammation, hypoxia, oxidative stress and homocysteinemia, are known to cause EDys [Brunner *et al.* 2005]. EDys frequently occurs in acute coronary syndromes [Gonzalez and Selwyn, 2003], heart failure, reperfusion injury, renal failure, systemic inflammatory disorders [Vlachopoulos *et al.* 2007] and ED [Bonetti *et al.* 2003]. EDys is the key event in the pathophysiology of ED and, importantly, men with penile vascular damage may have EDys in other vascular beds, as well [Kaiser *et al.* 2004]. Therefore, men with ED may be at increased risk for cardiovascular adverse events (AEs) and ED may be considered as a sentinel symptom in patients with occult CVD [Thompsom *et al.* 2005].

The age-related testosterone (T) decline may affect either arterial reactivity or sexual function [Allan and McLachlan, 2004]. Hypogonadism and ED in aging men are common disorders found in patients presenting to andrology clinics. Increasing evidence indicates that both disorders have important associations with the metabolic syndrome (MeS), diabetes and CVD, all conditions with an increased morbidity and mortality [Jones, 2007]. A low T-level is positively associated with the presence and severity of atherosclerosis and a reduction in plasma T might contribute to increased arterial stiffness, which in turn has been associated with increased cardiovascular risk [Hougaku *et al.* 2006]. The early recognition of these clinical conditions is important to allow treatment and hence reduce cardiovascular risk. The increased incidence of CVD in aging men compared with premenopausal women suggests an unfavourable effect of male sex hormone T on the cardiovascular system. However, numerous epidemiological and interventional studies reported a controversial relationship between T and CVD. T inversely correlates with the severity of atherosclerosis and has beneficial effects upon vascular reactivity, inflammatory cytokine, adhesion molecules, insulin resistance, serum lipids, and hemostatic factors [Fukui *et al.* 2007]. Interestingly, men with established coronary heart disease display reduced circulating T levels [Rosano *et al.* 2007] that are often associated with a certain degree of EDys independently of other vascular risk factors (VRFs), suggesting a protective role of endogenous T on the endothelium [Akishita *et al.* 2007]. Thus, a modern approach to ED should be geared not only towards ameliorating the symptom of erectile inadequacy, but also towards modifying the burden of any concomitant medical conditions in which EDys plays a pivotal role in worsening the course of disease and thus contributing to the severity of ED [Aversa *et al.* 2008a]. In this article, we shall discuss the role of T and PDE5-inhibitors to improve endothelial and erectile functions and their possible untoward effects on systemic endothelial function/dysfunction deriving from chronic exposure to these substances.

Endothelial function and dysfunction

In a normal physiologic state, healthy endothelium serves as an anticoagulant membrane, exerting predominantly fibrinolytic, anticoagulant and anti-aggregatory effects. These effects occur through the expression of anti-thrombin III (inhibiting fibrinogen to fibrin conversion), heparin-like molecules (which enhance anti-thrombin III activity), tissue factor pathway inhibitor (which inactivates the extrinsic pathway)[Kharbanda and Deanfield, 2001] and tissue-type plasminogen activator (tPA) from intact endothelial cells (ECs). Additionally, ECs bind thrombin, leading to protein-C activation and eventually inactivation of plasminogen activator inhibitor-1 (PAI-1) [Kharbanda and Deanfield, 2001]. In a haemostatic response, ECs produce key components of platelet activation and aggregation, including von Willebrand factor (vWf), fibronectin and thrombospondin, ultimately leading to the initiation of the coagulation cascade [Sagripanti and Carpi, 2000]. These dynamic thrombotic functions are important, as thrombus formation is a key element in atherosclerosis progression.

The endothelium regulates transmigration of circulating cells through a complex interplay of trans-signaling molecules. ECs recruit inflammatory cells via expression of cell adhesion molecules (CAMs) such as selectin and immunoglobulin superfamily adhesion molecules, and by responding to pro-adhesion signals from circulating cytokines [Panes *et al.* 1999]. Platelets also have a range of endothelial signalling abilities, including the release of vasodilating agents (such as adenosine diphosphate and serotonin), as well as vasoconstricting and procoagulant factors (such as endothelin-1 and vWf). The effects of these circulating cell signals can further be attenuated by some endothelially-derived substances such as nitric oxide (NO) and prostacyclin [Kharbanda and Deanfield, 2001]. The vascular endothelium also plays an obligatory role in vasodilation. These effects are mediated in large part by the action of endothelial derived nitric NO which has vasodilatory properties. NO is also known to inhibit platelet activation/aggregation, vascular smooth muscle proliferation, leukocyte adherence, and low density lipoprotein oxidation, all of which are known to contribute to CVD states. Endothelial nitric oxide synthase (eNOS), one of three nitric oxide synthase (NOS) isoforms, is responsible for the majority of endothelium derived production of NO from the substrate L-arginine [Vane *et al.* 1990]. Bioavailable NO can be increased by enhancing its production or reducing its inactivation. As a freely diffusible gas, NO acts not only within the lumen but also on the surrounding smooth muscle cells where it increases cyclic guanosine monophosphate (cGMP)-mediated vasodilation [Kharbanda and Deanfield, 2001]. In contrast, the endothelium also produces vasoconstricting hormones known as the endothelins [Maas *et al.* 2002]. A number of novel plasma markers have been associated with atherosclerosis and EDys, and the latter can be tested *in vivo* using several techniques that rely principally on measuring change in arterial diameter or flow in response to stimuli, and *in vitro* using circulating biomarkers, such as high-sensitivity C-reactive protein, P-selectin, CAMs and endothelial progenitor cells (EPCs) in clinical studies [Farouque and Meredith, 2001]. Longitudinal observations confirmed that EDys of the coronary and peripheral circulation is predictive of cardiovascular events, the sensitivity and specificity being greater for coronary artery EDys than for peripheral dysfunction [Vita and Keaney, 2002].

The mechanism underlying EDys induced by CRFs, such as diabetes, hypertension, smoking and dyslipidemia, involves two processes: the inhibition of dimethylarginine dimethylaminohydrolase, which catalyses the hydrolysis of asymmetric dimethyl arginine (ADMA), an inhibitor of eNOS [Boger, 2003]; and the uncoupling of eNOS activity [Watts *et al.* 2007]. Down-regulation of eNOS with these diseases results in reduced bioavailability of NO which determines EDys. The guanidino dimethyl arginine derivatives — symmetric (SDMA) and ADMA are derived from degradation of methylated proteins and are found in plasma [Krzyzanowska *et al.* 2008]. ADMA, in contrast to SDMA, has been shown to inhibit NOS, reduce NO levels and to be associated with cardiovascular events [Valkonen *et al.* 2001]. ADMA levels are ten times higher inside ECs [Elesber *et al.* 2006]. Both processes increase oxidative stress in the ECs [Cooke, 2005]. This increase in oxidative stress leads to further oxidative catabolism of NO, formation of peroxynitrite, and activation of the proinflammatory nuclear factor kappa B, which in turn induces cellular inflammation and adhesion molecule production [Cooke and Dzau, 1997]. One potent free radical, superoxide anion, inactivates NO resulting in the production of peroxynitrite — a potent oxidant that stimulates the production of vasoconstrictor prostanoids. NO production is also reduced in the presence of free radicals. Oxidative stress leads to the up-regulation of anti-oxidation enzymes, such as superoxide-dismutase, producing hydrogen peroxide [Browne *et al.* 2003]. These mechanisms, and the reduction in bone-marrow-derived endothelial progenitor cells (EPCs), could underpin a common pathogenesis for both ED and EDys [Baumhäkel *et al.* 2006]. In individuals with established ED, an elevated ADMA level has been shown to correlate with the severity of comorbidities, such as in patients with renal disease [Kielstein *et al.* 2001], insulin resistance and diabetes [Schiel *et al.* 2003], and CAD [Browne *et al.* 2003; Lu *et al.* 2003], indicating the impact that ADMA levels could have on endothelial function [Wierzbicki *et al.* 2006].

EDys is an important component of the metabolic or insulin resistance syndrome, as demonstrated by inadequate vasodilation and/or paradoxical vasoconstriction in coronary and peripheral arteries in response to stimuli that release NO [Cersosimo and DeFronzo, 2006]. Other distinct non-metabolic branches of

insulin-signaling pathways regulate secretion of the vasoconstrictor endothelin-1 in endothelium. Metabolic insulin resistance is characterized by pathway-specific impairment in phosphatidylinositol 3-kinase-dependent signaling, which in endothelium may cause imbalance between production of NO and secretion of endothelin-1, leading to decreased blood flow, which worsens insulin resistance [Kim *et al*. 2006]. Deficiency of endothelial-derived NO is believed to be the primary defect that links insulin resistance and EDys. NO deficiency results from decreased synthesis and/or release, in combination with exaggerated consumption in tissues by high levels of reactive oxygen (ROS) and nitrogen (RNS) species, which are produced by cellular disturbances in glucose and lipid metabolism. EDys contributes to impaired insulin action, by altering the transcapillary passage of insulin to target tissues. Reduced expansion of the capillary network, with attenuation of microcirculatory blood flow to metabolically active tissues, contributes to the impairment of insulin-stimulated glucose and lipid metabolism. This establishes a reverberating negative feedback cycle in which progressive EDys and disturbances in glucose and lipid metabolism develop secondary to the insulin resistance [Kim *et al*. 2006].

The recent discovery that circulating endothelial microparticles (EMPs) are a hallmark of EDys [Brodsky *et al*. 2004] and that circulating EPCs of bone marrow origin contribute to the regeneration of damaged endothelium has opened up exciting avenues of research [Goldschmidt-Clermont *et al*. 2005]. EPCs play a key role in promoting endothelial repair processes after different injuries and their circulating levels are intimately correlated with the degree of vascular response to vasoconstrictor stimuli; in other words, the higher the EPCs, the better the vasodilator arterial response to shear stress. A low number of circulating EPCs is supposed to be an independent risk factor for coronary heart disease. Recent studies demonstrated a reduction of EPCs in patients with chronic heart failure [Valgimigli *et al*. 2004] or endothelial dysfunction [LinksHeiss *et al*. 2004]. In subjects with the MeS, circulating EPCs are synergistically decreased by clustering components of the syndrome [Fadini *et al*. 2006], and their levels negatively correlate with the homeostasis model assessment value, a measure of insulin resistance. Virtually all risk factors for atherosclerosis have been associated with decreased levels of circulating EPCs, while absent or insufficient EPCs in patients with endothelial-cell injury may affect the progression of cardiovascular disease, with EPCs as an independent predictor of cardiovascular outcomes [Werner *et al*. 2007]. The finding that men with ED of any origin have reduced number of EPCs and that men with type-2 diabetes [Esposito *et al*. 2007] or who are overweight [Esposito *et al*. 2009] have increased levels of EMPs which are independently involved in the pathogenesis of ED, opens a new scenario for the application of these potential novel markers in the early detection of ED. However, there is still a matter of debate as to whether any reliable surrogate marker of EDys in cardiovascular medicine may be applied [Braunwald, 2008]. We can conclude that endothelial integrity has a paramount role in preserving a man from cardiovascular and genital organ injuries.

Hyperhomocysteinemia (HHcy) is considered one of the most important CRFs increasing considerably the risk of stroke and myocardial infarction. With respect to endothelial function, direct effects of HHcy on vascular endothelial cells have been demonstrated through the reduction of endothelial NO production. Also, the presence of mild elevations of plasma total homocysteine has been identified as an independent risk factor for early atherosclerotic vascular disease [Nygård *et al*. 1997]. Mild HHcy has a complex etiology, including insufficient intake of vitamins B6 and B12 and folate and genetic factors, of which an homozygote mutation of 5-methylenetetrahydrofolate reductase (MTHFR) thermolabile variant is probably the most important one (See Figure 1). The mechanism of premature CVD or ED in this context is not precisely known [Lombardo *et al*. 2004], but may be related to increased vulnerability to lipid toxicity, vascular smooth muscle-cell growth-factor properties of homocysteine, endothelial damage, or vasomotor dysfunction or to disorders of platelet aggregation and coagulation [Bellamy and McDowel, 1997].

Testosterone and endothelium

The cerebral vasculature is a target tissue for sex steroid hormones. Estrogens, androgens, and progestins modulate the function and pathophysiology of cerebral circulation [Krause *et al*. 2006]. Hypogonadism related-ED represents the link between internal medicine and sexual medicine, so far. T circulating-levels are determinant for correct endothelial function, and tend to a stepward decrease with aging and with
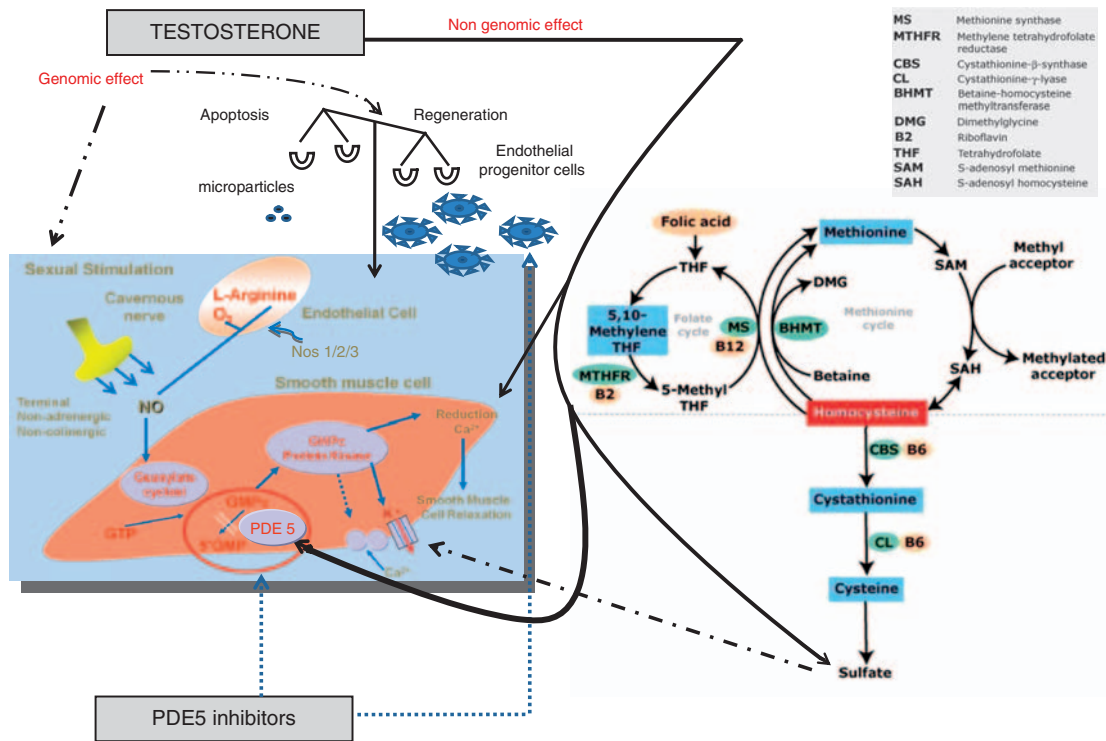
Figure 1. Schematic representation of different pathways related to penile erection in humans controlled by testosterone and phosphodiesterase type 5-inhibitors.

the presence of numerous comorbidities [Travison *et al.* 2007]. T plays a key role in coordinating and facilitating the delicate balance between the effect of endogenous vasoconstrictors and vasorelaxing agents of vascular tone [Aversa *et al.* 2005; Thompson and Khalil, 2003]. This process is supported by the fact that androgen receptors (ARs) have been localized within vascular endothelium and smooth muscle-cells. Thus, arterial functions may be directly subject to T influence and, most likely, two independent pathways of T-induced effects within the vessel wall can be assumed (i.e., genomic and nongenomic) [Orshal and Khalil, 2004; Wynne and Khalil, 2003]. The classic pathway of androgen action involves steroid binding to the AR, a ligand-activated transcription factor acting on the genome [Heinlein and Chang, 2002a; Kallio *et al.* 1996; Chang *et al.* 1995]. The genomic action of AR is modulated by a large variety of coregulators, which are proteins that fine-tune target gene expression by enhancing (coactivators) or restraining (corepressors) transcription [Lee and Chang, 2003; Heinlein and Chang, 2002a]. Although T circulates throughout the body, the factors responsible for variation in tissue androgen sensitivity remain to be further clarified. Intensity of

expression of the single human AR [Kang *et al.* 2003; Lutz *et al.* 2003] partly defines androgen sensitivity, but AR is almost ubiquitously expressed to some degree in tissues. Further biologic determinants of tissue androgen sensitivity, including the functional AR polymorphisms as well as tissue distribution and regulation of AR coregulators, androgen metabolic enzymes, and nongenomic mechanisms, remain to be better defined so that their net integrated effects can be more fully understood.

Androgen sensitivity could be modulated by a functional polymorphism of the AR that influences the strength of the genomic signal transduced from its interaction with an androgen as a bound ligand. One such functional AR polymorphism is the exon 1 triplet CAG (polyglutamine), whereby the repeat length is directly correlated with T levels [Heinlein and Chang, 2002b]. There is now considerable evidence for rapid, non-genomic effects of steroid, including androgens [Cho *et al.* 2003]. Non-genomic steroid action is distinguished from classic genomic effects by rapid onset (seconds to minutes) that is faster than genomic mechanisms; insensitivity to inhibition of RNA and protein synthesis; effects produced by steroids unstable to access

the nucleus (either covalently linked to membrane-impermeable macromolecules or in cells lacking a nucleus); and not usually blocked by classic antagonists resulting from different steroidal specificity from classic cognate nuclear receptors. As for other steroids, non-genomic androgen effects characteristically involve the rapid induction of conventional second-messenger signal transduction cascades, including increases in cytosolic calcium and activation of protein kinase A, protein kinase C, and MAP-kinase, leading to diverse cellular effects, including smooth muscle relaxation, neuromuscular and junctional signal transmission, and neuronal plasticity [Aversa *et al.* 2005]. Most non-genomic effects involve a membrane receptor, and putative-binding sites are described for all major classes of steroids, including androgens [Herve, 2002; Gerdes *et al.* 2000]. No membrane AR has been characterized, but preliminary evidence of a low-affinity microsomal membrane-binding site for alkylated androgens [Chirino *et al.* 1989] and an endothelial cell plasma membrane dehydroepiandrosterone (DHEA)-binding site [Williams *et al.* 2002] still require functional proof of specific receptor status. A plasma membrane sex harmone binding globuline (SHBG) receptor capable of modulating androgen action at the level of plasma membranes and initiating intracellular cyclic 3'-5' adenosine monophosphate (cAMP) signaling has been described in humans. The SHBG receptor remains to be fully characterized, and it is not clear whether it has any physiological role in species like rodents that lack circulating SHBG [Rosner *et al.* 1999].

Recent animal and *in vitro* studies have further documented that T up-regulates the expression of arterial AR mRNA and is associated with an inhibitory effect on neo-intimal plaque formation [Hanke *et al.* 2001]. Additionally, positive acute hemodynamic effects of T on coronary vasomotion and stress-test induced ischemia were reported [Rosano *et al.* 1999]. Vascular ARs may mediate these effects of T on the arterial wall, and T has been shown to produce coronary, aortic, and brachial vasculature dilatation by activation of both endothelial-dependent and -independent mechanisms [Chou *et al.* 1996]. Endothelium-dependent effects of T are likely mediated, at least in part, through NO production, whereas mechanisms of endothelium-independent effects involve one or more types of smooth muscle ion conductance channels [Littleton-Kearney and Hurn, 2004]. The

interaction of T with its specific nuclear receptors may trigger not only long-term genomic effects, but also acute non-genomic vasodilator responses [Wynne and Khalil, 2003]. T may activate the endothelium and stimulate the NO-cGMP and/or the hyperpolarization-mediated vascular relaxation pathway and may thus represent potential beneficial effects of T against coronary artery atherosclerosis. Additional endothelium-independent effects of T may involve inhibition of the signaling mechanism of vascular smooth muscle contraction such as intracellular concentration $[Ca^{2+}]$ and protein kinase C, whereas a significant portion of the vasorelaxing effect of T appears to be endothelium independent because no significant difference is observed between the relaxing effect of the hormone in isolated vessels with or without endothelium [Crews and Khalil, 1999]. Also, inhibition of NO-synthase, prostaglandin synthase, and guanylate cyclase do not appear to affect the vasorelaxing effect of T, suggesting that the T-induced vascular relaxation may involve inhibition of the mechanism of vascular smooth muscle contraction [Murphy and Khalil, 1999; Yue *et al.* 1995]. Several studies have shown that acute administration of T induces a rapid relaxation in vascular tissues of different species including humans [Costarella *et al.* 1996; Perusquia *et al.* 1996], suggesting a non-genomic effect of this hormone on vascular reactivity [English *et al.* 2002]. Different mechanisms have been proposed to explain T-induced vasodilatation [Tep-Areenan *et al.* 2002] but it remains a matter of debate which is the effective mechanisms and which are the mediators involved of the T-induced vasorelaxation. T might induce relaxation in human isolated corpora cavernosa strips by activation of smooth muscle adenosine triphosphate-sensitive $K^{(+)}$ channels. This finding suggests that T, in addition to its known endothelial action, might regulate erectile function locally by its action on the smooth muscle of the human corpus cavernosum [Yildiz *et al.* 2009]. By contrast, contractile studies suggested that T may enhance thromboaxane A2 (TXA2)-induced coronary vasoconstriction in guinea pigs [Schrör *et al.* 1994]. Androgens may also regulate the expression and density of TXA2 receptors in cultured rat aortic and guinea pig coronary smooth-muscle cells [Higashiura *et al.* 1997]. TXA2 acts through membrane surface receptors to aggregate platelets by both constricting or modulating the proliferation vascular smooth-muscle cells [Aiayi *et al.* 1995]. Any systemic vascular

effect of T, therefore, is likely to be a balance of vasodilatation by endothelial and non-endothelial effects and vasoconstriction resulting from TXA2 and possibly other mediators [Honda *et al.* 1999].

Recently, hydrogen sulphide (H_2S) has been shown to act as gaseous modulator on rat vascular system both *in vivo* [Zhao *et al.* 2003] and *in vitro* [Hosoki *et al.* 1997]. H_2S is an endogenous gas produced in mammals from L-cysteine by two different enzymes: cystathionine β-synthetase (CBS), predominantly present in the central nervous system, and cystathionine γ-lyase (CSE), predominantly localized in the cardiovascular network [Levonen *et al.* 2000; Yap *et al.* 2000; Van der Molen *et al.* 1978]. The finding that activation of K-channels is involved in T-induced vasodilatation [Deenadayalu *et al.* 2001; Ding and Stallone, 2001] led to the hypothesis of a possible involvement of H_2S as a mediator involved, since drugs that block K_{ATP} channels, such as glibenclamide, have been shown to block the relaxant effect caused by exogenous H_2S [Cheng *et al.* 2004; Zhao *et al.* 2001]. In a recent study, Bucci *et al.* elegantly suggested that the T non-genomic vascular effect might involve the L-cysteine/H_2S pathway. In particular, the data presented demonstrated that, in rat aorta, the non-genomic effect of T is linked to a positive modulation of CSE/CBS activity. The H_2S produced acts on K_{ATP} channels, contributing to the vasodilator effect of T. Thus, H_2S involvement in the vascular activity of T may help to explain the beneficial effects of T on the cardiovascular system [Bucci *et al.* 2008]. On this basis, preliminary animal studies have suggested the involvement of H_2S in facilitating erectile function [Srilatha *et al.* 2007, 2006]. However, the involvement of a functionally intact L-Cys/H_2S pathway in human penile erection has not yet been demonstrated. In a recent work, by using human corpora obtained by a standardized surgical procedure, it has been demonstrated that human penile tissue expresses both CBS and CSE, and tissue homogenates efficiently convert L-Cys to H_2S. Functional studies, performed *in vitro*, confirm that the L-Cys/H_2S pathway plays a functional role in human tissue. Indeed, either sodium hydrogen sulfide (NaHS), an exogenous source of H_2S, or L-Cys, the substrate for CBS/CSE, relaxed HCC strips in a concentration-related manner. Pharmacological modulation of CBS and CSE by using a CSE inhibitor and/or a CBS inhibitor confirmed the involvement of the L-Cys/H_2S pathway both in

vitro and in vivo in rats. Collectively, these observations indicate that a functional L-Cys/H_2S pathway may be involved in mediating penile erection in humans and other mammals [D'Emmanuele di Villa Bianca *et al.* 2009]. (see Figure 1).

Moreover, the recent finding that hypotestosteronemia is associated with a low number of circulating EPCs in young hypogonadotropic hypogonadal patients [Foresta *et al.* 2006] and that T replacement is able to revert EPC reduced counts through a direct stimulatory effect on the bone marrow, [Foresta *et al.* 2008] clearly suggests a new potential field of application for T replacement therapy. Pilot studies suggest that T may have a role as an antiatherogenic therapy, by preserving endothelial and smooth-muscle cell integrity; also, recent evidence demonstrated that androgen deprivation for prostate cancer may reduce insulin sensitivity, thus suggesting a key role for T in the development of insulin resistance/MeS also [Smith *et al.* 2006].

PDE5-inhibitors and endothelium
The PDE5-inhibitors (PDE5-i) have revolutionized the management of ED since they appeared to offer advantages over other medical approaches in terms of ease of administration and costs, and oral drug treatment with PDE5-i is now widely advocated as first-line therapy. These agents act by potentiating the action of intracavernosal NO, thereby leading to a more sustained erection. Sildenafil was the first PDE5-i to be released and has been studied extensively. Subsequently two other agents – vardenafil and tadalafil – have been introduced. All of these drugs have been shown to be effective across a wide range of ED etiologies and have been shown to improve erectile function, penetration and maintenance of erection, resulting in more successful intercourse. Their effects are greater at higher doses [Lugg *et al.* 1995]. Sildenafil and vardenafil are short-acting agents, while tadalafil is a long-acting agent thus allowing the user more flexibility in planning sexual activity [Aversa *et al.* 2006]. Although the various classes of PDE5-i differ with respect to selectivity and pharmacokinetic profiles, their efficacy and safety are almost comparable in broad populations of men with ED. PDE5-i have no effect on the penis in the absence of sexual stimulation. The ability of a drug to select the target tissue (penis) while bypassing other tissues (vascular system) depends upon the fact that human

corporeal smooth muscle is known to be rich in PDE5, which is abundant in the aorta and in some part of the peripheral vascular smooth muscle [Mercapide *et al.* 1999]. Experimental findings support the fact that chronic administration of PDE5-i may regulate the transduction pathway leading to the activation of endothelial-NOS but has no effect on NO bioavailability or on the cGMP pathway, thereby eliminating a possible concern for tachyphylaxis [Behr-Roussel *et al.* 2005].Since PDE5-i are widely used in treating male ED, it has been recently hypothesized that they may exert also important systemic effects, at the endothelial level, as well [Reffelmann and Kloner, 2006].

Recent research focused on PDE5-i has proven to be of great impact in the treatment of numerous human extra-sexual diseases [Aversa, 2008b]. In fact, specific inhibitors of members of the PDE super family are currently being investigated for the treatment of asthma, acute ischemic stroke, cancer, systemic inflammation, pulmonary hypertension, and many other conditions and they have been approved for clinical use in the treatment of claudicatio intermittens, congestive heart failure, chronic obstructive pulmonary disease [Lin, 2003]. The distribution of PDE activity has been determined with anti-PDE1 and anti-PDE5 antibodies in the human cardiac ventricle and saphenous vein, and *in vitro* studies were performed on the isolated human cardiac ventricle, corpora cavernosa, saphenous vein, and mesenteric artery as well as on rabbit aorta, dog coronary artery, dog trabecular tissue, and rabbit and human platelets. In fact, sildenafil selectively increases cGMP levels in coronary vascular smooth muscle tissue but produces no change in cyclic adenosine monophosphate (cAMP) levels, which is consistent with the drug's selectivity for PDE5 [Wallis *et al.* 1999]. An interesting study by Turko *et al.* showed that sildenafil stimulates cGMP binding to the allosteric sites of PDE5 by interacting at the catalytic site of this enzyme, but the drug does not compete with cGMP for binding at the allosteric sites. In that study, it was concluded that the selectivity and potency of sildenafil was likely to be provided by a non-conserved residue or residues of specific aminoacids in the PDE5 catalytic domain [Turko *et al.* 1999].

As already outlined above, the event that triggers EDys is represented by the reduction of the overall antioxidant pool [Parodi *et al.* 2007] with the consequent reduced response to oxidative stress and the activation of several pro-atherogenic processes: reduction of NO bioavailability; increased levels of circulating free fatty acids, with subsequent sub-endothelial storage of lipid depots and increased smooth-muscle cell proliferation of the media layer of the vascular wall [Marks *et al.* 1995]. Pro-inflammatory and -infective processes may in turn contribute to activate and amplify the acute endothelial injury thus perpetuating such a vicious circle. In early atherogenic lesions, EDys causes adhesion and migration of monocytes and T-lymphocytes in the vascular inner layer in response to increased endothelial production of intercellular molecules, i.e. selectin, VCAM-1 and ICAM-1 that may quench NO [Basta *et al.* 2004]. This process may concur to determine impaired arterial inflow to the penis, thus contributing to persistent ED.

Growing evidence indicates that PDE5-i have a beneficial effect on inflammatory activation and surrogate markers of EDys. Although exact mechanisms are not fully known, the basis for these anti-inflammatory effects is the increased activity of the NO-cyclic guanosine monophosphate (NO-cGMP) axis [Vlachopoulos *et al.* 2005]. Through their up-regulation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, cytokines not only increase formation of superoxide, but they also up-regulate the expression of PDE5. PDE5-i antagonize these inflammatory effects by enhancing the NO-cGMP pathway, which, apart from augmenting smooth-muscle-cell relaxation, inhibits NADPH-oxidase expression/activity [Hotston *et al.* 2007]. Beneficial acute and chronic effects of sildenafil on arterial function in men with ED are well known [Desouza *et al.* 2002]. Given the unfavourable effect of inflammation on arterial function, this effect could be partly attributed to a robust anti-inflammatory action of daily sildenafil [Aversa *et al.* 2008c]. In a pilot study, a 4-week treatment with tadalafil every other day produced a favourable effect on endothelium-dependent vasodilatation of cavernous arteries compared with an on-demand dosage in men with ED of any origin. In that study, positive effects on markers of endothelial function and inflammation, including VCAM and ICAM, ET-1, and high-sensitivity C-reactive protein, as well as on insulin were also found [Aversa *et al.* 2007a]. Daily tadalafil also significantly decreased hypoxia-induced up-regulation of tumor-necrosis factor alpha (TNF-*a*) and interleukin-1 beta (IL-1*ß*) expression in pulmonary arteries and improved

erectile function in diabetic men, representing a preferred dosing option when compared with on-demand administration [Buvat *et al.* 2006]. Thus, an emerging role in the management of ED patients seems to be the possible use of daily dosing with a threefold purpose: First, recovery of non-responders to on-demand therapy; second, rehabilitation of erectile function which means endothelial rehabilitation; and third, modification of hormonal patterns, thus adding clinical evidence to the possibility of alternative regimens producing multiple systemic benefits [Bruzziches *et al.* 2008]. There is growing evidence that patients presenting with ED should be investigated for CVD, including diabetes, even if they have no symptoms. HHcy, known to be an important risk factor for EDys, seems to be an important determinant in ED especially in diabetic patients [Al-Hunayan *et al.* 2008]. The relationship between HHcy and low arterial blood supply to penile arteries in patients with ED [Demir *et al.* 2006] suggests that adopting strategies to reduce HHcy may offer the potential of improving the functioning of the entire vascular system thus turning out a men who is a PDE5-i non-responder into a responder [Lombardo *et al.* 2004]. On the basis of currently available evidence, it is possible to hypothesize that EDys and systemic cardiovascular pathology may be modulated by daily PDE5 inhibition, resulting in improved erectile function owing to effects upon both local and systemic targets. Unlike pulmonary hypertension, for which sildenafil may provide a net cost saving compared with inhaled or intravenous medications [Vida *et al.* 2007], daily PDE5-i use may be actually limited by high costs, given the average course frequency of five to six times per month. Should lower-dose daily administration confirm attractive efficacy and safety profiles compared with on-demand use, and equivalent net monthly cost approach, once-a-day PDE5-i use will become an important treatment option especially in complicated patients [Aversa *et al.* 2007b].

The endothelium links internal to sexual medicine

Since the metabolic aspect of ED is rather neglected or not sufficiently treated, it is our opinion that ED should mark the starting point for the evaluation and prevention of significant severe diseases (such as diabetes, dyslipidemia, atherosclerosis, hypertension, CAD, neuropathy, etc.) hitherto unknown by the patients

[Foresta *et al.* 2009], all of which contribute to CVD and are associated with EDys. These abnormalities frequently cluster in individuals, and the term MeS is now widely used to define this cluster. On the other hand, many established therapies for CVD, such as β-blockers, angiotensin converting enzyme inhibitors (ACE-i), and statins, have been investigated for their impacts on the process of atherosclerosis and EDys [Shindel *et al.* 2008]. Recent studies have demonstrated that statin treatment increases the number of EPCs and improves EPC function. [Llevadot *et al.* 2001]. Moreover, statin therapy is able to promote vascular repair after balloon injury mediated by EPC [Walter *et al.* 2002]. By contrast, it has been reported that the statin dose during chronic and continuous treatment independently predicts reduced numbers of circulating as well as isolated EPCs in patients with CAD [Hristov *et al.* 2007]. A significant effect of statins in lowering total T and SHBG levels in a population of men with type-2 diabetes has been recently demonstrated [Stanworth *et al*, 2009]. These findings have important implications for the diagnosis of hypogonadism not only in diabetic men receiving statin treatment but also other drugs that are able to directly inhibit T synthesis, i.e. antiandrogens, antifungals. There is growing evidence that ED can be considered as a useful surrogate marker for CAD, with studies showing that a large proportion of men develop the problem before cardiac symptoms become evident [Aversa and Bruzziches, 2007]. One study showed that ED symptoms occurred before symptoms of CAD in 67% of men in a consecutive series presenting with chest pain and angiographically documented CAD. Of particular note, all patients with type-1 diabetes and ED developed sexual dysfunction before CAD onset [Montorsi *et al.* 2003a]. Gazzaruso *et al.* also evaluated the presence of ED in 133 uncomplicated type-2 diabetic men with angiographically verified silent CAD and in 127 type-2 diabetic men without myocardial infarction at exercise ECG, 48-h ambulatory ECG and stress echocardiography. The findings showed a strong, independent association between ED and silent CAD in apparently uncomplicated type-2 diabetic patients [Gazzaruso *et al.* 2004]. The obvious reason why ED may present before other signs of CVD is because penile artery diameter is smaller (1–2 mm) than the coronary artery (3–4 mm) or carotid artery (5–7 mm), so the symptoms associated with atherosclerosis occur sooner (the 'artery size' hypothesis).

These studies have demonstrated that cardiovascular assessment of men with ED enables the detection of CVD and has been showed that ED presented well before angina symptoms of CAD in two-thirds of men with a mean time interval of almost 3 years [Kaiser *et al.* 2004; Montorsi *et al.* 2003b]. Another study revealed that 45% of men with ED had previously undiagnosed hyperlipidemia, 5% had undiagnosed diabetes and 7% had undiagnosed hypertension [Billups and Friedrich, 2000]. Kaiser *et al.* also looked at whether patients with vascular ED and no other clinical CVD had structural and functional abnormalities at other levels. The vascular parameters of 30 patients with ED (and 27 age-matched normal controls) were measured. Results showed that patients with ED have a peripheral vascular defect in endothelium-dependent and -independent vasodilation that occurs before the development of other overt functional or structural systemic vascular disease and is independent of other traditional risk factors [Kaiser *et al.* 2004]. Detecting atherosclerosis at this early stage could potentially prevent life-threatening events by facilitating appropriate intervention. This provides justification for investigating ED patients for other cardiovascular symptoms. Preliminary diagnostic testing for silent CAD by means of adenosine stress myocardial perfusion scintigraphy in diabetic patients with ED revealed that 45% of them had coronary alterations in the presence of concomitant penile artery damage as detected by penile ultrasound [Corona *et al.* 2008]. However, for the future, it will be important to perform studies in order to assess the sensitivity, specificity and cost-effectiveness of using ED as a marker for CVD [Kirby *et al.* 2005]. Recent data suggest tadalafil could exert prolonged beneficial effects on vascular endothelial function if taken regularly [Caretta *et al.* 2005]. In this view, once-a-day dosing with tadalafil should be an attractive alternative especially for those ED patients with overt EDys. We can speculate that precocious treatment of these men with daily long-acting PDE5-i, i.e. tadalafil, may represent a future strategy for preventing or reducing the extent of coronary dysfunction and/or myocardial infarction. A daily-based approach also may improve organic ED at the level of vascular endothelial function. Another possible explanation as to why chronic PDE5-i may induce endothelial rehabilitation comes from a recent study by Ayala *et al.* [Ayala *et al.* 2007] that demonstrated improvements in insulin action by chronic sildenafil in a murine model of diet-induced obesity and insulin resistance. In their experiment, this improvement occurred even in the absence of an exogenous NO donor, suggesting that the endogenous supply of NO in the high fat-fed state did not limit the effect of sildenafil on insulin action. Chronic PDE5 inhibition also resulted in increased energy expenditure, suggesting that improved energy balance and weight reduction might be partially responsible for the enhanced insulin action without any adverse effects on cardiac morphology or blood pressure measured *in vivo*, supporting human studies showing no association between long-term use of sildenafil and risk of ischemic events [Pegge *et al.* 2006]. These data have been confirmed with administration of chronic, short-acting [Aversa *et al.* 2008c] and long-acting PDE5-i tadalafil in men with CRFs even in the absence of ED [Rosano *et al.* 2005]. A pilot study by McMahon [McMahon, 2004] evaluated improvements in response rates once non-responders to on-demand tadalafil were shifted to daily dosing and concluded that up to 30% of non-responders may be 'salvaged' by daily dosing. Additional data on endothelial response to chronic tadalafil have been obtained in another pilot study involving men with ED and comorbidities. Results suggest a beneficial effect on endothelial function via decreasing plasma levels of surrogate markers (i.e., endothelin-1, C-reactive protein, CAMs). In this study, the authors added another possible mechanism underlying penile rehabilitation: improvement of morning erections, which may provide better end-organ oxygenation, especially in the diabetic population. This latter aspect was confirmed in another study [Proietti *et al.* 2007] carried out in male sclerodermic patients with ED. Besides improvement in penile vascular circulation, daily tadalafil markedly reduced endothelin-1 plasma levels and increased morning erections after 12 weeks of treatment. Moreover, in ED-patients with systemic sclerosis, it was hypothesized that the reduction of adrenomedullin levels obtained after daily tadalafil, was responsible for the reduction of Raynaud's phenomenon number and severity of attacks over the time [Rosato *et al.* 2009]. Although this appears to be a fascinating hypothesis that opens new treatment perspectives, larger studies are needed in order to assess the possible clinical implications of chronic therapy with PDE5-i for cardioprotection and endothelial rehabilitation in patients with comorbidities. Most notably, these drugs are largely used for obstructive sleep

apnea-induced ED. Because NO promotes upper airway congestion, muscle relaxation, and pulmonary vasodilation, it has been demonstrated that a single 50 mg dose of sildenafil at bedtime may worsen respiratory and desaturation events [Roizenblatt *et al.* 2006] so that caution is mandatory in this group of patients.

The relationship between diabetes, the MeS and T deficiency is complex. Male hypogonadism is generally characterized by abnormally low serum T-levels. Typical symptoms include change in mood and cognitive function disorders [Kenny *et al.* 2002], decreased bone mineral density [Snyder *et al.* 1999], increased visceral adiposity and body mass index (BMI) [Tan and Pu, 2002], decreased muscle mass and strength [Kenny *et al.* 2001], and sexual dysfunction [Morales and Heaton, 2001]. Free T-levels begin to decline at a rate of 1% per year after the age of 40 years. It is estimated to affect between 19 and 34% of men over the age of 60 [Kalyani and Dobs, 2007]. Cross-sectional studies have found that between 20 and 64% of men with diabetes have hypogonadism, with higher prevalence rates found in the elderly. Hypogonadism can be a risk factor for the development of diabetes and the MeS through various mechanisms including changes in body composition, androgen receptor polymorphisms, glucose transport, and reduced antioxidant effect. Conversely, diabetes and the MeS can be risk factors for hypogonadism through some similar but mostly distinct mechanisms, such as increased body weight and leptin levels, decreased sex hormone binding globulin levels, suppression of gonadotrophin release or Leydig cell T production, cytokine-mediated inhibition of testicular steroid production, and increased aromatase activity contributing to relative estrogen excess [Nieschlag *et al.* 2004]. Defects in the hypothalamic-pituitary-gonadal axis may also result from type-2 diabetes, visceral obesity (which is strongly associated with insulin resistance), CAD and MeS and from treatments with a wide range of medications [Kapoor *et al.* 2006]. Androgen deficiency can also occur at any time during a man's life, but occurs more frequently with advancing age (LOH, late onset hypogonadism). Short-term interventional studies have also demonstrated that T replacement therapy produces an improvement in insulin sensitivity in men. Thus, hypotestosteronemia may have a role in the pathogenesis of insulin-resistant states and androgen replacement therapy could

be a potential treatment that could be offered for improvements in glycemic control, insulin resistance, cholesterol and visceral adiposity and reduction in cardiovascular risk, particularly in diabetic men [Heinlein and Chang, 2002b]. Treatment of ED in men with diabetes has been changed by the introduction of PDE5-i. However, men with diabetes tend to respond less positively to these agents, at least as currently prescribed. This decreased responsiveness may be related to the severity of EDys that usually occurs in these patients. There has been much recent interest in the potential relationship between LOH and diabetes, but there is no evidence of a causal relationship and the evidence in favor of treating borderline hypogonadism in diabetes is limited.

Combination therapy in internal and sexual medicine

The role of T salvage in the setting of 'sub-clinical' hypogonadism of patients with ED has been evaluated in the presence and absence of PDE5-i effectiveness [Morelli *et al.* 2004]. Preclinical investigations reported by Traish *et al.* [Traish *et al.* 1999] provided convincing evidence that PDE5-i are ineffective in improving erectile function in androgen-deficient animals and that the re-administration of androgen facilitates PDE5-i action. As already outlined, T may directly control the expression and activity of PDE5 in human penile tissues [Traish *et al.* 2003]. Androgen deficiency or hypogonadism reduce the cavernosal expression of PDE5 mRNA, protein and enzyme activity, and T supplementation restores PDE5 expression and activity which represents the substrate for the inhibitory action of PDE5-i. The effects of androgens on penile tissues in experimental models demonstrated that androgen deprivation induces: smooth-muscle cell degeneration (apoptosis), adipose tissue deposition with associated fibrosis of corpus cavernosum [Traish and Guay, 2006]; reduction in the expression of eNOS and nNOS; decrease of arterial inflow and increase of venous out-flow in the corpus cavernosum; enhanced response to mediators of vasoconstriction and smooth muscle contraction such as α-adrenergic agents; decrease of NO-mediated smooth muscle relaxation during sexual stimuli; and down-regulation of expression of PDE5 enzyme. This latter aspect seems to be crucial in determining metabolic and structural imbalance in the corpus cavernosum, resulting in venous leakage and ED; this suggests a rationale

for combination therapy with PDE5-i combined with T preparation for treating ED refractory to monotherapy [Shabsigh *et al.* 2006]. These clinical observations suggest a critical role for T in human erectile function. Aversa and co-workers were the very first to demonstrate that in the clinical setting, men with ED and low free-T may have an impaired relaxation of the penile smooth muscle, thus providing clinical evidence for the importance of androgen in regulating erectile function [Aversa *et al.* 2000]. More importantly, in all patients a strong direct correlation between resistive index values and free-T levels has been reported. Again, this relationship was maintained also when adjusted for age, SHBG, and estradiol. These results indicate that in men with ED, low free-T may correlate independently of age with the impaired relaxation of the cavernous smooth muscle cells. In a subsequent study, it has been shown that the treatment with transdermal T-patch and sildenafil on-demand, significantly increased scores in the erectile function domain of the International Index of Erectile Function (IIEF) [Aversa *et al.* 2003]. Other studies demonstrated that T therapy is able to improve erectile function and the response to PDE5-i in patients with ED and hypogonadism and also in men with LOH symptoms [Shabsigh *et al.* 2004; Kalinchenko *et al.* 2003]. Administration of intramuscular T and sildenafil was found to be efficacious in renal transplant patients and in patients on renal dialysis [Tas *et al.* 2006], and oral T has been reported to reverse ED associated with type-2 diabetes in patients failing on sildenafil therapy alone [Lin *et al.* 2001]. On the other hand, few studies suggested that in ED-associated hypogonadism, T alone may not be sufficient for early restoration of erectile potency [Shabsigh, 2003; Yassin *et al.* 2006] thus suggesting that the combination with a PDE5-i is initially recommended. In conclusion, T plus PDE5-i combination therapy improves the response to PDE5-i in patients previously not responding to PDE5-i therapy alone and in whom T levels at baseline are in the hypogonadal or normal-low adult range, i.e. late-onset hypogonadism, without any side effects on prostate size and PSA values in the long-term [El-Sakka *et al.* 2005]. Noteworhthy, T-induced remodelling of penile tissue structure is a process that may require a longer period of T administration, as long as 4 weeks for expected results on erection.

Regarding the hypothesis on steroid hormone changes induced by chronic treatments with PDE5-i for sexual disturbances, several studies indicate that resumption of sexual function with different oral therapies is able to increase T levels [Jannini *et al.* 1999]. It has been demonstrated as a beneficial effect of the administration of sildenafil or tadalafil on steroid hormones, i.e. total- and free-T that raised more significantly in those subjects using tadalafil *versus* sildenafil probably because of the more robust activation of the hypothalamic-pituitary axis due to higher number of sexual intercourses per month allowed by the long-acting drug [Carosa *et al.* 2004]. Successive studies have demonstrated that due to its indirect action, the raising effect on T is transient and is lost twelve months after continuative tadalafil assumption; T:estradiol ratio increased due to a significant reduction of estradiol levels which may account for by tadalafil persistent efficacy over the time [Greco *et al.* 2006a]. This study suggested for the first time a possible direct inhibitory effect of tadalafil on aromatase activity in humans independently from the body mass index and the quantity of adipose tissue. Preliminary studies from our laboratory aimed to investigate whether tadalafil could directly modulate aromatase expression in differentiated human adipocytes in culture demonstrated that it directly affect aromatase expression, hence it could positively modulate the T:estradiol circulating ratio *in vivo* [data not published]. Thus, the aromatase activity inhibition due to chronic exposure of tadalafil might be responsible for tadalafil sustained effectiveness in vivo during daily tadalafil low-dose treatment.

Conclusion

It is clear that the modification of reversible causes, i.e. inadequate lifestyle, cigarette smoking, alcohol or recreational drug abuse [Aversa *et al.* 2008b], hyperglycemia and hypertension, must represent the first approach to improve endothelial function and to promote general well-being and sexual health. Almost 20–30% of ED cases may be attributed to subnormal T levels [Morelli *et al.* 2005] and T deficiency is frequently associated with chronic diseases, that are in turn associated with increased deposition of visceral fat; this latter serves as an endocrine organ, producing inflammatory cytokines and thus promoting EDys and concurring to determine vascular disease. In fact, a considerable body of evidence exists suggesting a link among reduced T plasma levels and the presence of diabetes, MeS and systemic

vascular diseases. Adequate T concentrations are also crucial for the regulation of a correct endothelial function, for the expression of penile PDE5 isoenzyme [Greco *et al.* 2006b; Morelli *et al.* 2004] as well as for the adequate production of H_2S. Men with ED and low T levels are potential candidates to benefit from combination therapies if response to monotherapy is not sufficient [Rosano, 2000]. However, if we consider overall hormonal alterations of sex steroids in men complaining ED, it is noteworthy to remember that up to 41% of these men may present with alterations of T:estradiol ratios [Aversa *et al.* 2006a]. The beneficial effects of T supplementation along with chronic PDE5-i administration on endothelium in deficient men with or without ED appears to be a promising therapy to boost the effects on remodeling of vascular wall determined by single vasoactive agents used to treat internal medicine diseases [Aversa, 2008], and may represent a 'salvage' therapy especially in difficult-to-treat ED. Although the safety of this class of agents has been indisputably established for the treatment of ED on an on-demand basis, there remains a paucity of controlled data on the long-term endothelial effects and possible hormonal untoward effects deriving from chronic use.

Conflict of interest statement
None declared.

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