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# "New pharmacological prospectives in erectile function"

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# *CHAPTER* 1

## Introduction:

## physiology and pathophysiology of erectile function

# INTRODUCTION

Erectile dysfunction, known as impotence, is by no mean a modern phenomenon. It has been present throughout the ages. The word "impotence" derives from latin word "*impotencia*" that means lack of power and it has been already mentioned in the Old Testament of the Bible. In the ancient Egypt, people believed that impotence could be dependent on two causes: a natural one, where a man is not able to accomplish the sexual act, and a supernatural one; on the other hand, according to Greek mythology, impotence in adult life was caused by sexual anxiety during the childhood and in the middle ages it was believed to be caused by witches. Nevertheless, restrictive social attitudes and taboos have severely inhibited scientific investigation of human sexual function. In the 20$^{th}$ century a sort of sexual revolution came up and in recently many studies contributed to understand sexual anatomy and physiology and to introduce new concepts about sexual therapy, further lessening social barriers to open discussion about human sexual behaviour.

Perhaps, progress in male sexual medicine accelerated in 1970s when, for the first time, sexual dysfunction was considered to be an endocrine or psychological problem. In these years, new surgical and pharmacological techniques were introduced for the treatment of erectile dysfunction (Michal *et al*., 1973; Michal *et al*., 1977). A specific development in sexual medicine occurred in 1992, when the National Institutes of Health in the United States recommended the use of the term *erectile dysfunction* rather than *impotence* and also defined erectile dysfunction as the "consistent inability to attain and/or maintain a penile erection, sufficient for an adequate sexual relation.

In 1990s, thanks to the contribution of many studies elucidating mechanisms of penile physiology, it has been showed that the erection is regulated by a balance between relaxant and contracting factors and that the unbalancing of these mediators is causing erectile dysfunction.

## 1.1 Anatomy

The human penis is composed of the paired corpora cavernosa and the single corpus spongiosum, which surround the urethra. The first controls the rigidity/flaccidity of the penis, while the second regulates the diameter of the urethra for urination/ejaculation. The proximal portion of the corpora cavernosa is firmly anchored to the pubic bone, but the distal end is covered by the glans penis. The corpora cavernosa are characterized by a spongy tissue structure of connective trabecular tissue surrounding vascular spaces named sinusoids. In addition the corpora cavernosa are surrounded by a fibroelastic covering, the tunica albuginea. During the erection, blood fills up sinusoids and its flow is regulated by helicine arteries, which are branches of the cavernous artery. Venules are instead, responsible for the drainage of the sinusoids from the subtunical venous plexuses, which traverse the tunica and empty into the dorsal and cavernous veins.

The two corpora cavernosa and the corpus spongiosum are surrounded by Buck's facia. The remaining superficial layers of the penis consist of subcutaneous cellular tissue, the superficial penile fascia (Dartos fascia) and the skin. The penis primarily is vascularised by pudendal artery, a branch of the internal iliac artery and it is divided into the bulborethral, dorsal and cavernosal arteries. All together these vessels supply the blood to the penis. Particularly the bulbourethral artery supplies the urethras and corpus spongiosum, while the cavernosal arteries, entering the centre of corpora cavernosa, give rise to the elicine arteries, which in turn supply the sinusoidal spaces. The paired dorsal penile arteries proceed down the penis along with the dorsal nerves and supply superficial structure and glans penis as well as the corpora cavernosa via circumflex arteries.

Instead, the superficial venous system consists of superficial dorsal veins, which drain the skin and the subcutaneous tissue above the buck's fascia. The deep venous system starts with the subtunical venules draining the sinusoids, which merge to form the emissary

veins. These veins cross the tunica albuginea and drain into the circumflex veins which in turn join the deep dorsal vein beneath Buk's fascia, which ends in the periprostatic venous plexus. The corpus spongiosum is drained by the bulbar and spongiosal veins which have many channels that communicate with the corpora cavernosa. The subtunical and emissary veins are compressed during erection.

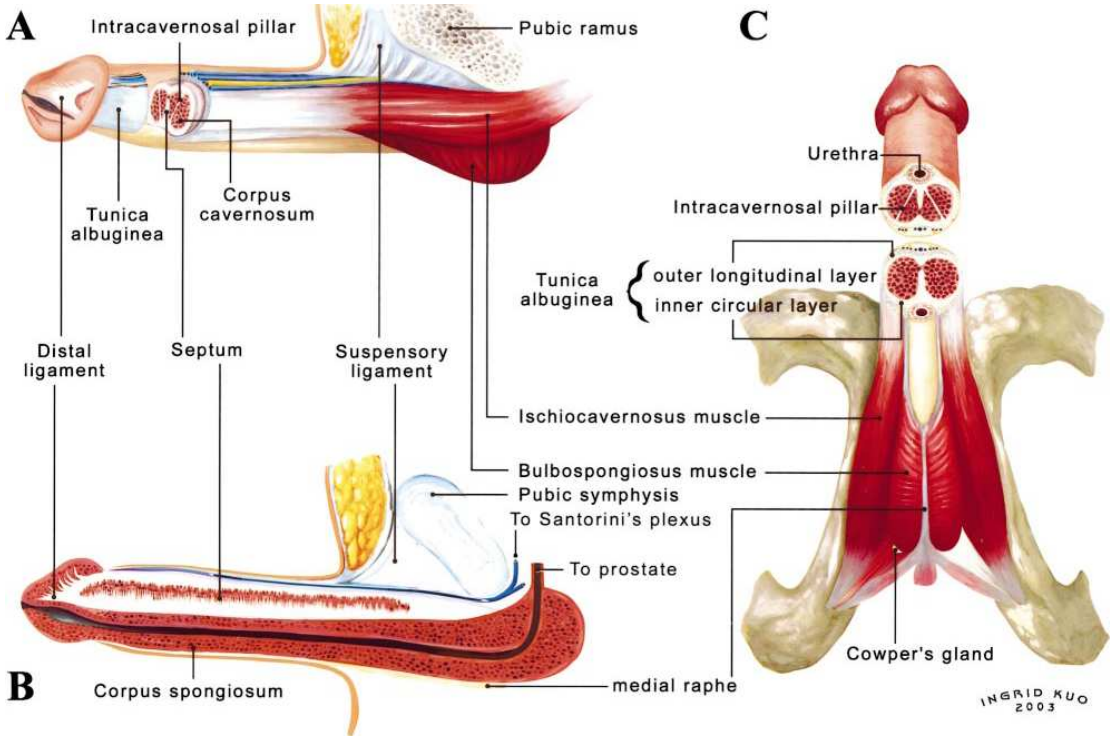



**Figure 1. Schematic illustration of human penis.**

**(A) Lateral aspect of the penis**. The penis leans on and is supported by a suspensory ligament, and terminates with glans penis. The corpora cavernosa, surrpunded by the tunica albuginea, are held by the bulbospongiosus muscle in which the fibers are mostly transverse. The corpus cavernosum is entrapped in the ischiocavernosus muscle with the muscle fibers aligned in the longitudinal direction.

**(B) Medial aspect of the penis.** The distal ligament, an inelastic fibrous structures, forms the trunk of the glans penis **.** The urethra is contained in the corpus spongiosum.

**(C) Ventral aspect of the penis.** The ischiocavernosus muscle is paired with and situated at the lateral boundary of the perineum. The anterior fibers of the bulbospongiosus muscle partially radiate to encircle the corpus cavernosum and mostly insert into the ventral thickening of the tunica.

## 1.2 Physiology of penile erection

Erection is the final common pathway of the integrative synchronized action of psychological, neuronal, hormonal, vascular, and cavernous smooth muscle systems.

### *1.2.1. Hemodynamic and mechanism of erection and detumescence*

Penile erection is an example of microcirculation where there is a predominance of adrenergic tone in the flaccid state of the penis (or turgid state) while its reduction occurs during the erect state (or rigid state). Initially the dilatation of arterioles and arteries causes an increase in blood flow and volume augmentation. Blood inflow provokes a shift from the turgid state to the full rigid state. The expansion of sinusoidal spaces traps the incoming blood in the *corpora cavernosal* due to the so called veno-occlusive mechanism: the compression of the subtunical venular plexus between the tunica albuginea and the peripheral sinusoids, in association to the stretching, reduces the venous outflow. In fact the stretching of the tunica occludes the emissary veins between the inner circular and the outer longitudinal layers and further decreases the venous outflow to a minimum. The blood trapped within the corpora cavernosa produces the erect state of the penis, which is associated to an intracavernous pressure of approximately 100 mmHg (the full erection phase). During sexual activity, the bulbocavernosus reflex is activated and the ischiocavernosus muscles contract to forcefully compress the base of the blood-filled corpora cavernosa (rigid-erection phase) (Figure 2b).

Erection thus involves sinusoidal relaxation, arterial dilatation, and venous compression (Lue *et al*.,983). The importance of smooth muscle relaxation has been demonstrated in animal and human studies (Saenz de Tejada *et al*., 1989; Ignarro *et al*., 1990), while detumescence is characterized by three phases, as reported in an animal studies (Bosch *et al*., 1991). The first phase entails a transient increase in intracorporal

pressure, indicating the beginning of smooth muscle contraction against a closed venous system. The second phase shows a slow pressure decrease, suggesting a slow re-opening of the venous channels with resumption of the basal level of arterial flow. The third phase shows a fast pressure decrease with fully restored venous outflow capacity (Figure 2a).

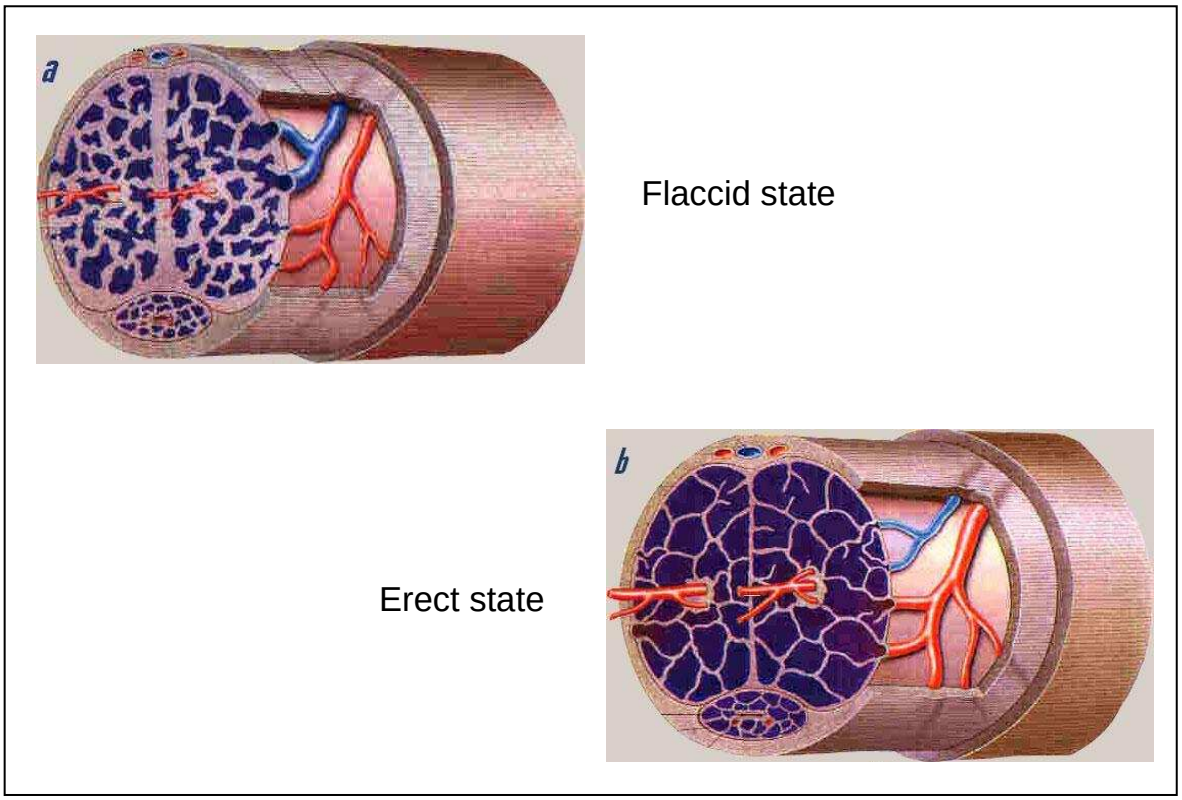



**Figure 2. Hemodynamic and Mechanism of Erection and Detumescence**

**(A)** In the flaccid state the arteries, arterioles and sinusoids are contracted. The intersinusoidal and subtunical venular plexus are open, allowing free flow to the emissary veins.

**(B)** Initially a dilatation of the arterioles and arteries cause an increase of blood flow, followed by an expansion of sinusoidal spaces and volume augmentation. Once gathered in the sinusoidal spaces, the blood is trapped in the corporal cavernosal do to the so-called veno-occlusive mechanism. This process lead to the compression of the subtunical plexuses between the peripheral sinusoids and the tunica albuginea, thus reducing the venous outflow.

### *1.2.2. Neuroanatomy and neurophysiology of penile erection*

Penile erection involves a complex interaction between the central nervous system and local factors. Primarily, it is a neurovascular event modulated by the individual's psychological and hormonal status. Sexual stimulation elicits nerves impulses that are carried to the penis to release neurotransmitters from the cavernous nerve terminals.

The neuronal system involved in erection is often divided into spinal and supraspinal networks. It is generally accepted that the spinal system directly controls erection and that the supraspinal network modulates this control mechanism through different ascending and descending pathways.

*a) Spinal pathways*

Both autonomic (sympathetic – T12-L2 – and parasympathetic – S2-S4) and somatic (sensory and motor) nerves innervate the penis. The sympathetic and parasympathetic nerves from the neurons in the spinal cord and peripheral ganglia, merge to form the cavernous nerves, which enter the corpora cavernosa and corpus spongiosum to affect the neurovascular events during erection and detumescence. The somatic nerves are primarily responsible for sensation and the contraction of the bulbocavernosus and ischiocavernosus muscles. In particular, sympathetic pathways are anti-erectile, sacral parasympathetic pathways are pro-erectile, and contraction of the perineal striated muscles upon activity of the pudendal nerves improves penile rigidity. Spinal neurons controlling erection are activated by information from peripheral and supraspinal origin.

*b) Supraspinal pathways*

Marson *et al*., (1993) and then Sachs *et al*., (1988) identified the medial preoptic area (MPOA) and the paraventricular nucleus (PVN) of the hypothalamus and hippocampus as important integration centers for sexual function and penile erection.

Many studies, by using positron emission tomography (PET) and functional MRI (fMRI), demonstrated that central neuronal activation is responsible for three types of erection: psychogenic, reflexogenic and nocturnal (Robert *et al*., 2005). Psychogenic erection is the result of audiovisual stimuli or fantasy. Impulses from the brain modulate the spinal erection centers (T11-L2 and S2-S4) to activate the erectile process. Reflexogenic erection is produced by tactile stimuli to the genital organs. The impulses reach the spinal erection centers, then some of them follow the ascending tract, resulting in sensory perception, while others activate the autonomic nuclei to send messages via the cavernous nerves to the penis in order to induce erection. Nocturnal erection occurs mostly during rapid-eye-movement (REM) sleep. In human during REM sleep, an increased activity in the pontine area, the amygdalas and the anterior cingulate gyrus but decreased activity in the prefrontal and parietal cortex have been showed. Additionally, during REM sleep, the cholinergic neurons in the lateral pontine tegmentum are activated while the adrenergic neurons in the locus ceruleus and the serotoninergic neurons in the midbrain raphe are silent. This differential activation may be responsible for the nocturnal erections during REM sleep.

### *1.2.3. The mediators involved in Penile Erection*

The physiological mechanism of penile erection is characterized by a coordinated and intricate interplay between the pathways of vasorelaxation and vasoconstriction.

The penis is maintained in a flaccid state by a predominance of α-adrenergic tone. In fact, this state is characterized by the contraction of penile arterial and corporal smooth muscle cells through activation of post-junctional $\alpha_1$-receptors mainly by increasing of intracellular calcium. In addition, noradrenaline inhibits vasodilatatory neurotransmitter release by prejunctional $\alpha_2$-adrenoceptors.

Instead penile vasodilatation is mainly mediated by L-arginine/NO pathway through activation of guanylyl cyclase in smooth muscle component, followed by an increase in cGMP which in turn lowers intracellular calcium (Figure 3).

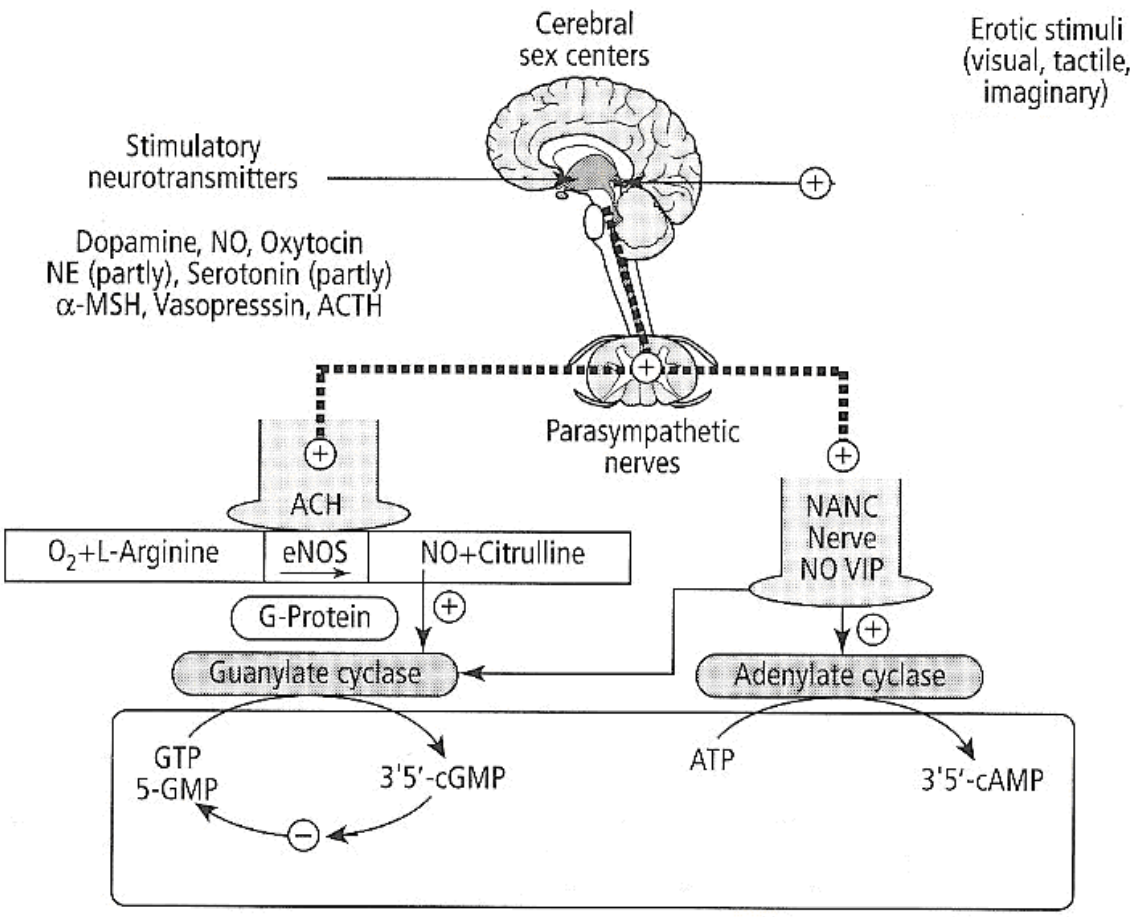



**Figure 3: Physiology of erection and the impact of PDE5**

1.2.3.a *Contraction*

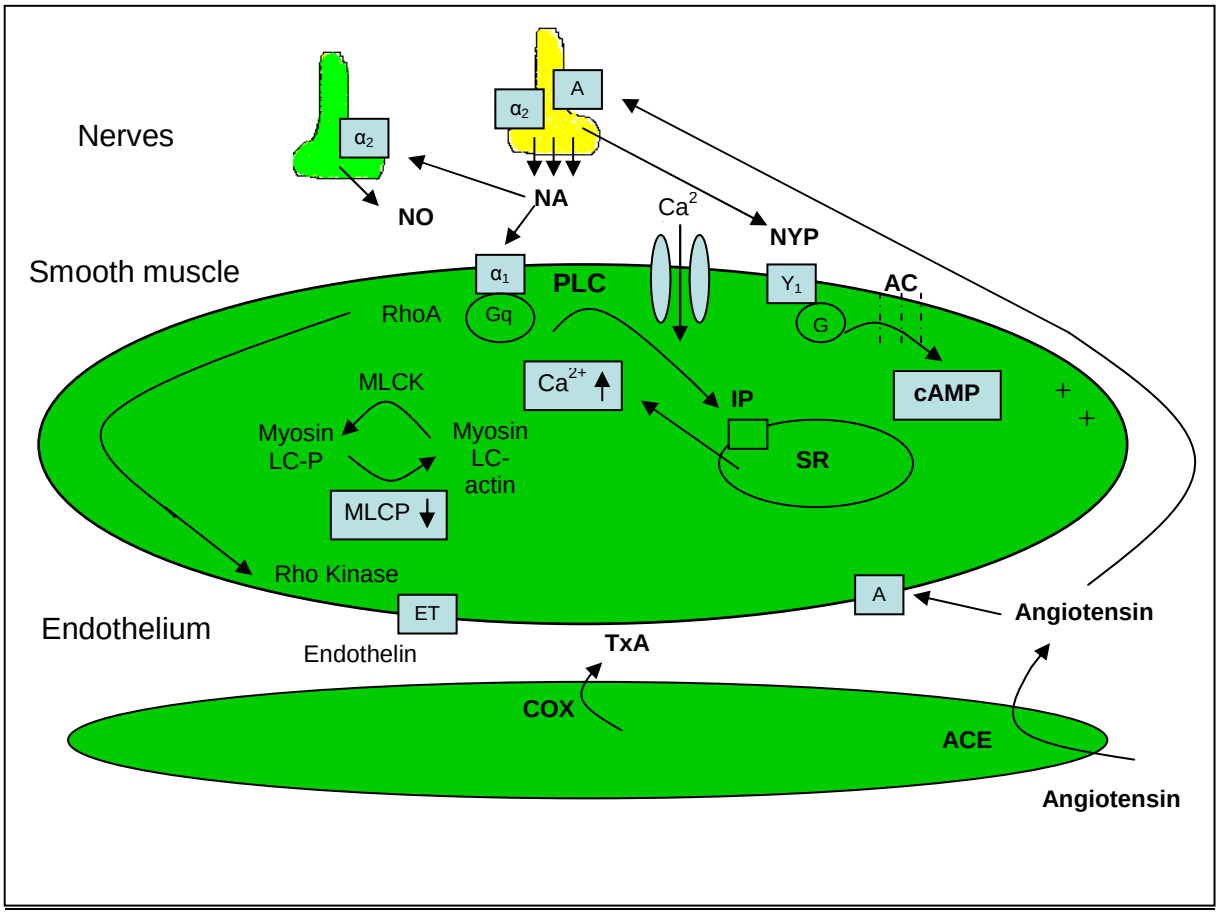



**Figure 4. Mechanisms of penile vascular smooth muscle contraction in the flaccid state.**
The release of noradrenaline (NA) and neuropeptide Y (NPY) from sympathetic nerves and increased formation of angiotensin II and endothelin in the endothelial cells are the causes of the contraction of the penile smooth muscle.

✓ The adrenergic system

The cavernous trabecular smooth muscle cells and penile vasculature receive an adrenergic innervation (Tamura *et al*., 1995). As indicated by the erection induced after injection of α-adrenoceptor antagonist, sympathetic tone is the mainly responsible for maintenance the penis in the flaccid state (Wagner *et al*., 1989; Diederichs *et al*., 1990;

Giuliano *et al*., 1993). In 1980 Levin and Wein showed that in human corpus cavernosum α-adrenoceptor are almost ten times higher than β- receptor (Levin & Wein, 1980). Particularly, the human penile tissue exhibits a profile for $\alpha_1$-and $\alpha_2$-adrenoceptor expression quantitatively and qualitatively similar (Goepel *et al*., 1999). mRNAs for the three $\alpha_1$-adrenoceptor subtypes ($\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$) have been identified with a predominance of $\alpha_{1A}$ (El-Gamal *et al*., 2006), while the contribute of $\alpha_2$-receptor is exclusively due to the $\alpha_{2A}$-subtype (Goepel *et al*., 1999).

There are several clinical studies about the possible use of α-adrenoceptor antagonist in the treatment of erectile dysfunction and also some of these are crucial to understand the involvement of the α-adrenoceptor in the physiology of penile erection: noradrenaline binds to postjunctional $\alpha_{1A}$-adrenoceptors and cause smooth muscle cells contraction, which in turn increases $IP_3$ and intracellular calcium levels; calcium then binds to calmodulin and activates myosin light chain kinase (MLCK) as well as inhibits myosin light chain phosphatase (MLCP); moreover noradrenaline, by binding prejunctional $\alpha_2$-adrenoceptor, inhibits the release of nitric oxide, while limit the overflow of noradrenaline by binding $\alpha_2$-autoceptor (Somlyo *et al*., 2000; Kimura *et al*., 1996).

In addition, in 2003, Cirino *et al*. demonstrated the presence of $\beta_3$-adrenoceptors in smooth muscle cells of human corpus cavernosum. This study showed that, in human penile tissue, the activation of $\beta_3$-adrenoceptors leads to a cGMP dependent but NO-indipendent relaxation blocked by a specific receptor antagonist. Besides, they demonstrated that human corpus cavernosum exhibits basal $\beta_3$-receptor-mediated vasorelaxant tone and that $\beta_3$-receptor activity is linked to inhibition of the Rho-kinase pathway (Cirino *et al*., 2003).

✓ Endothelin

Endothelins (ETs), ET-1, ET-2 and ET-3, are a family of endogenous peptides mainly secreted by endothelial cells (Yanagisawa M, *et al.*, 1988). These peptides exert a potent vasoconstrictor activity by acting through two different receptors, named $ET_A$ and $ET_B$.

Particular attention has focused on the role of endothelins as potential modulators of corpus cavernosum basal tone and their importance in maintaining smooth muscle cell contractility when the penis is in the flaccid state. Indeed, mRNA for ET-1 is expressed by endothelial cells of human penile tissue (Saenz de Tejada I, *et al*., 1991). Several studies have showes ET-1 as a potent agonist for vascular smooth muscle cell contraction (Christ *et al*., 1995; Saenz de Tejada *et al*., 1991.). ET-2 and ET-3 also causes contraction of smooth muscle in human penile tissue but they are less potent than ET-1 (Saenz de Tejada *et al*., 1991).

The physiologic effects of ET-1 are mediated by increase of intracellular calcium levels in cells. ET-1, when released from endothelial cells into the surrounding tissue, binds its receptor at the target cells and facilitates an increase in calcium through inositol triphosphate and voltage-gated calcium channel based pathways (Marsden *et al*., 1989; Berridge *et al*., 1987; Rasmussen *et al*., 1987).

In separately published reports, Christ *et al* and Zhao and Christ have shown ET-1 to be a potent agonist for smooth muscle contraction in tissues, suggesting its importance as a mediator for maintaining smooth muscle cells basal tone in the penis(Christ GJ, *et al*. 1995; Zhao & Christ, 1995)

✓ Angiotensin and angiotensin converting enzyme

Angiotensin II (Ang II) is the main active metabolite of the renin-angiotensin cascade and it is produced by angiotensin-converting enzyme, which converts Ang I in Ang II.

Circulating Ang II influences blood pressure and systemic peripheral resistance. In mammalian cells it exerts its activity by binding two subtypes of plasma membrane receptors, $AT_1$ and $AT_2$ (Touyz & Schiffrin 2000). In erectile tissue, there is a local production of Ang II as angiotensin-converting enzyme is localised in the endothelium of human corpus cavernosum strips (Kifor *et al*., 1997; Park *et al*., 1997). Functional and binding studies in isolated corpus cavernosum strips showed that the angiotensin receptor subtype AT1 mediates the contraction evoked by exposure to Ang II (Kifor *et al*., 1997; Park *et al*., 1997). It has been demonstrated that, in men, Ang II plays an important role in regulation of flaccid state and detumescence of penis. Becker et al. confirmed the functional importance of Ang II in the penile erection by evaluation of peptide plasma levels. In particular, they demonstrated that in blood samples taken during flaccidity, tumescence, and rigidity, cavernous Ang II levels did not differ significantly; while during the detumescence phase, the increase in Ang II plasma levels was significantly higher if compared to other penile phases. (Becker *et al*., 2001). These results provide evidences for the physiological importance of this peptide in the termination of penile erection.

- ✓ The Rho-kinase pathway

As previously described, the flaccid state of the penis is controlled by various contractile mediators by increasing of intracellular calcium and activating calcium sensitization. In fact the contraction is induced at constant intracellular calcium concentration by certain agonists are due to inhibition of the myosin phosphatase, whereas the inhibitory signal for calcium sensitization is provided by RhoA to Rho-kinase. RhoA, a monomeric G-protein activated by GTP and inactivated when the bound nucleotide is GDP, mediates calcium sensitization in erectile function. Indeed, activated RhoA-kinase phosphorylates the regulatory subunit of MLCP and inhibits phosphatase activity. Actually the importance of

calcium-sensitizing RhoA/Rho-kinase pathway is well known and it plays prominent role in the cavernosal vasoconstriction in order to maintain the flaccid state. This role has been demonstrated in 2001 by Chitaley et al. In a study carried out on rats, they showed, that the treatment with a Rho-kinase inhibitor *in vitro* induces relaxation while *in vivo* it caused a penile erection independent of nitric oxide (Chitaley *et al*., 2002).

1.2.3.b *Relaxation*

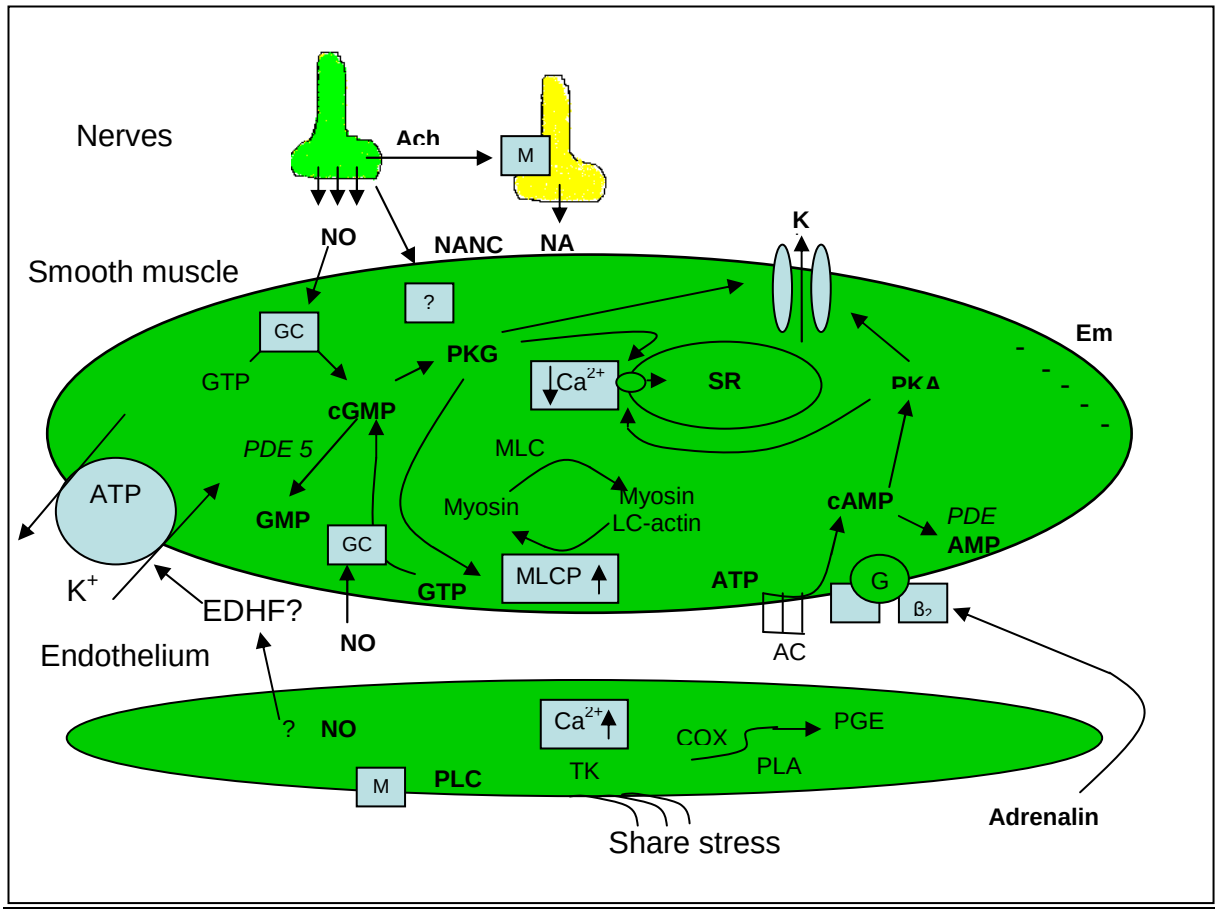



**Figure. 5. Mechanisms of penile vascular smooth muscle relaxation in the erectile state.**
The initial event in erection is vasodilation caused by nitric oxide (NO) released from the parasympathetic nerves and other NANC neurotransmitters. The increased flow through the penile arteries due to shear stress on the endothelial cells probably leads to release of NO, prostaglandins (PG), and a non-NO non-PG factor such as endothelium-derived hyperpolarizing factor (EDHF).

- ✓ L-arginine/NO pathway

Substantial evidence has supported nitric oxide (NO) as the central component of signal transduction system that acts in the penis to mediate erectile response. The enzyme responsible for NO production is NO synthase (NOS), expressed in main biological tissues as three different isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Forstermann *et al*., 1998). The iNOS isoform, which primarily is expressed in immune system, is not perceived to be involved in normal penile erection. In fact iNOS represent another source of NO in smooth muscle cells but it requires cytokines stimulation for its expression (Figure 6). Instead, both eNOS or nNOS are constitutive isoform requiring calcium-binding protein calmodulin, oxygen, and reduced NADPH for catalytic activity. eNOS is located in caveolae (50–100 nm invaginations of the plasma membrane) where it is complexed with caveolin-1 (CAV-1), which decorates the cytoplasmic surface of caveolae and forms a physiological inhibitor of eNOS (Garcia-Cardena *et al*., 1997; Bucci *et al*. 2000). Following activation of endothelial cells by a cadre of mediators, CAV-1-mediated inhibition is diminished by the recruitment of several proteins that promote an activation complex(Garcia-Cardena *et al*., 1997). The first protein shown to be involved in the regulation of eNOS was CaM. Another protein involved in the regulation of eNOS is heat shock protein 90 (HSP90), which directly binds to and activates eNOS in vitro causing NO release (García-Cardeña *et al*., 1998). Recently, HSP90 has been shown to influence the phosphorylation and coupling of eNOS to HSP90 and CaM . Direct interaction between CAV-1, CaM and HSP90 is evident in coprecipitation experiments from cells and, in experiments using purified proteins, eNOS forms a bridge between CAV-1 and HSP90 (Gratton *et al*., 2000). Thus, HSP90 influences eNOS activity in several ways.Another post-translational modification that regulates eNOS activity is protein phosphorylation (García-Cardeña *et al*., 1996). eNOS is phosphorylated primarily

on serine residues and, to a lesser extent, on tyrosine and threonine residues. The serine/threonine kinase Akt (protein kinase B) directly phosphorylates eNOS at Ser1177 (human eNOS) and Ser1179 (bovine eNOS), which increases eNOS activity several-fold (Gallis *et al*., 1999).

NO is also constitutively produced and released from autonomic nerve terminals and endothelial cells in the corporal tissue. It has been also shown that NO mediate neurogenic non-cholinergic non-adrenergic (NANC) relaxation in human penile resistance arteries, in large penile arteries and in veins (Simonsen *et al*., 2002; Toda *et al*., 2005). In particular NO derives from its precursor, L-arginine, under catalytic action of NOS and it diffuses into smooth muscle cells where binds intracellular guanylate cyclase (Burnett *et al*., 1995; Burnett *et al*., 1997). This event induces the activation of guanylate cyclase that catalyzes conversion of guanosine triphosphate (GTP) to cGMP; this mediator then operates through a cGMP-dependent protein kinase to regulate the contractile state of the corporal smooth muscle (Hedlund *et al*., 2000). Indeed, in 2000 Minhas and co-workers demonstrated that cGMP inhibits the presynaptic release and contractile effects of norepinephrine (Minhas *et al*., 2000).

NO, in corporal smooth muscle cells, also induced a relaxation which is independent of cGMP action. Gupta *et al*., in 1995, showed that NO stimulates Na+/K+-ATPase channels, resulting in direct hyperpolarization of the corporal smooth muscle cell, which prevents the opening of voltage-dependent calcium channels and thereby attenuates tissue contraction.

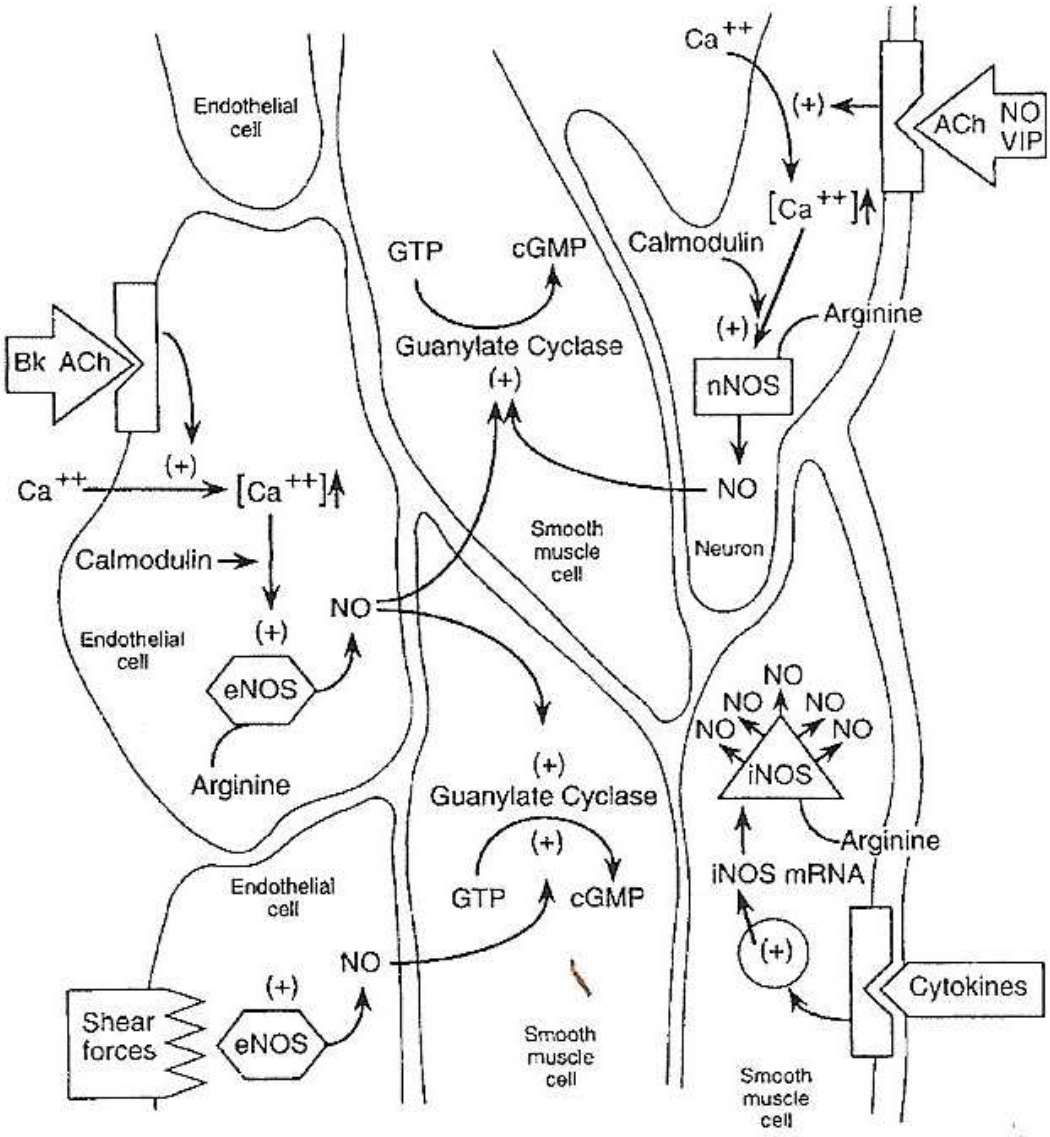



**Figure 6**: **Diagrammatic representation of nitric oxide (NO) synthesis, regulation, and action in the penis**. Constitutive formation of NO derives from its precursor, L-arginine, under the catalytic action of the NO synthase (NOS) isoforms nNOS and eNOS, respectively, on the basis of the cellular localizations of the enzyme to neurons and endothelial cells. Conventional signal transduction involving NO constitutively is characterized by messenger molecules that commonly activate NOS isoforms by signaling influx of calcium and its binding with calmodulin.

✓ Prostanoids

It is well established that human corpus cavernosum can locally synthesize and metabolize several prostanoids (Miller and Morgan 1994; Anderson & Wagner 1995; Porst *et al*., 1996; Minhas *et al*., 2000). The effects of five primary active prostanoid metabolites, PGD, PGE, PGF, PGI, and thromboxane $A_2$, are also mediated through specific receptors namely DP, EP, FP, IP and TP receptors. Prostanoid receptors are G-protein coupled with different transduction systems (Coleman *et al*., 1994; Pierce *et al*., 1995; Narumiya *et al*., 1999). $PGF_{2\alpha}$ and thromboxane $A_2$ may be involved in contraction of erectile tissue, stimulating FP and TP receptors and initiating phosphoinositide turnover. Indeed $PGE_1$, stimulating EP receptor, induced relaxation of human corporal smooth muscle by hyperpolarization after activation of $Ca^{2+}$-dependent potassium channel (Lee *et al*., 1999). Escrig *et al*., in 1999, showed that repeated $PGE_1$ treatment enhances responses to nerve stimulation in the rat penis by up-regulating constitutive NOS isoforms (Escrig *et al*., 1999).

Prostanoids exert their effects mainly through cAMP. In fact forskolin, which directly stimulates adenylate cyclase and consequently intracellular cAMP, significantly increases the production of cAMP by $PGE_1$, which suggests a possible synergistic effect (Palmer *et al*., 1994; Traish *et al*., 1997a). Traish *et al*., also demonstrated that the augmentation of the forskolin-induced cAMP generation by $PGE_1$ was mediated by EP receptors and more likely it could be ascribe to interactions between the adenylyl cyclase and G-proteins (Traish *et al*., 1997b).

✓ Vasoactive intestinal polypeptide

The human penis as well as animal one is richly supplied with nerves containing vasoactive intestinal polypeptide (VIP) (Dail *et al*., 1993). These nerves are densely

concentrated in the penis around the pudendal arteries and in erectile tissue but they are also present in vas deferens and epididymus (Polak *et al*., 1981). VIP has a potent vasorelaxant effect on human corpus cavernosum. The effects of VIP is also mediated through specific receptors namely DP, EP, FP, IP and TP receptors. VIP receptors are of two different types, 1 and 2, both linked via stimulatory G-protein to adenylyl cyclase (Fahrenkrug 1993; Harmar *et al*., 1998). The stimulatory effect of VIP on adenylyl cyclase leads to an increase in cAMP, which in turn activates cAMP-dependent protein kinase. In corporal tissue from human, rats and rabbits VIP increased cAMP concentrations without affecting the cGMP levels (Hedlund *et al*., 1995; Miller *et al*., 1995). VIP is a potent vasorelaxant of human corpus cavernosum at very low doses but it is not clear if VIP released from nerves is responsible for the relaxation of penile smooth muscle cells *in vitro* or *in vivo* (Andersson and Wagner 1995). The effect of exogenous VIP was inhibited by VIP antiserum. Adaikan *et al*., showed that intracavernous injection of VIP in human volunteers caused some degrees of penile enlargement but not erection in 5 of 7 subjects. Besides, VIP plasma levels measured in 12 patients with various types of erectile dysfunction (6 with psychogenic impotence, 2 with induratio penis, 4 with impotence of vascular origin) did not increase during pharmacologically induced erection in any patients treated with $PGE_1$ (Adaikan *et al*. 1986a,b). In addition VIP concentrations in peripheral venous blood were similar to those detected in corpus cavernosal blood (Koehn *et al*., 1993). These data suggested that VIP does not cause erection by itself but it could be acting in synergy with other vasodilatory agents. Nevetheless, a clinical study showed, that in healthy adult male volunteers there wasn't any increase in VIP plasma levels neither in systemic nor in cavernous blood when the flaccid penis became rigid. Conversely, during penile detumescence mean cavernous VIP levels increased whereas VIP remained unaltered in systemic circulation (Becker *et al*., 2002). These findings highlight VIP as a

possible mediator in the mechanism of male sexual arousal, although its role in the penis physiology needs to be clearly addressed.

- ✓ Hydrogen sulphide

Hydrogen sulphide ($H_2S$), the colourless gas with a strong odour of rotten eggs, was only known as a toxic environmental pollutant. About twenty years ago $H_2S$ has been proposed as gaseous neurotransmitter involved in physiological and/or pathological processes (Li et al., 2005; Zhong G *et al*., 2003; Bhatia *et al*., 2005). This gas is produced in many tissues, particularly in the brain, in the cardiovascular system, in the liver and in the kidney. It is synthesised from L-cysteine (L-cys) by cystathionine β-synthase (CBS) and cystathionine γ-liase (CSE), both pyridoxal-5'-phosphate-dependent enzyme. CBS and CSE have been detected in human and other mammalian cells and their expression is tissue specific (Levonen *et al*., 2000; Zhao *et al*., 2001). Recent studies demonstrated the involvement of $H_2S$ in erectile function in rodents and monkeys (Srilatha *et al*., 2007). In 2008 d'Emmanuele di Villa Bianca *et al*. proved the involvement of L-cys/$H_2S$ pathway in penile erection *in vivo*. They showed that human penile tissues expressed CBS and CSE as both protein and mRNA, and that CSE modulates the L-cys/$H_2S$ pathway in peripheral cavernous nerves. In addition they demonstrated that human tissues can synthesize $H_2S$ from L-cys via catalytic actions of CBS and CSE. In this study it has been also revealed that NaHS (stable donor of $H_2S$) and L-cys relaxed human corpus cavernosum strips in an endothelium-independent and concentration-dependent manner. Finally, *in vivo* studies performed on rats, they showed that intracavernous administration of NaHS caused a dose-dependent increase in intracavernous pressure, implying that exogenous $H_2S$ can cause penile erection (d'Emmanuele di Villa Bianca *et al*., 2008).

## 1.3 Erectile dysfunction

Erectile dysfunction (ED) is defined as the consistent or recurrent inability of a man to attain and/or maintain a penile erection sufficient for sexual activity (2nd International Consultation on Sexual Dysfunction-Paris, June 28th–July 1st, 2003). ED can be due to a number of different pathophysiological process. Already twenty years ago, Helen Kaplan estimated that erectile failure was psychogenic in 90% and organic in 10 % of cases. Today, the current opinion is that in most patients ED is of mixed aetiology that may be either predominantly psychogenic or organic. In fact actually ED is classified into three different types: psychogenic (caused by psychological or emotional factors rather than physical ones), organic (caused by the inability of penile smooth muscle cells to relax) and mist (when there is a combination of both, organic and psychogenic ED).

Recently, several clinical and epidemiological studies have been performed in order to define the risk factors for ED and they showed the possible role of age, obesity, smoking and cardiovascular diseases in the development of ED. These events can induce multiple disorders, including nerve damage, endothelial damage, alteration in receptor expression/function, or in the transduction pathways implicated in the relaxation and contraction of smooth muscle cells.

### *1.3.1 Age*

The prevalence of ED increases significantly with age, as there are clear changes in the physiology of male sexual activity (Lunefeld *et al*., 2002; Seftel *et al*., 2003). Indeed, in the Massachusetts Male Aging Study (MMAS) 39% of men had some degree of ED at the age of 40 (Feldman *et al*., 1994). This prevalence gradually increased to reach 67% at the age of 70. The relevant age-related increase of ED prevalence is predominantly due to age

as a risk factor. In fact it is difficult to discriminate between aging and concomitant diseases.

It is well known that aging cause alteration in anatomical structures. In fact changes of the smooth muscle cells component, elastic fibres, or collagen content provoke mechanical alteration of penis, reducing its elasticity and compliance.

Aging can induce also alteration in the transduction pathways implicated in the penile physiology. In fact, Garban *et al*., (1995) found that the NOS activity decreased in penile tissue in senescent rats (Garban *et al*., 1995). In another study, NOS mRNA expression in old rats was found lower than in young ones (Dahiya *et al*., 1997). Instead in the aging rabbit, endothelium-dependent corpus cavenosum relaxation was attenuated; however, eNOS was up regulated both in vascular endothelium and corporal smooth muscle cells. These data suggest that erectile dysfunction in the aging rabbit cavernosum appears to be related to endothelial dysfunction and is characterized by eNOS up regulation and aberrant intracellular calcium fluxes (Haas *et al*., 1998).

Besides, ED is often associated with other conditions that are common in aging men, and the most important involves the cardiovascular system.

### *1.3.2 Lifestyle*

Several studies reported that lifestyle factors are frequently associated with ED and they include a sedentary life, and obesity, smoking and alcohol or drug abuse.

a) Obesity

An increase in body mass represents a significant risk factor involved in the development of ED.

In 1999 Chung *et al*., evaluated ED in 325 patients classified into two groups according to the body weight: <120% and >120% of the ideal body weight. They showed that in patients with obesity there was a significant decrease in the quality of erectile function. Furthermore, about 43% of obese patients presented a significantly increased prevalence of vascular risk factors, suggesting that obesity does impose a risk to vasculogenic impotence, promoting development of chronic vascular disease. A controlled study in obese men also revealed that increase of physical activity reduced the progression of ED (Esposito *et al*., 2004). In another large study, during five years, the incidence of ED was estimated in men aged 50, 60, 70 years residing in the city of Tampere or eleven surrounding municipality in Finland. It has been demonstrated that there were not difference of ED according to the level of education, marital status, urban/rural place of residence and amounts of alcohol and coffee consumption, while obesity and current smoking significantly increased the incidence of ED (Shiri *et al*., 2004).

a) Smoking

Smoking is an important risk factor in the development of impotence (Mannino *et al*., 1994). In 2000 Feldman *et al*., conduced a study based on Massachusetts Male Aging Study, in a random-sample cohort study. They investigated the relationship between baseline risk factors for coronary heart disease and subsequent ED, on the premise that subclinical arterial insufficiency might be manifested as ED. They showed that cigarette smoking at baseline almost doubled the likelihood of moderate or complete ED at follow up. Cigar smoking and passive exposure to cigarette smoke also significantly predicted incident ED, as overweight and a composite coronary risk score did. Weaker prospective associations were seen for hypertension and dietary intake of cholesterol and unsaturated fat (Feldman *et al*., 2000). In fact cigarette smoking has been considered an independent

risk factor in the development of atherosclerotic lesions in the internal pudendal and common penile arteries of young men with ED. In this study, evaluating the relationship between cigarette smoking and the atherosclerosis of the hypogastric-cavernous arterial bed, it has been showed that the atherosclerosis was significantly increased in patients that had been smoking for many years (Mannino *et al*., 1994). Smoking also has been associated with several changes at molecular level; in particular it causes an impaired penile flow. In rats passive smoking causes age-independent moderate systemic hypertension and marked decrease in penile NOS activity and nNOS content (Xie *et al*., 1997).

a) Alcohol or drug abuse

Several classes of medications, recreational drugs and alcohol have been linked to the manifestation of ED. About alcohol, its primary action is increasing blood volume, which in turn causes higher blood pressure. Thus, in theory, alcohol consumption should assist erection and not cause erectile dysfunction. Also, elevated quantity of alcohol operates as a sedative and may also help curb performance anxiety that is a major cause of ED in younger men. Even drugs and some medications exhibit their adverse events, like either central inhibitory neuroendocrine mechanisms and/or local neurovascular actions, or they have an impact on the hormonal (luteinizing hormone, testosterone and prolactin) and neurotransmitter pathways (serotoninergic, dopaminergic or adrenergic systems).

### *1.3.3 Cardiovascular risk factors*

More than 20 years ago, it has been showed that chronic arterial disease, compromising the blood flow in the cavernous arteries, could be a significant cause of ED (Michal *et al*., 1982). Heart disease and its associated risk factors, such as hypertension and low serum

high density lipoprotein cholesterol, are significantly correlated with ED (Feldman *et al*., 2000). Several studies suggest ED as a predictive factor for coronary disease (Shamloul *et al*., 2004; Billups *et al*., 2005). Vasculogenic impotence has been reported to be the first sign of a generalized arteriopathy. Muller *et al*. reported a very high incidence (85%) of cavernous artery insufficiency in a group of 117 hypertensive patients (Muller *et al*., 1991). In a rabbit model of atherosclerotic ED, it was shown that chronic cavernosal ischemia impaired not only the endothelium-, but also neurogenic-dependent corpus cavernosum relaxation and NOS activity (Azadzoi *et al*., 1998). Hypercholesterolemia also impaired endothelium mediated relaxation of rabbit corpus cavernosum smooth muscle (Azadzoi and Saenz de Tejada; Azadzoi *et al*., 1998 ).

### *1.3.4 Diabetes*

Several studies confirmed the etiological role of diabetes in ED. In fact has been reported that ED develops in 35-75 % of diabetic patients (Hatzichristou *et al*., 1994) and it has been shown to occur three times more frequently in diabetic than in non diabetic subjects (Feldman *et al*., 1994). The cause is multifactorial, but it almost commonly reflects endothelial dysfunction and autonomic neuropathy (Saenz de Tejada *et al*., 1989). Penile NOS activity and content were reduced in rat models of both type I or II diabetes with ED (Vernet *et al*., 1995). However, in streptozotocin-induced diabetic rats, NOS binding increased (Sullivan *et al*., 1996), and NOS activity in penile tissue was significantly higher than in controls, despite a significant drop of mating behaviour and indications of defective erectile potency (Elabbady *et al*., 1995). It has also been showed that the nocturnal penile tumescence was altered in diabetic patients, indicating a change in normal physiology function. In 1990, Blanco *et al*., demonstrated that there was a functional penile neuropathic condition of the cholinergic nerves in the corpus cavernosum

of diabetic patients and concluded that ED in diabetes may be a secondary effect of a neuropathic condition. Molecular studies on human penile tissue have also shown that the diabetic ED is related to the effects of advanced glycation end products and NO formation (Seftel *et al*., 1997). In fact, advanced glycation end products were found to be more elevated in the penile tissue, but not in serum, in diabetic patients than healthy patients. However the involvement of advanced glycation end products has been demonstrated by the high levels in the tunica and corpus cavernosum of diabetic patients (Seftel *et al*., 1997).

There is only one study about the possible role of androgens in diabetics. This study demonstrated that in diabetics there was an unchanged level of luteinizing hormone but there was a decrease in testosterone levels accompanied by an increase in testosterone binding globulin capacity and then a markedly decrease of the apparent free testosterone concentration (Ando *et al*., 1984). Finally it has been showed that an impairment of VIP-releasing innervations in penile tissue may be an important factor in the development of impotence in diabetes (Crowe *et al*., 1983).

### *1.3.5 Medical disorder*

Several systemic diseases are associated with ED.

a) Neurologic disease

Multiple neurologic diseases can be the cause of ED. As well known, the sexual stimulation takes place in the brain: the higher cortex, the limbic and paralimbic regions and other subcortical regions participate in motivational, motor, or emotional sexual stimulation. Impulses from brain to genitalia are carried by either lumbar and sacral spinal roots and autonomic system. Lesions in these areas may result in ED. Indeed various

neurological conditions are associated with ED: multiple sclerosis, temporal lobe epilepsy, stroke, Parkinson and Alzheimer's disease, all disorders at brain level, or spinal cord injuries (Giuliano *et al*., 2000; Saenz de Tejada *et al*., 2005; Goldestein *et al*., 1982). Peripheral neurologic disorders related to ED include afferent neuropathies, such as dorsal penile affections (diabetic neuropathy), or afferent lesions, or lesions of the cavernous nerves after radical pelvic surgery.

b) Chronic renal failure

More than 50% of men with chronic renal failure is affected by ED. This process is multifactorial and includes hyperprolactinemia, hypertension, smooth muscle dysfunction secondary to circulating toxins, neuropathy and atherosclerosis (Nogues *et al*., 1991). In patients requiring dialysis, occlusion of cavernous arteries and veno-occlusive dysfunction were found in 78% and 90% of patients respectively (Kaufman *et al*., 1994).

c) Respiratory disease

Up to 30% of men with chronic obstructive pulmonary disease suffer of ED. The pulmonary disease, and consequently hypoxia, may represent the primary etiologic factor in these cases (Fletcher *et al*., 1982). Other authors suggested an association between ED and sleep syndrome, as well as sleep apnoea (Moreland *et al*., 1999).

d) Hepatic insufficiency

ED has been associated with chronic liver dysfunction in more than 50% of case and it may reach up to 75% in alcoholic liver cirrhosis (Cornely *et al*., 1984). Zonszein et al., hypothesizing a mechanism, suggested that ED in hepatic failure can be caused by hyperprolactinemia, by a decrease of testosterone due to an increased sex hormone binding globuline synthesis, and by elevated level of estrogens (Zonszein *et al*., 1995).

### *1.3.6 Other man dysfunctions*

a) Peyronie's disease

Peyronie's disease is characterized by the formation of a fibrous nodule in the tunica albuginea. These plaques impede tunical expansion during erection, resulting in penile bending. Many possible etiological factors have been suggested but penile trauma appears to be the most likely. Trauma to the penis move up an inflammatory process that stimulates the fibroblast to produce collagen and then the plaque.

b) Priapism

Priapism is defined as unwanted erection not associated with sexual desire or stimulation. It is generally secondary to blunt trauma of the penis or perineum. Two different types of priapism can be distinguished: ischemic, or no flow priapism, and high flow priapism. The former is characterized by a reduction or loss of the normal venous outflow from the penis; this event leads to a failure of detumescence, increasing anoxia, necrosis and fibrosis in the tissue. The latter, much less common, causes an abnormal high arterial blood flow into the penis.

## 1.4 Diagnosis of erectile dysfunction

Sexual dysfunction may stem from various causes, including physiological or relationship factors, medical illness or medications taken for illnesses. Often such factors work in concert. For this reason, the first and most important step in the treatment of ED is represented by examination and diagnosis. In fact the doctor evaluates two paramount factor contributing toward the successful diagnosis of ED: history and physical exam. At first the patient may ask about his personal history (about his past and present medical problems, about medications or drugs being used, and about any history of psychological problems, including stress, anxiety, or depression) and then about his sexual history (the nature of the onset of the dysfunction, the frequency, quality, and duration of any erections, and whether they occur at night or in the morning; the specific circumstances when erectile dysfunction occurred; the patient's motivation for and expectations of treatment; whether problems exist in the current relationship). Instead the physical exam include examination of the genital area and a digital rectal examination to check for prostate abnormalities.

### *1.4.1 Questionnaires*

Questionnaires are not mandatory in the evaluation of ED, but they often serve as an important adjunct to the proper diagnosis. There are a variety of general questionnaires in use such as Sexual Health Inventory for Men (SHIM), International Index of Erectile Function (IIEF), Sexual Encounter Profile (SEP) and Male Sexual Health Questionnaire (MSHQ). All questionnaire present simple, straightforward, yet comprehensive question about erectile activity over 3-6 month time frame (Cappelleri *et al*., 2005; Rosen 1997; Rosen 2004). IIEF and SEP are currently the most used questionnaire to measure of efficacy in any study evaluating ED treatments.

### *1.4.2 Physical Examination*

The physical examination represents an essential component of sexual dysfunction evaluation. In most cases, this examination does not identify the aetiology or cause of ED, but will help in formulation of diagnosis. The physical examination includes a general screening for medical risk factors or disorders, with specific attention to cardiovascular, neurologic and genitalia system (Tab. 1)

| |
|---|
| check for gynecomastia |
| check for body hair distribution |
| check the penis size and suprapubic fat pad |
| check the femoral pulses |
| check for inguinal hernia |
| check for sexually-transmitted disease |
| check the penis for foreskin |
| check the foreskin, if present for ability to retract, warts, cancer and other lesions. |
| check the glans for balanitis and other similar conditions, warts and cancer. |
| check position of the urethra |
| check the tunica for plaques |
| check the urethra for warts, and strictures |
| check the penile skin for lesions |
| check the testis size and consistency |
| check the testis lie |
| check for varicocele |
| check for the presence of two testis |
| check the spermatic cord for the vas and any pathology |
| check the rectum for prostate size, volume, lesions, tenderness |
| check the rectum for hemorrhoids, anal cancer, anal stenosis, anal tone |

**Tab. 1**: Brief points to cover in the physical examination of the male with ED.

### *1.4.3 Laboratory test*

Routinely laboratory tests include, first of all, blood sugar and fasting lipid profile, PSA screening and urine analysis (glucose, ketones and proteinuria). Then hormones associated with sexual desire are monitored: free testosterone, testosterone, prolactin and dehydroepiandrosterone (DHEAS) may be part of ED evaluation. Luteinizing hormone (LH), follicle stimulating hormone (FSH) and sex hormone binding protein (SHBG) are determined for further differential diagnosis in the case of low testosterone values, to ascertain primary or secondary hypogonadism. Instead estradiol, growth hormone and thyroid parameters are determined in individual cases to diagnose further hormonal deficits.

### *1.4.4 Invasive tests*

These tests were used since 1980s. Now invasive tests have been surmounted by previously mentioned tests. The invasive tests more used are:

- ✓ Pharmacological testing

Intracavernous injection of a vasodilator (generally $PGE_1$) is used to differentiate vasculogenic to non-vasculogenic ED. In fact positive results of this test only demonstrate the integrity of veno-occlusive mechanism (Figure 7). If a full erection is not seen within ten minutes, or if the erection lasts only a short time, the patients is allowed to stimulate himself. Failure after stimulation is considered to be a good indicator of the presence of vasculogenic ED (Muhlhall *et al*., 1997) (Figure 7).

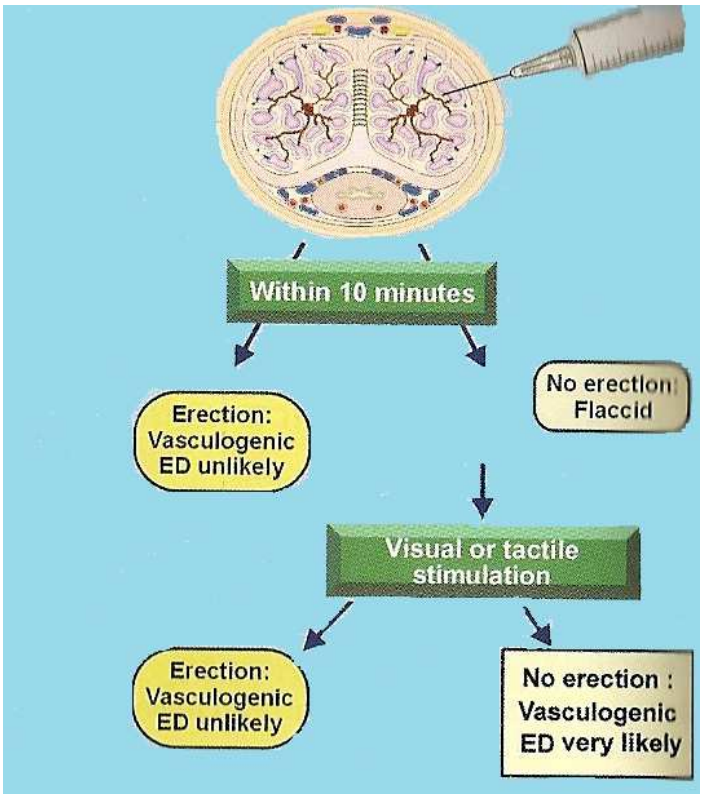



**Figure 7: Intracavernous injection of vasodilator drugs.**

✓ Color Doppler Imaging (CDI)

After intracavernous injection of $PGE_1$ the penis can be scanned by color duplex ultrasonograpy (Figure 8) (Connolly *et al.* 1996). The velocity of blood flow in intracavernous artery during diastolic and systolic phase is monitored within five minutes of injection and repeated frequently.

Doppler is a good technique to make the differential diagnosis of no flow and high flow priapism.

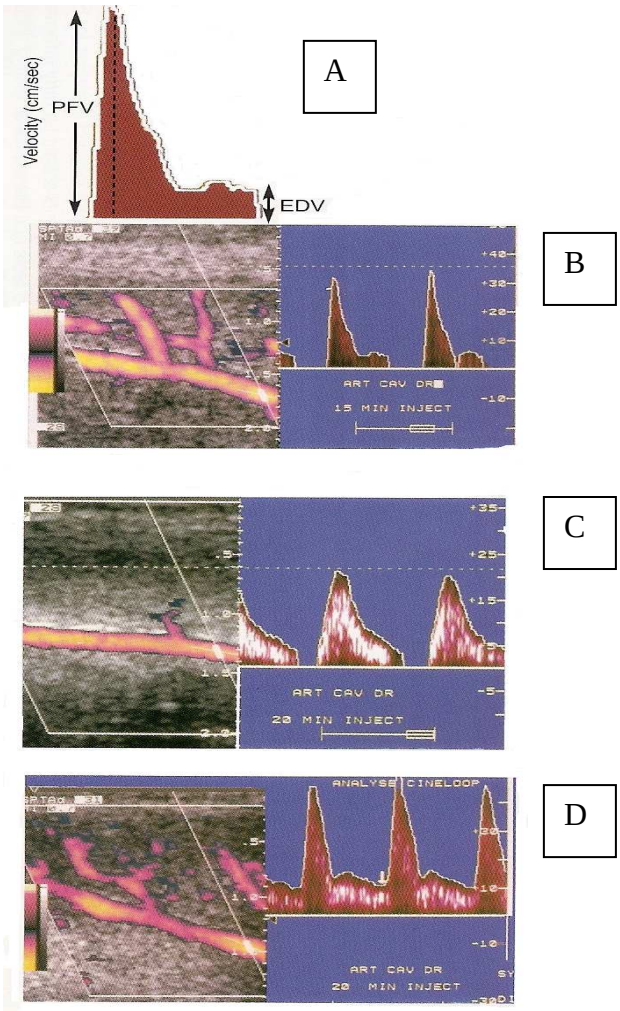



**Figure 8: Color Doppler Imaging (CDI).**
**(A)** Arterial waveform in the penis recorded by color duplex ultrasonography showing standard parameters measured (PFV, peak flow velocity; EDV, end-diastolic velocity). **(B)** Normal vascular response: PFV >35 cm/s, EDV nil. **(C)** Arterial insufficiency: PFV <25 cm/s. **(D)** Veno-occlusive dysfunction: 20 minutes after injection and sexual stimulation. PFV >35 cm/s and EDV >5 cm/s.

✓ Nocturnal penile tumescence (NPT)

The NPT test checks whether a man is having normal erections during sleep. Most men have 3 to 5 full erections during deep (rapid eye movement, or REM) sleep. Men who do not have erections because of psychological problems can still have erections during deep sleep. Occasionally, some sleep problems or serious depression can prevent these normal nighttime (nocturnal) erections. An electronic monitoring device is more expensive than using the snap gauge, but it is more accurate and gives more detailed information about erections during sleep (Figure 9 A,B). This device records how many, how long, and how rigid the erections are during sleep. If good erections occur during sleep, the cause of the erection problems probably is not physical.

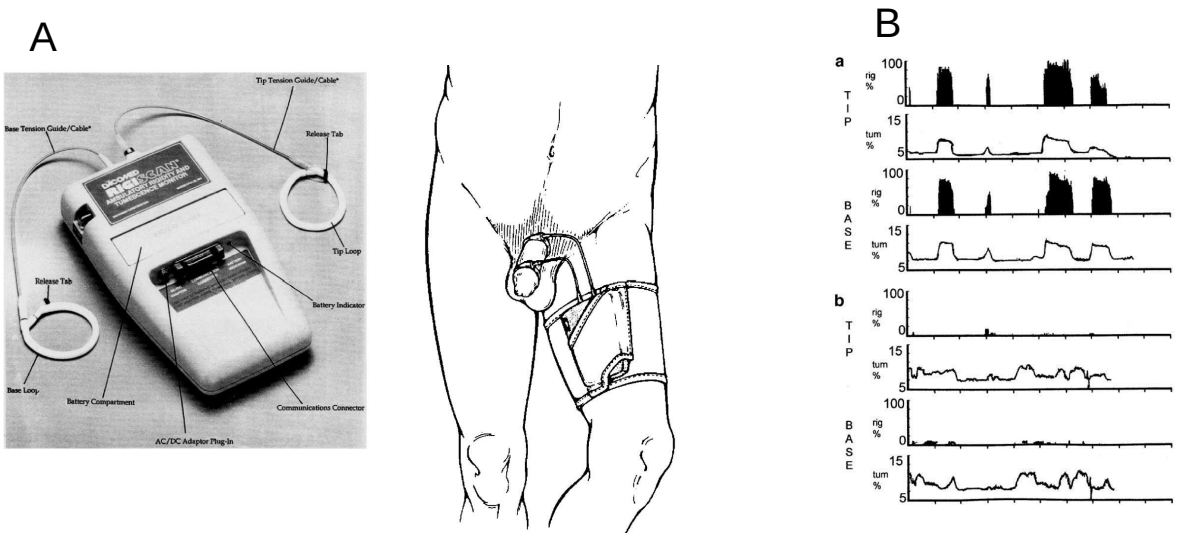



**Figure 9: (A)** The Rigiscan: a device for the evaluation of nocturnal penile tumescence. The apparatus records through the ring's rigidity at the base and tip of the penis**. (B)** (a) A Rigiscan trace from a nocturnal penile tumescence test from a normal healthy volunteer. The trace shown refers to 1 night. Four erective episodes are visible, with good duration and excellent rigidity both at base and tip of the penis. (b) A Rigiscan trace from a nocturnal penile tumescence test from a severe ED patient. The trace shown is a 1 night record. The absence of erective episodes is suggestive of organic erectile dysfunction.

- ✓ Bulbocavernous reflex Latency Times (BCR) and Somatosensory Evoked Potentials (SSEP)

This method is used to prove the integrity of somatic penile innervations. A ring electrode on the distal shaft of the penis is used to apply impulses via a constant current stimulator, and the reflex latency time is measured via the needle electrodes placed in the bulbocavernous muscle.

## 1.5 Treatment of erectile dysfunction

Pharmacological therapies can acts centrally or peripherally on the penis, or both.

Once, pharmacologic treatment of ED was limited to a few substances, most of which had to be injected directly into the corpora cavernosa.

### *1.5.1 Intracorporal pharmacological therapy*

Three drugs have been used most frequently: $PGE_1$ (the most widely used), papaverine and phentolamine.

✓ Papaverine

Papaverin, a non oppioid derivate from *Papaver Somniferum*, was the first drug to be used. As a good smooth muscle relaxant, *in vitro* has been showed that papaverine evokes relaxation of human corpus cavernosum strips, penile arteries cavernous sinusoids, and penile veins (Virag *et al*. 1984).

At cellular level papaverin is a non-specific phosphodiesterase inhibitor that causes increase in intracellular cGMP and cAMP, resulting in a fall in the cytoplasmatic calcium concentration leading to smooth muscle relaxation and penile erection (Figure 10).

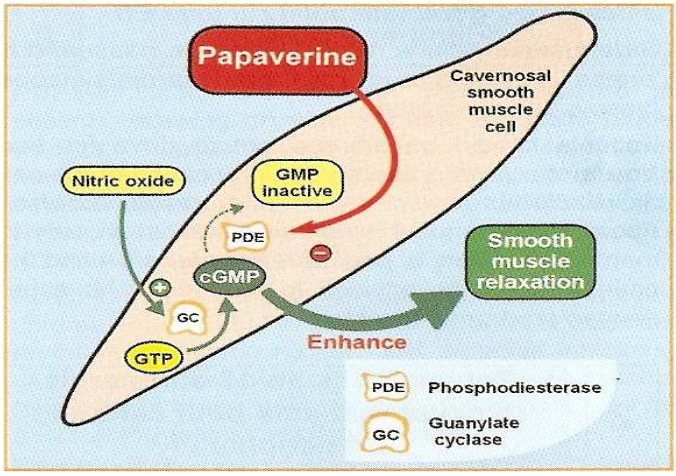



**Figure 10: Molecular mechanism of papaverine action.**
It inhibits both cAMP and cGMP resulting in smooth muscle relaxation.

✓ Prostaglandin E1 (PGE1)

The effect of PGE1 on the human corpus cavernosum was described for the first time in 1975 by Karim and Adaikan who demonstrated its effect in HCC *in vitro* (Karim & Adaikan, 1975). PGE1 acts primarily via specific receptor on the surface of the smooth muscle cell to stimulate the enzyme adenylate cyclase. This enzyme converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) (Lin, 1995). Therefore injection of PGE1 causes a rise in intracellular cAMP, causing a fall in intracellular calcium and subsequently smooth muscle relaxation (Lee *et al*., 1999) (Figure 11).

PGE1 was also shown to suppress the collagen synthesis by TGF-β1 in cultured HCC, suggesting that $PGE_1$ may play a key role in modulation/prevention of collagen synthesis with subsequent fibrosis of corpus cavernosum (Moreland *et al*., 2003).

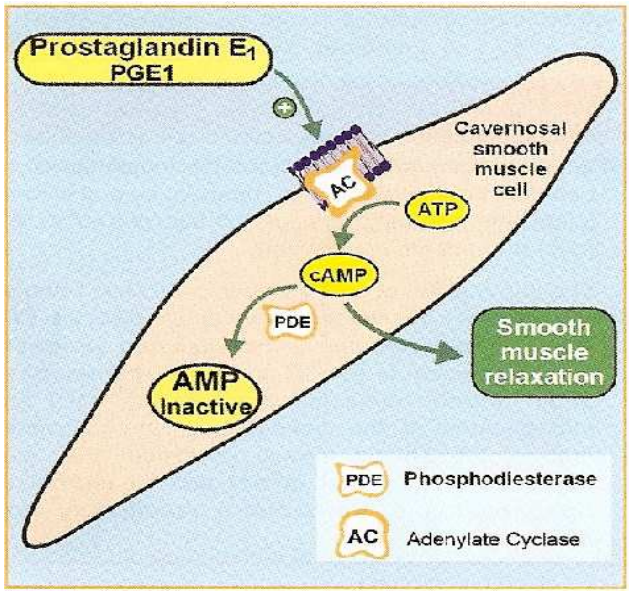



**Figure 11: Molecular mechanism of $PGE_1$ action**.
It stimulate adenylate cyclase causing a fall in intracellular calcium and thus smooth muscle relaxation.

✓ Phentolamine

Phentolamine is a competitive non-selected $\alpha_1$ and $\alpha_2$-adrenoceptor blocker that acts both on presynaptic and postsynaptic α-adrenoceptors. In addition it acts opening potassium channel, antagonizing endothelin and causing a stimulatory effect on NO synthase (Gupta *et al*., 1998; Traish *et al*., 1998) (Figure 12). Phentolamine when injected alone has a modest therapeutic effect; however, it has a synergistic action when combined with drugs such as papaverine and $PGE_1$.

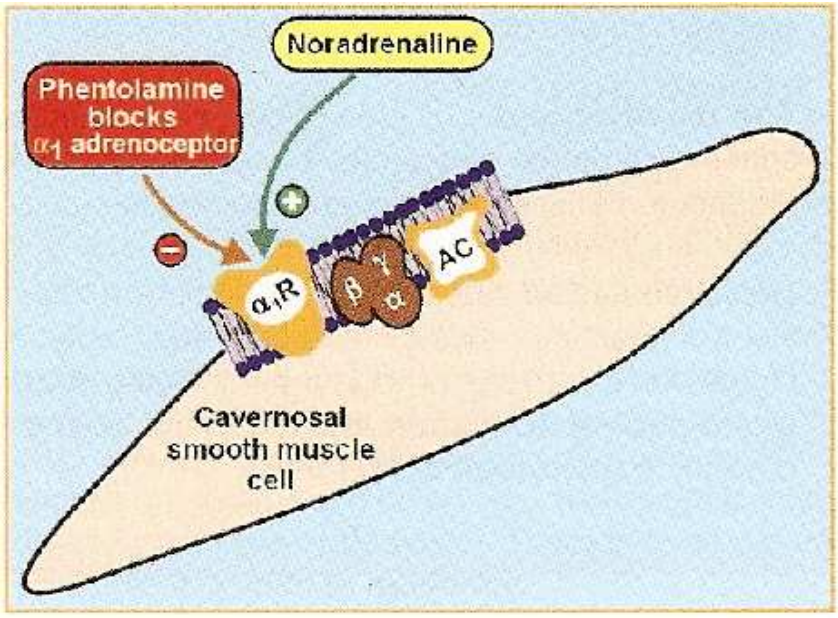



**Figure 12: Molecular mechanism of phentolamine action.**
As inhibitor of both $\alpha_1$ and $\alpha_2$ adrenoceptors, it blocks the tonic sympathetic neuronal activity which normally produces smooth muscle contraction.

### *1.5.2. Oral therapy*

The first oral therapy was characterized by drugs containing yohimbine, and in some cases the serotonin re-uptake inhibitor trazodone (Figure 13). Instead new generation of drugs acts directly on smooth muscle cells inhibiting PDE5.

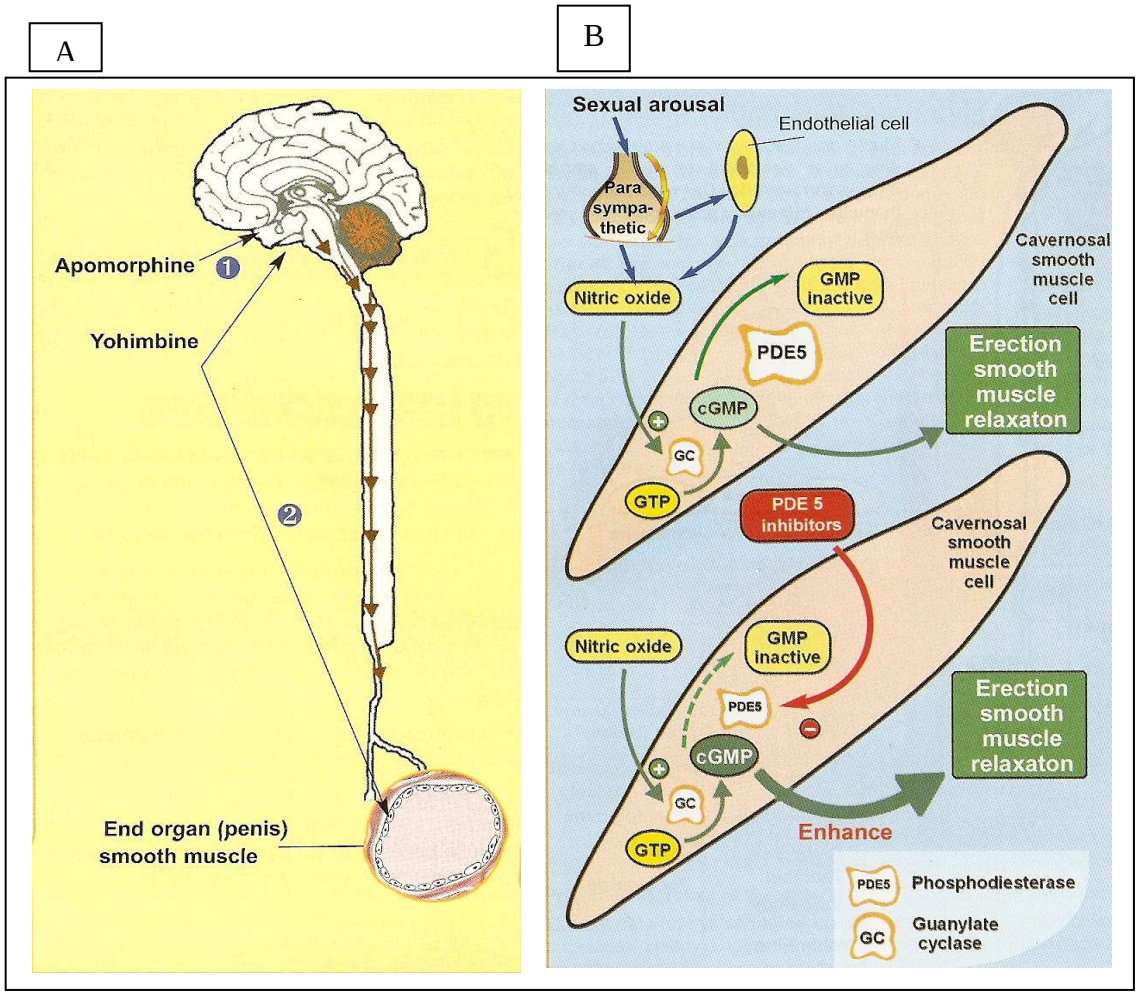



**Figure 13: Centrally and peripherally acting drugs.**
**(A)** Apomorphine acts centrally on D2 dopaminergic receptors in the hypothalamus. Yohimbine is an $\alpha_2$ adrenergic blocker that acts centrally and peripherally. Both decrease the penile smooth muscle intracellular $Ca^{++}$ inducing smooth muscle relaxation and promoting erection. **(B)** PDE5 inhibitors decrease the degradation of cGMP following in smooth muscle relaxation and enhancing erection.

✓ Yhohimbine

Yohimbine is an alkaloid from the bark of *Coryanthe johimbe* K.Schum, of Rubiacee family. It is a central $\alpha_2$-adrenoceptor blocker and its activity being 50-100 times higher on presynaptic receptors than on postsynaptic ones (Ernst *et al*., 1998). It has a peripheral action acting on $\alpha_2$-receptor of the penile arteries and on presynaptic $\alpha_2$-adrenoceptor, which are localized on $\alpha_1$-adrenoceptor of sympathetic nerve endings (Saenz de Tejada, 1995). Therefore yohimbine facilitates erection at central level because acting on $\alpha_2$-adrenoceptors inhibits erection-suppressing impulses mediated by sympathetic $\alpha_1$-adrenoceptor. Instead, on penile tissue it inhibits presinaptically $\alpha_1$-adrenoceptor activity and facilitates the relaxation of smooth muscle cells. In addition, in 2005, d'Emmanuele di Villa Bianca *et al*. showed that apomorphine has a peripheral relaxant direct effect as well as an antiadrenergic activity on HCC strips. Since HCC possesses more D1-like (D1 and D5) than D2-like (D2, D3 and D4) receptors, while both D1- and D2-like receptors are mainly localized on smooth muscle cells, the relaxant activity is most probably mediated by D1-like receptor partially through NO release from endotheliumn (d'Emmanuele di Villa Bianca *et al*., 2005).

✓ Apomorphine

The activity of apomorphine was first known for its effect at central level in Parkinson's disease. Apomorphine is a central dopamine receptor agonist with effect on $D_1$ and $D_2$ receptors. Predominant sites of its action are located in the paraventricular nucleus, stria terminalis, medial pre-optic area and amygdaloid nucleus (Chen *et al*., 1996). Apomorphine induced-erections are both testosterone and NO dependent. (Chen *et al*., 1997; Melis *et al*., 1993).

✓ PDE5 inhibitors

Actually PDE5 inhibitors are the reference class in ED oral therapy. They enhance penile smooth muscle relaxation and penile erection in response to sexual stimulation. These drugs, inhibiting PDE5, decreases the degradation of cGMP. The augmentation of cGMP concentration increases smooth muscle cells relaxation which dilates sinusoids resulting in increased blood flow and enhancing erection.

### *1.5.3. Surgical treatment*

✓ Vascular surgery

The vascular surgery is only indicated in high selected patients to correct penile arterial insufficiency, to correct venous leakage due to veno-occlusive dysfunction by reducing venous outflow during erection, or both, when the two pathologies coexist.

✓ Penile prostheses

Penile prostheses are the last therapy when all other means have failed or are contraindicated. The intervention consist in an implantation of a pair of flexible silicon cylinders in the corpora cavernosa. The cylinder are connected to a pump device placed in the scrotum that can inflate or deflate the prosthesis.

## 1.6 Aim of studies

Erectile dysfunction (ED), defined as the consistent or recurrent inability of a man to attain and/or maintain a penile erection sufficient for sexual activity (2nd International Consultation on Sexual Dysfunction-Paris, June 28th–July 1st, 2003), can be caused by a number of different pathophysiological process. For these reasons, the present study can be divided into three steps in which we have analyzed various aspects of physiology and physiopathology of erectile function to give a contribution in the development of novel therapeutic approaches for treatment of ED and sexual arousal disorders.

We have first analyzed man dysfunctions that develop into ED, and in particular Peyroenie's disease (PD). PD is a connective tissue disorder involving the growth of fibrous plaques in the tunica albuginea of the penis with curvature and distortion, causing penile pain and erectile dysfunction during the erection (Gelbard *et al*., 1999). PD consists in a molecular and physiopathological disorder that is poorly understood mainly because of to the lack of an appropriate animal model. For this reason in the first part of this study we have focused on the characterization of a new model of PD naturally occurring in the mouse strain Tight skin (*Tsk*). *Tsk* mice, for their mutation of fibrillin I gene, has been previously proposed as a model of scleroderma and fibrosis (Jimenenz *et al*., 1984; Green *et al*., 1976).

PD represent a particular case of ED. Indeed, it is now widely accepted that ED is predominantly a vascular disease. In particular the erectile function is regulated by a balance between vasorelaxant and vasoconstrictor factors. The breaking of this balance can be cause of ED. To identify new pathways involved in penile erection, that could be target for new therapies, our attention has been placed on urotensin II, an endogenous peptide previously described as an important vasoactive peptide (Ames *et al*., 1999; Bottril

*et al*., 2000). We have evaluated if and how urotensin II pathway could be involved in penile erection.

Finally we have analyzed various methods for ED diagnosis. ED and its grade and the efficacy of ED treatment, are actually evaluated by medical questionnaire scores, a non-objective method. The unique objective and non-invasive method is the rigiscan, but it is poorly reproducible. The most used therapy in ED treatment is actually represented by PDE5 inhibitors which, inhibiting PDE5, increase the action of cGMP and thereby enhance penile cavernosum and vascular smooth muscle relaxation and erection. The target of these drugs, PDE5, has been founded in other tissues than the penis, such as vascular smooth muscle, smooth muscle of the gastrointestinal tract and in platelets. For these reasons we have assessed if platelet cGMP could act as a biomarker of PDE5 activity in ED clinical studies.

# *CHAPTER 2*

## A new animal model of Peyronie's disease

## 2.1 Peyronie's disease

Peyronie's disease (PD), or *induratio penis plastica*, is an inflammatory condition characterized by connective tissue disorder for the formation of fibrous plaques, nodules within the tunica albuginea (Gelbard *et al*., 1993; Devine *et al*., 1988)

The formation of these plaques alters penile anatomy, impeding expansion of the tunica albuginea during erection, resulting in penile binding. (Figure 14) (Bevilacqua *et al*., 2000 a; b).

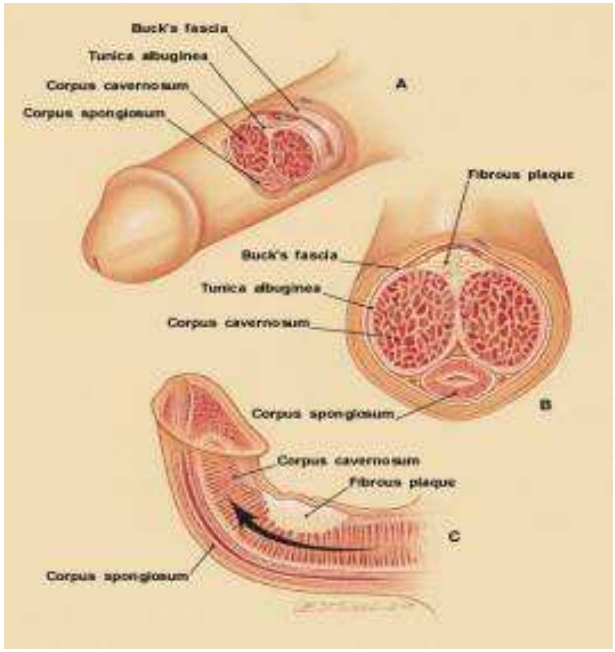



**Figure 14:** A fibrous plaque of Peyronie's disease on the top portion of shaft. Bending of penis upward is in response to presence of scar that prevents full expansion

This condition affects 5-9 % of the men (Vernet *et al*., 2005). Subsequent to the progression of the PD, and than to inflammation and cessation of the pain, the plaques may ossify in 15-25 % of cases (Vernet *et al*., 2005).

Peyronie's patients can be classified into three categories: (1) patients with asymptomatic plaques, (2) patients with plaques that exacerbate penile bending, (3) patients whose Peyronie's disease is associated with ED (Krane, 1997).

The mechanisms of the development of this disease are still unclear for the absence of an appropriate animal model. In fact, the animal model actually used have been obtained by injection of beta transforming growth factor (TGFβ) (El-Sakka *et a*l., 1997; El-Sakka *et a*l., 1998) or fibrin (Davila *et al*., 2003) in the rat tunica albuginea. Unluckily, these acute animal models do not reflect the natural course of human disease.

The aetiopathogenesis of PD is unclear. In fact in 1957, Furey initially suggested (and most investigators now concur) that minor sexual trauma is the major cause of PD (Furey, 1957). Afterward it has been demonstrated that repetitive microtrauma during intercourse may be the initiating factor, causing a localized autoimmune response in susceptible individuals (Mulhall, 2003; Jarow & Lowe, 1997). PD is now considered a multifactorial pathology based on the interaction of a genetic predisposition, trauma and tissue inflammation (Bevilacqua *et al*., 2000 a,b). Cytokine (particularly TGFβ and platelet-derived growth factor (PDGF)) dysregulation and overexpression, production of free radicals (ROS), and ultimately excessive extracellular matrix deposition leads to plaque formation (Mulhall, 2003). At the present time pharmacotherapy has not been effective or widely accepted, and surgery to either remove the plaque or insert penile prostheses remains the mainstay of the treatment.

We have looked for a spontaneous animal model that may mirror the natural history of human PD.

## 2.2 Materials and methods

### *2.2.1 Animals*

About twenty years ago Jacson Laboratory (Bar Harbor, ME) obtained male Tsk (B6.Cg-Fbn1$^{Tsk}$+/+ Pldn$^{pa}$/J) and female *pallid* (B6.Cg-Pldn$^{pa}$/J) mice. For our experiment these animals were from Siena colony, obtained using *Tsk* mice and homozygous pallid female, because *Tsk* females were poor breeders. In this study male congenic C57B1/6J wild type (WT) mice served as controls. All the animal analyzed had no sexual experiences and were housed in groups of two to four in macrolon cages at room temperature (22-24°C), with relative humidity (40-50%) and with food and water *ad libitum*.

### *2.2.2 Histology*

Male *Tsk* mice were monitored for about two years and sacrificed at different ages, 2, 4, 6, 8, 10, 12, 24 months (n= 8 for each groups). The penis of each animal was excised and fixed in buffered fomalin (5%) for 24 hours. All tissues were than dehydrated, cleared in toluene and embedded in paraffin. Finally trasversal section (6µm) were cut and stained with hematoxylin/eosin and Masson's trichrome stain.

### *2.2.3 Biochemical assay for hydroxyproline*

To determine tissue collagen, 6 penises from each animal group, at various ages, were weighed, homogenised in 5% TCA (1:9, w:v) and centrifuged for 10 min at 4000 × *g*. The pellet was then washed twice with distilled water and hydrolyzed for 16 h at 130 °C in 6N HCl. The pH of the samples was adjusted to 6.0 by adding 10N and then 0.1N sodium hydroxide. Distilled water was added to a final volume of 1.2 ml. Hydroxyproline was then

determined in tissue hydrolysates according to the method of Kivirikko *et al*. and data were expressed as μg/penis (Kivirikko *et al*., 1967).

### *2.2.4 Immunohistochemistry of TGFβ, type I and type II collagen*

To evaluate the presence of TGFβ the penis of mice of each group were stained by immunoperoxidase method. The tissues were fixed in formalin and cut in section of about 6 μm. Tissue sections were pre-treated with 3% hydrogen peroxidise for eliminating endogenous peroxidase activity. The samples were heated in a microwave for 20 minutes in citrate buffer of pH 6.0, and than allowed to cool slowly to room temperature for restoring immunoreactivity to tissue antigens retrieval. All the sections were incubated with 3% bovine serum albumin for 30 minutes at room temperature to block non-specific antibody binding. They were incubated overnight at 4°C with the primary antibody (Ab) (dilution 1:20), rabbit polyclonal Ab to mouse TGFβ. To determine type I collagen, the section slices were rinsed and incubated with goat polyclonal Ab to type I collagen (diluition 1:20). To detect type II collagen, instead, samples were incubated with rabbit polyclonal Ab to type II collagen (diluition 1:200).

For TGF-β and type II collagen, the section slices were rinsed and incubated with sheep anti-rabbit IgG for 30 min at room temperature. For type I collagen, the section slices were rinsed and incubated with rabbit anti-goat IgG for 30 min at room temperature. The staining was revealed by adding peroxidase–antiperoxidase complex, prepared from rabbit (or goat) serum. Detection was accomplished by incubating in diamino-benzidine freshly dissolved in 0.03% $H_2O_2$ in 50 mM Tris-HCl pH 7.6 as negative control for the immunostaining the primary antibodies were replaced by non-immunised rabbit (or goat) serum. The sections were counterstained with ematoxilyn.

### *2.2.5 RNA isolation and cDNA synthesis*

Total RNA was extracted from 6 mouse penises, at different ages, using TRi Reagent , according to the manufacturer's instructions. RNA was resuspended in RT-PCR Grade Water and the amount and purity of RNA was quantified spectrophotometrically by measuring the optical density at 260 and 280 nm. Integrity was checked by agarose gel electrophoresis.

Two micrograms of total RNA was treated with TURBO DNAse for 30 min (TURBO DNA-free kit) and reverse transcribed using the RETROscript kit according to the manufacturer's instructions. Two hundredths of the final volume of reverse transcription was used for real-time RT-PCR

### *2.2.6 Quantitative Real Time PCR for iNOS and TGFβ*

For iNOS we performed the amplification using Pre-made Assay in a 7500 abi Thermal Cycler. The amplification for TGFβ, instead, was done by Taqman chemistry using probes, primers and cycling condition previous described by Overbergh. (Overbergh *et al*., 1999).

### *2.2.7 Real Time PCR for collagens, PDGF*

Real-time RT-PCR was performed in triplicate for each sample on the MJ Opticon Monitor 2 with specific locked nucleic acid (LNA) probes from the Mouse Universal Probe Library Set. Primers were designed by using the free online ProbeFinder software (available at the Universal ProbeLibrary Assay Design Center: www.universalprobelibrary.com) that shows a pair of specific primers for each probe from the Universal ProbeLibrary set (see Table 2). The combination of primers and probes provides specific amplification and detection of the target sequence in the sample. PCR

reactions were performed in a volume of 25 µl and contained 12.5 µl of FastStart TaqMan Probe Master, 300 nM forward and reverse primers, 200 nM UPL probes and 5 µl of cDNA.

Reactions were incubated at 95 °C for 10 min and then amplified for 40 cycles, each cycle comprised of an incubation step at 94 °C for 15 s followed by 60 °C for 1 min.

The real-time RT-PCR assay included a no-template control and a standard curve of four serial dilution points (in steps of 10-fold) of each of the test cDNAs.

The analysis of the results was based on the comparative Ct method (ΔΔCt) in which Ct represents the cycle number at which the fluorescent signal, associated with an exponential increase in PCR products, crosses a given threshold. The average of the target gene was normalized to *18S rRNA* as the endogenous housekeeping gene (Winer *et al*., 1999).

| Transcript | Primer forward reverse | Probe# |
|---|---|---|
| 18S (m11188.1) | Forward 5′-aaatcagttatggttcctttggtc-3′ | 55 |
| | Reverse 5′-gctctagaattaccacagttatccaa-3′ | |
| | | |
| NOS-2 (NM010927) | Forward 5′-ctttgccacggacgagac-3′ | 13 |
| | Reverse 5′-tcattgtactctgagggctgac-3′ | |
| | | |
| Col1a1 (NM007742.2) | Forward 5′-catgttcagctttgtggacct-3′ | 15 |
| | Reverse 5′-gcagctgacttcagggatgt-3′ | |
| | | |
| Col3a1 (NM009930.1) | Forward 5′-tcccctggaatctgtgaatc-3′ | 49 |
| | Reverse 5′-tgagtcgaattggggaga-3′ | |
| | | |
| Pdgfb (NM011057.3) | Forward 5′-cggcctgtgactagaagtcc-3′ | 32 |
| | Reverse 5′-gagcttgaggcgtcttgg-3′ | |
| | | |
| Pdgfrb (NM008809.1) | Forward 5′-tcaagctgcaggtcaatgtc-3′ | 67 |
| | Reverse 5′-ccattggcagggtgactc-3′ | |
| | | |
| Pdgfa (NM008808.2) | Forward 5′-ccagcgactcttggagataga-3′ | 73 |
| | Reverse 5′-gggctctcaggcttgtctc-3′ | |
| | | |
| Pdgfra (NM011058.1) | Forward 5′-gtcgttgacctgcagtgga-3′ | 80 |
| | Reverse 5′-ccagcatggtgatacctttgt-3′ | |
| | | |
| Tgfb1 (NM011577.1) | Forward 5′-tggagcaacatgtggaactc-3′ | 72 |
| | Reverse 5′-gtcagcagccggttacca-3′ | |

Tab 2. Primers Sequence and Probe Catalog Number

### *2.2.8 Statistical analysis*

Data are presented as mean ± standard deviation. The significance between groups was calculated using one-way analysis of variance. A p value less than 0.05 was considered significant.

## 2.3 Results

### *2.3.1 Macroscopic morphologic analysis*

The *Tsk* mice were monitored for about two years and sacrificed at different ages to evaluate the development and progression of pathology.

The gross morphology of the penis at the age of 2 months (Figure 15 A), 6 months (Figure 15 B), 8 months (Figure 15 C) and 12 months (Figure 15 D) is reported in Figure 15.

As can be seen there was an increase of fibrotic plaque with age. The disease in *Tsk* mice slowly developed reaching the maximum at 12 months. No further progression was observed from 12-13 months onwards.

Instead we have obtained different results from the penis of male congenic C57B1/6J wild type that showed a normal appearance at different age (Figure 15 E,F).

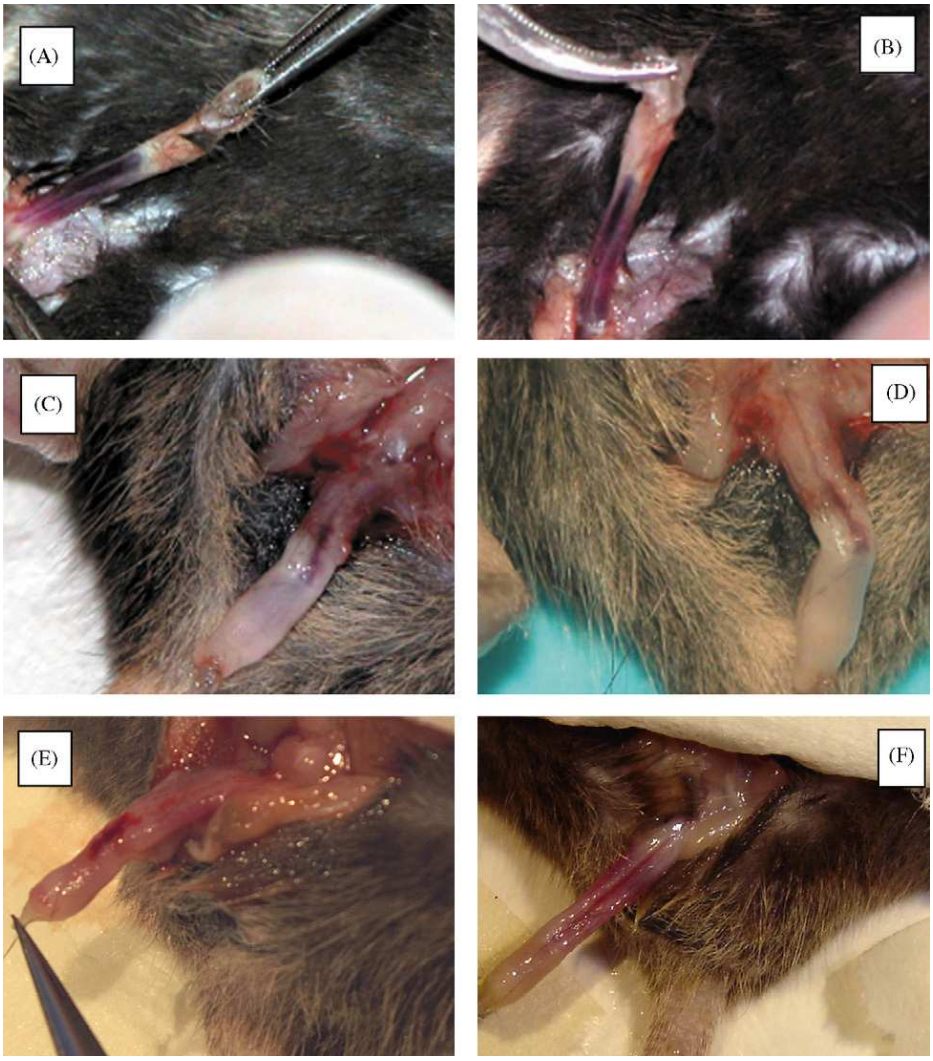



**Figure 15**: **Gross anatomy of the *Tsk* mouse penis at 2 (A), 6 (B), 8 (C) and 12 (D) months of age**. The penises from 2 month-old mice show a normal appearance (A). The white areas in (B), (C) and (D) are fibrotic hypovascularized segments in the distal part of the penis. Fibrosis is appreciable from 6 months of age (B) onwards and progressively increases at 8 (C) and 12 (D) months of age. The latter group of mice (D) shows an extensive fibrosis and bending of the penis. The penises of 2 (E) and 12 month-old C57Bl/6 mice (F) show a normal appearance.

### *2.3.2 Histopathological analysis*

The histopathological studies were performed on penis tissues at different ages.

As can be seen in Figure 16 A, there was a normal appearance of the tunica albuginea in the penis of mice sacrificed at two months.

At six months of age, instead, the thickened of the inner layer bundles and the presence of inflammatory infiltrate of mononucleated cells in several areas, caused an evident disorganization and disassociation of the outer layer bundles (Figure 16 B).

In eight month-old *Tsk* mice the inner layers bundles of the tunica were markedly thickened and diffused areas of chondroid metaplasia were usually detectable (Figure 16 C).

These characteristics were more evident at twelve months of age when irregularly shaped and homogenous fibrous plaques were associated with areas of hyalinization, chondroid metaplasia or heterotopic ossification (Figure 16 D).

The sections of penis were stained by Masson's trichrome (Figure 17)

The penis tissue slides from two month old *Tsk* mice showed a normal amount and distribution of elastic and collagen fibres in the tunica albuginea (Figure 17 A).

At six months there was an increase of deposition of collagen bundles thickening the tunica albuginea (Figure 17 B).

The penis tissue slides from eight month old mice give us and idea about a development of pathological condition. In fact the smooth muscle structures in the pillars were not evident and the bundles appeared disrupted and dissociated (Figure 17 C).

These lesion is maximal in the penis of mice at twelve moths. In these tissues plaques were similar to those observed in the human Peyronie's disease. This pathological phase is characterized by an excessive collagen accumulation, disrupted

collagen bundles and areas of heterotopic ossification with bone mineralization (Figure 17 D).

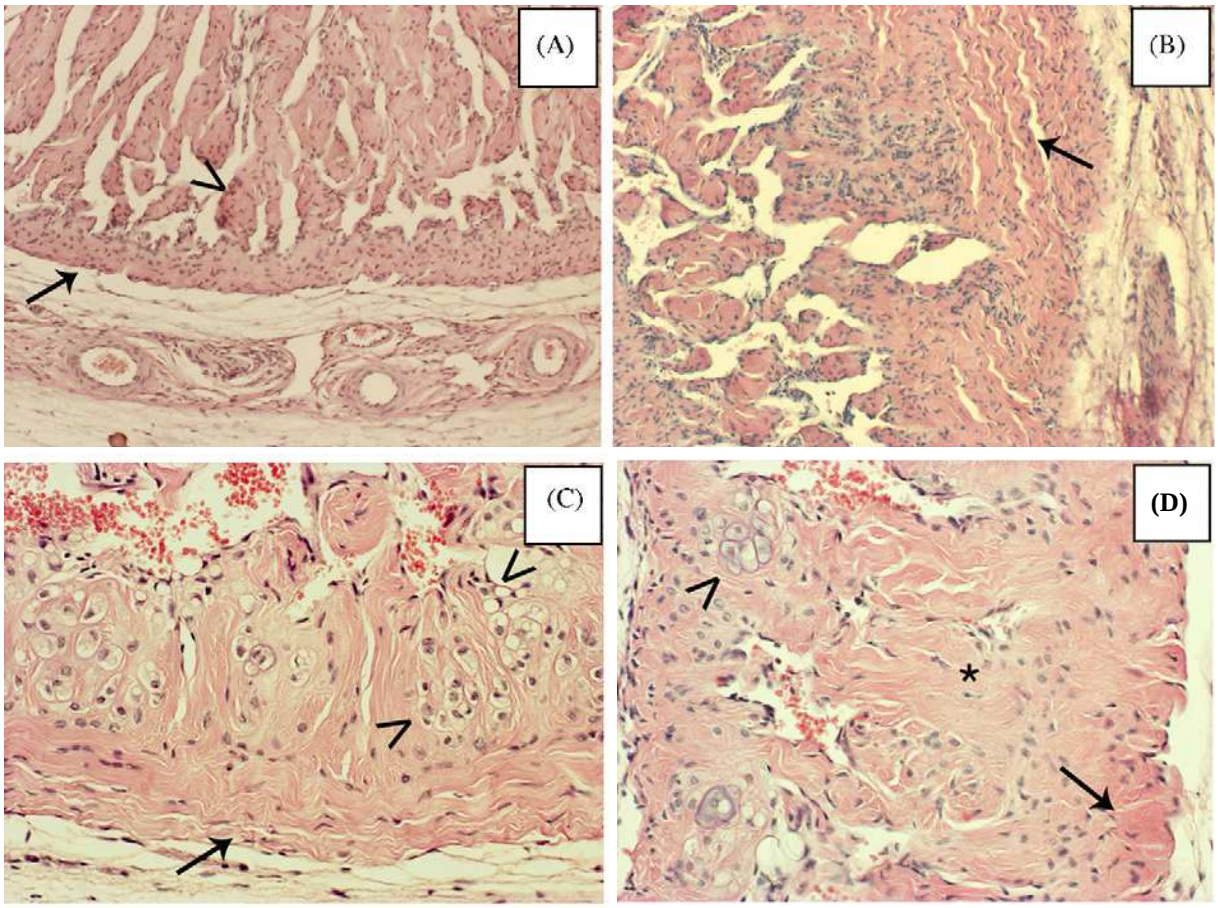



**Figure 16: Histopathological steps in the development of PD-like lesions in *Tsk* mice.**
**(A)** Normal appearance of the TA at 2 months of age. In this transverse section, note the fine circularly oriented bundles of the inner layer of the Tunica (arrowhead), which surround and penetrate the cavernous tissue. The outer layer bundles are longitudinal (arrow). **(B)** Longitudinal section of the TA at 6 months of age. The outer layer bundles are disorganized and disassociated (arrow). The inner layer bundles are thickened and an inflammatory infiltrate of mononucleated cells is present in several areas. **(C)** Longitudinal section of the TA at 8 months of age. The inner layer bundles of the Tunica are thickened and show diffused areas of chondroid metaplasia, which extend into the corpus (arrowhead). The outer bundles are disorganized and disassociated (arrow). **(D)** Transverse section of the TA at 12 months of age. The inner layer bundles of the Tunica are greatly thickened and form irregular homogeneous plates (asterisk) or nodules.

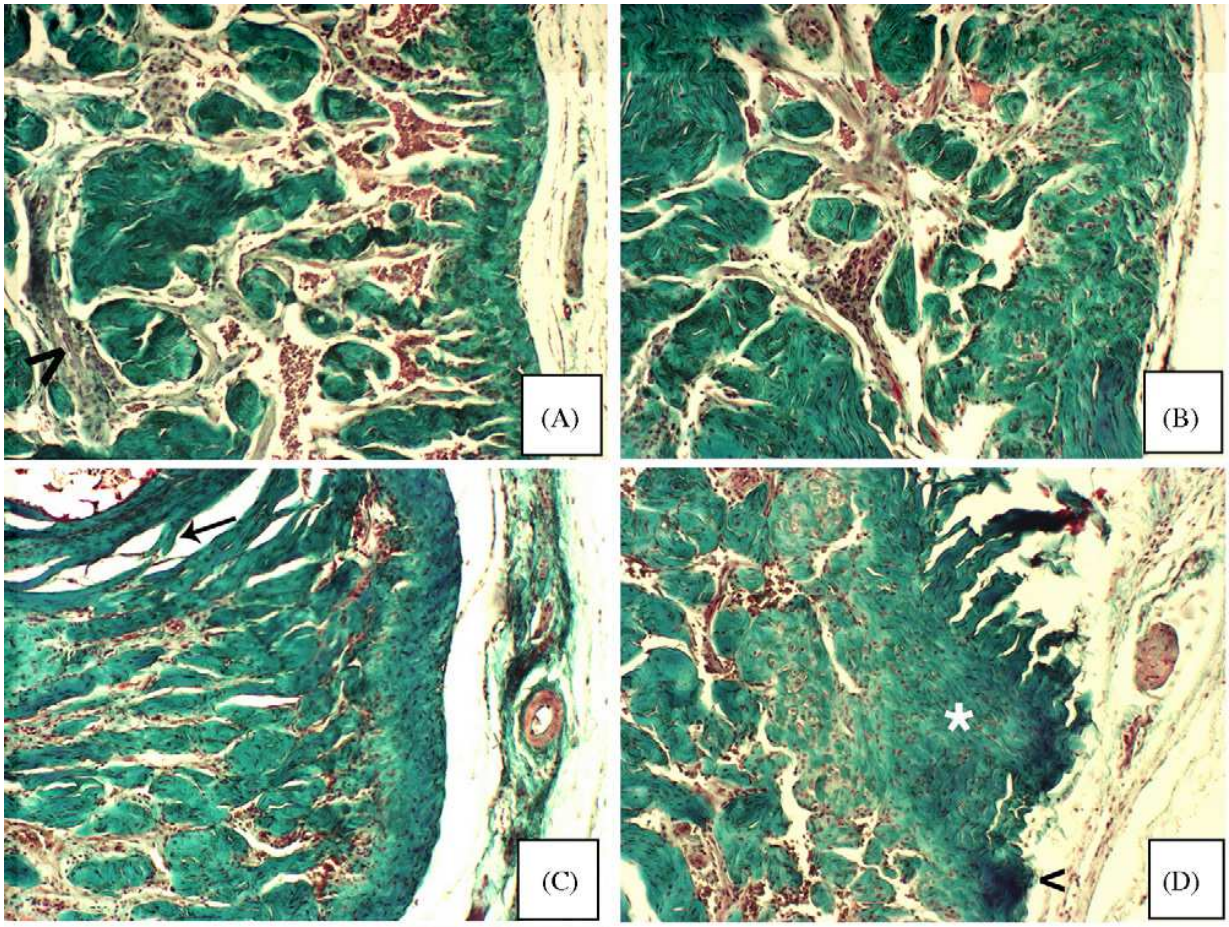



**Figure 17: Histopathological steps in the development of PD-like lesions in *Tsk* mice.**
**(A)** Section of a TA with a normal appearance in a 2 month-old *Tsk* mouse. The intracavernosal pillars extending from the inner layer of the Tunica are composed of smooth muscle structures that intermingle with connective tissue (arrowhead) in which elastic and collagen fibers are intertwined. **(B)** Section of the TA in a 6 month-old *Tsk* mouse. The Tunica is thickened due to the increased deposition of collagen bundles. **(C)** Section of the TA in a 8 month-old *Tsk* mouse, disrupted and disassociated collagen bundles (arrow) are present. The smooth muscle structures in the pillars are not evident. **(D**) Section of the TA in a 12 month-old *Tsk* mouse. Plaque, similar to that characterizing human Peyronie's disease, is present in the tunica albuginea of the corpora cavernosa (asterisk).

### *2.3.3 Biochemical assay for hydroxyproline*

The results of biochemical analysis of the total penis collagen are reported as hydroxyproline (HO-proline) for eight and twelve months old for both wild type and *Tsk* (Figure 18 A) and as mRNA for collagen I and III in six, eight, ten and twelve months old *Tsk* mice (Figure 18 B).

In the panel A the values are expressed as percent of increase in HO-proline in respect to two months old mice because no difference in tissue HO-proline content was observed between the penis of C57B1/6J wild type and *Tsk* mice at two months of age. At eight months of age penile tissues of *Tsk* mice showed an average of 50% ($p<0.01$) and 34% ($p<0.01$) higher content in HO-proline than the penis of two months old *Tsk* mice (265.8±17.2 µg/penis) and eight months old WT mice (320.9±24.6 µg/penis), respectively. At twelve months of age the penis of *Tsk* mice showed a mean content in HO-proline (434.7±20.4 µg/penis), which was 43% ($p<0.01$) greater than that of WT mice.

RT-PCR for type I and II collagens in penile tissue showed an increase of collagen content in eight months old *Tsk* mice (Figure 18 B). This effect was preceded, at six months of age, by a significant increase of type I and II collagen mRNA expression. Instead it was followed by a decrease of type III collagen mRNA expression particularly evident at twelve moths of ages (Figure 18 B).

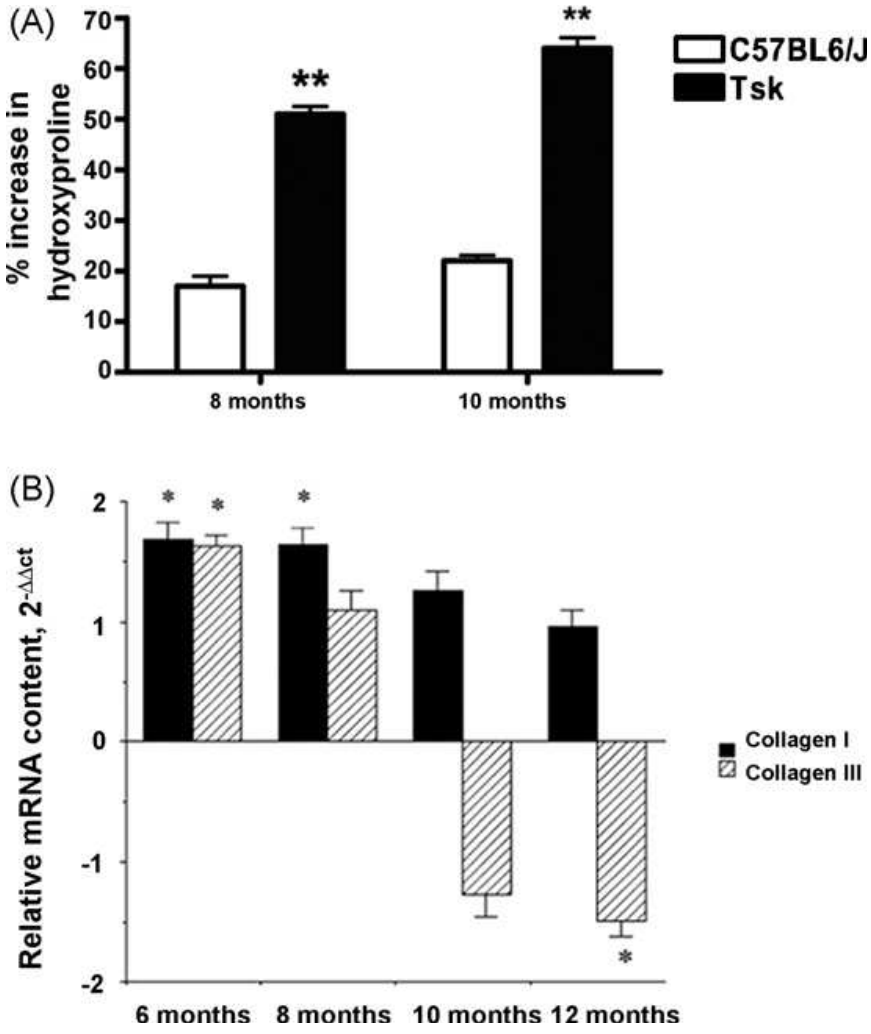



**Figure 18: Total tissue collagen measured as hydroxyproline and quantitative RT-PCR of type I and III collagens. (A)** Values are expressed as the percent changes (mean±S.D.) from the mean values obtained in 2 month-old C57Bl/6J WT and *Tsk* mice, respectively. Tissue hydroxyproline values in 2month-old WT and Tsk mice were 273.2±20.4 and 265.8±17.2 µg/penis, respectively. Data were from 6 penises of each strain of mouse at the different ages. **p < 0.01 *vs* their respective 2 month old reference mice.
**(B)** A significant increase in expression of types I and III collagens appear evident at 6months of age. This is followed by decreased expression of type III collagen from 10 months of age onwards. *p < 0.01 *vs* their respective 2 month-old reference mice.

### *2.3.4 Biochemical analysis for TGFβ*

The results obtained by immunostaining for TGFβ showed a soft positive reaction around the small vessels in the tunica albuginea of *Tsk* mice at two months of age (Figure 19 A). More marked positive staining was observed at six and eight months (Figure 19 B and Figure 19 C, respectively). Instead the immunostaining for TGFβ was less intense in penile tissues of *Tsk* mice at twelve months of age (Figure 19 D).

The positive immunostaining for TGFβ, instead, was totally absent in the penis of C57B1/6J wild type animals at various age.

In addiction quantitative RT-PCR was performed for TGFβ; the results are reported in Figure 19 E.

According to the immunohistochemical results the maximal increase of TGFβ was observed at six and eight months of age.

The level of TGFβ slowly declined thereafter reaching a significant difference at twelve months with respect to those observed at eight months.

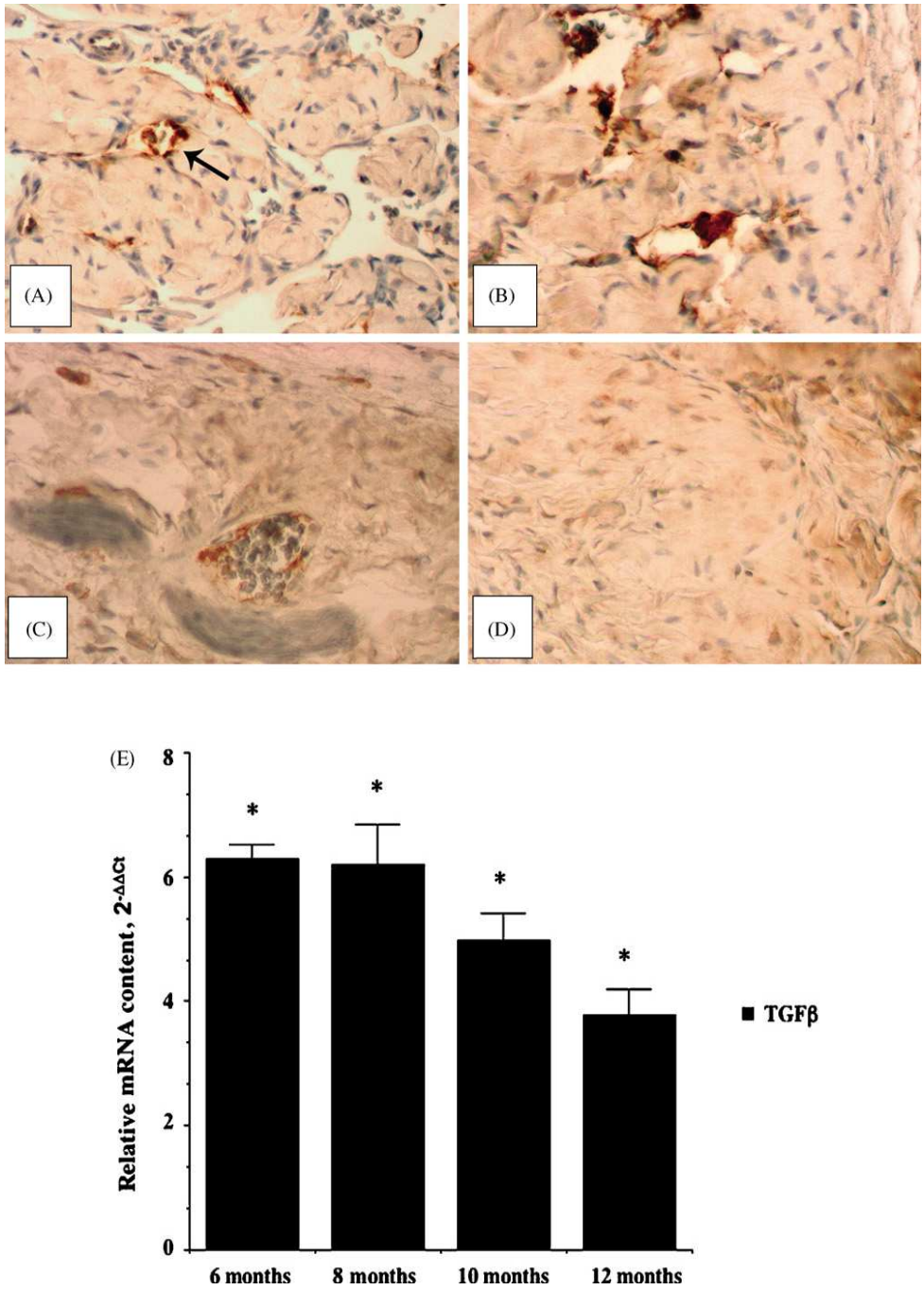



**Figure 19: (A–D) Representative immunohistochemical reaction for TGFβ in the tunica albuginea (TA) of *Tsk* mice at various ages.** TA of *Tsk* mice at 2 (A), 6 (B), 8 (C) and 12 (D) months of age. TGFβ staining is mainly detected around the small vessels (arrow). TGFβ expression is evident from 2 months of age (A) onwards. Maximum staining is observed at 6 (B) and 8 (C) months of age. A less intense staining of TGFβ is still present at 12 months of age (D). Counterstained with haematoxylin, original magnification ×200. **(E) Quantitative TGFβ expression.** TGFβ expression in *Tsk* penises at different ages is reported. The TGFβ signal is significantly increased at 6 and 8 months of age compared to 2 month-old *Tsk* mice. A significant decrease in TGFβ expression appears evident from 12 months. *$p < 0.01$ vs their respective 2 month-old reference mice.

### *2.3.5 RT-PCR analysis for iNOS*

The results of RT-PCR analysis of iNOS expression are reported as mRNA for six, eight, ten and twelve months old for *Tsk* mice (Figure 20). The values are calculated relatively to that obtained from two months old mice.

RT-PCR revealed an increase of iNOS expression with disease progression in *Tsk* mice.

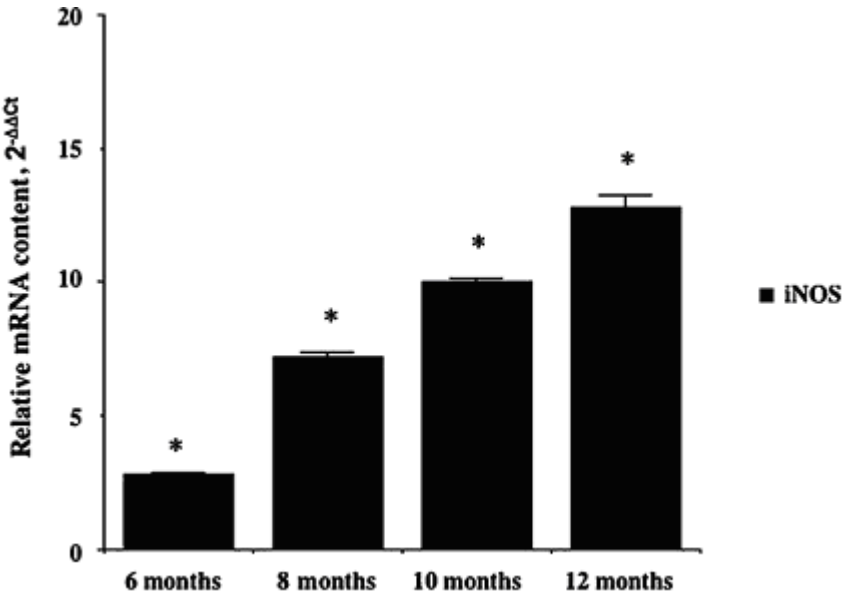



**Figure 20: Quantitative RT-PCR of iNOS**.
iNOS mRNA expression progressively increases with age.
*p < 0.01 *vs* their respective 2month-old reference mice.

## 2.4 Discussion

Peyronie's disease is an acquired inflammatory condition of the penis characterized by the development of plaque in the tunica albuginea. This pathological condition is associated with penile curvature, erectile dysfunction and, in some cases, pain. Smith *et al*., sowed that the prevalence of PD affect 3.2 % of the male population, (Smith *et al*., 2005; Schwarzer *et al*,. 2001). About two-thirds of these are between 45 and 60 years old, although an age range of 18 to 80 years has been reported.

Over 250 years after the first description by Francois de la Peyronie, as the exact causes of PD remain enigmatic, it is not surprising that many treatment option have been tried for this condition (Ralph *et al*,. 1992). Many of the theories seek to explain the pathogenesis of PD: while trauma is considered to be the provocative stimulus, other theories include failure of fibrin clearance, collagen alterations, genetic predisposition, autoimmune factors, free radical production, and cytogenetic aberrations (Smith *et al*., 2005). Many of these theories are based on an animal (*in vivo*) and cell culture (*in vitro*) models. The in vivo models are attained by the injection of both TGFβ (El sakka *et al*., 1997; El Sakka *et al*., 1998) or fibrin (Davila *et al*., 2003) into the rat tunica albuginea. The cell culture model was explained by Mulhall *et al.* whose derived cultured cells from plaque tissue; this *in vitro* analysis showed reliable phenotypic, genotypic and functional alterations in pathological tissue compared to normal tunica-derived or neonatal foreskin derived fibroblasts (Mulhall *et al.*, 2002). Nevertheless these model was not able to flawlessly replicate the *in vivo* environment. These studies suggests the major role of TGFβ signalling pathway in the development of disease but it is easy understood that actually the difficulty in PD diagnosis and therapies is the lack of an appropriate experimental model.

With the study reported here we described a spontaneous model of PD in the *Tsk* mice that with age could reproduce the natural development of human PD. This represents the

first spontaneous animal model. The *Tsk* mutation, consisting with a tandem duplication of the fibrillin I (FbnI) gene resulting in a larger transcript, discovered in 1976 (Christner and Jimenez 2004). As the FbnI gene participates at the microfibril formation, the *Tsk* mouse has been proposed as a model of scleroderma and fibrosis (Jimenenz SA *et al*., 1984; Green *et al*., 1976).

Male *Tsk* mice were monitored for twelve months to evaluate the homologies and the differences with the human disease. As in the human pathology, in the *Tsk* mice the disease develops slowly with age and progresses until twelve months when the gross and microscopic appearance of penis are similar to those reported in PD patients. No further progression was observed from twelve months of age onwards. In the first stage of the disease, during six months of age, there was the presence of inflammatory infiltrates beneath the tunica albuginea, disorganization of the outer layer and thickening of the inner layer due to increased collagen deposition. From eight months on there is an increase of disorganization and accumulation of collagen until twelve months, the later stage, characterized by the presence of thickened plaques of fibrous tissue in the tunica albuginea. Like in the human disease the formation of fibrotic plaques alters penile anatomy causing different pathological degrees (Bevilacqua *et al*., 2000). The development of PD in *Tsk* mice is characterized by a gradual increase in synthesis and deposition of collagen. In particular we have observed a relative increase of collagen of type I, that may greatly contribute to the penile stiffness, to the detriment of the more elastant type II collagen. The collagen accumulation in the penis of *Tsk* mice may be driven also in cooperation with other cytokines. In fact RT-PCR revealed high level of TGFβ especially in the earlier stage of the disease. Probably various pathways, which involve different cell type, growth and vasoactive factor, are implied in the collagen deposition and consequent fibrosis process.

At this regard we have demonstrated that iNOS could be involved in PD ; in fact RT-PCR for iNOS revealed an increase of mRNA level during the development of the pathology.

In conclusion, the *Tsk* mouse, as it replicates similar condition to the human disease, may represents a new animal model of PD naturally occurring, thus by using this especially model it is could be possible to develop in features some therapeutical approaches for PD disease.

# *CHAPTER 3*

## Urotensin II: a novel target in penile function

## 3.1 Urotensin II

Urotensin II (UII) is a disulphide-bridged peptide that was originally isolated from the goby Gillichthys Mirabilis urophysis (Bern *et al*., 1979) (Figure 20 a).

UII isoforms were identified in many species of fish (Bern *et al*.; Colon *et al*., 1996), as well as in amphibians (Conlon *et al*., 1992) and mammals, including man (Coulouarn *et al*., 1999). It has been shown that UII acts by a specific receptor, belonging to the superfamily of G-protein-coupled receptors (GPCRs). This receptor was previously known as an orphan G-protein coupled receptor (GPR14) and now designed as UT receptor (Figure 19 b) (Foord *et al*., 2005). UII amino-terminus region diverges between species while all isoforms share a conserve, cyclic hexapeptide, core-sequence motif of CFWKYC. The fact that U-II isopeptide is conserved over a wide range of evolutionary levels, indicates that the cyclic region retains full biological activity as a UT receptor ligand.

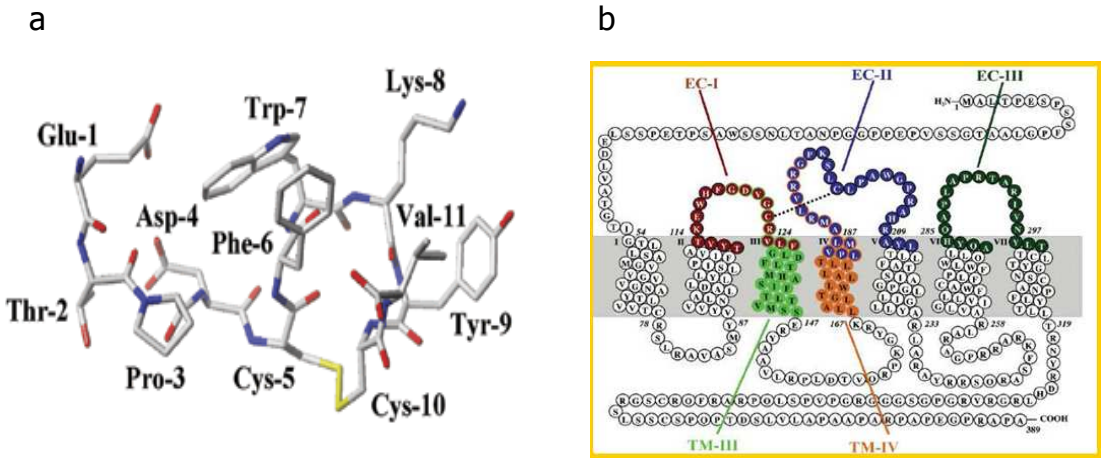



**Figure20: Representation of UII and UT receptor.**
**(a)** Structure of human (h-)UII: peptide contains a disulphide bridge between the two cysteine residues that cyclises the peptide into its biologically active conformation. The cyclic hexapeptide core, the conservative portion, is receded by an acid residue and followed by a neutral residue in h-UII. **(b)** Schematic structure of UT receptor: UII-binding determinants are located in the extracellular loops and the top portion of the transmembrane domains.

UII and UT receptor are expressed in variety of peripheral organs (liver, kidney, endocrine glands) and especially in cardiovascular tissues (cardiomyocyte, endothelium, and vascular smooth muscle cells) (Conlon *et al*., 1996; Ames *et al*., 1999).

Several studies have revealed that UII produces potent but variable constrictor effects in some but not in all vascular beds. Indeed, UII was described as the most potent vasoconstrictor identified by now (Ames *et al*., 1999); in fact Douglas et al. showed that UII is more potent than endothelin-1 (Douglas *et al*., 2000). On the other hand, it has been also demonstrated a vasodepressor and regionally selective vasodilator effect of UII (Bottril *et al*., 2000; Gardiner *et al*., 2001). The role of UII in cardiovascular system seems to be both species and vascular bed-dependent (Douglas 2000; Camarda 2002). In fact *in vitro* studies performed by Gibson *et al*. in 1987, showed that on intact rat aortic strips precontracted by noradrenaline low concentration (0.1-0.5 nM) of UII induced vasodilation, while higher concentrations (1-10nM) caused further contractions. After removal of the endothelium the vasodilation was absent but the contractile response remained (Gibson, 1987). The contractile effect of UII was also observed in isolated rat carotid arteries and left anterior descending coronary arteries, whereas no effect was observed in isolated small mesenteric arteries (Bottril *et al.*, 2000). When these vessels were first precontracted with metoxamine, UII caused endothelium-dependent relaxation (Bottril *et al*., 2000). In non human primates, UII caused vasoconstriction in large vessels such as aorta (Ames *et al*., 1999), coronary arteries (Ames *et al*., 1999) and pulmonary arteries (Zhu *et al*., 2004). In addition, even in man arteries isolated from heart (Maguire *et al*., 2000; 2004) and lung (MacLean *et al*., 2000; Stirrat *et al*., 2001; Bennet *et al*., 2004) was observed a vasoconstrictor effect of UII. Instead in human small resistance vessels such as abdominal resistance arteries Stirrat *et al*. (2002) observed an UI-induced relaxation, while in human skin microvasculature, there was no response to UII (Hillier *et*

*al*., 2001). Besides, as the presence of the peptide in pmolar concentrations in blood in healthy individuals, the U-II/UT receptor system seems to play an important role in cardiovascular function (Cheung 2004; Douglas 2004). Several studies reported that cardiovascular effect of UII include positive inotropy in human and rat myocardium (Russel *et al*., 2001; Russel and Molenar 2004; Gong *et al*., 2004), although negative ionotropy has been showed in canine cardiomyocites and in rabbit papillar muscle (Fontes-Sousa *et al*., 2007). On the other hand, in vivo studies showed that, in anesthetized monkey, systemic administration of U-II caused circulatory collapse as result of the profound vasoconstrictor effect of the peptide (Ames 1999). Conversely, infusion of U-II did not cause any significant change in heart rate, mean arterial pressure or cardiac index in healthy male volunteers (Affolter *et al*., 2002). In anesthetized rats intravenous injection of U-II showed an hypotensive effect partially mediated by NO (Abdelrahman and Pang 2002).

Two vascular effect of UII are regulated by the balance of the UII/UT receptor system on the vascular smooth muscle and on the endothelium. It has been demonstrated that inhibition of phospholipase C (PLC) inhibited the contractile response induced by UII (Saetrum *et al*., 2000). On these basis it has been showed that the contractile effect is mediated by an increase of phosphoinositide (PI) turnover, with a subsequent increase in intracellular $Ca^{++}$ (Ames *et al*., 1999; Filipeanu *et al*., 2002). Influx of intracellular calcium through the activation of L-type calcium channel, is also responsible for the UII induced increase in intracellular calcium and hence vasoconstrictor actions (Maguire and Davenport 2002) (Figure 22). Instead vasodilatatory response to UII appear to be dependent on the presence of endothelium. The endothelium-dependent vasorelaxation observed on precontracted rat coronary arteries, was abolished in the presence of the eNOS inhibitor L-NAME (Bottril *et al*., 2000). It has been showed that vasodilation is caused by

UII-mediated increases in intracellular calcium in endothelial cells, resulting in release of the endothelial-derived relaxing factors: both NO and endothelium-derived hyperpolarizing factor (EDHF) (McDonald *et al*., 2007) (figure 22). In addition to endothelial factors, variations in both the vasodilatatory and constrictor response to UII might reflect the expression of the UT receptor which, in part, will depend on the size and location of the vessel (Onan *et al*., 2004).

It is well known that corpus cavernosum is an highly vascularised tissue whose function is dependent upon a balance between the vasodilatory and the vasoconstrictory tone. In penile erection there is a strong involvement of the vascular system and the L-arginine/NO pathway plays a major role (Ignarro 1990).

Thus we aimed to address part of our study in this issue, in particular we investigated: i) the involvement of UII/UT receptor pathway in the penile erection and ii) molecular mechanisms induced UII vascular effect.

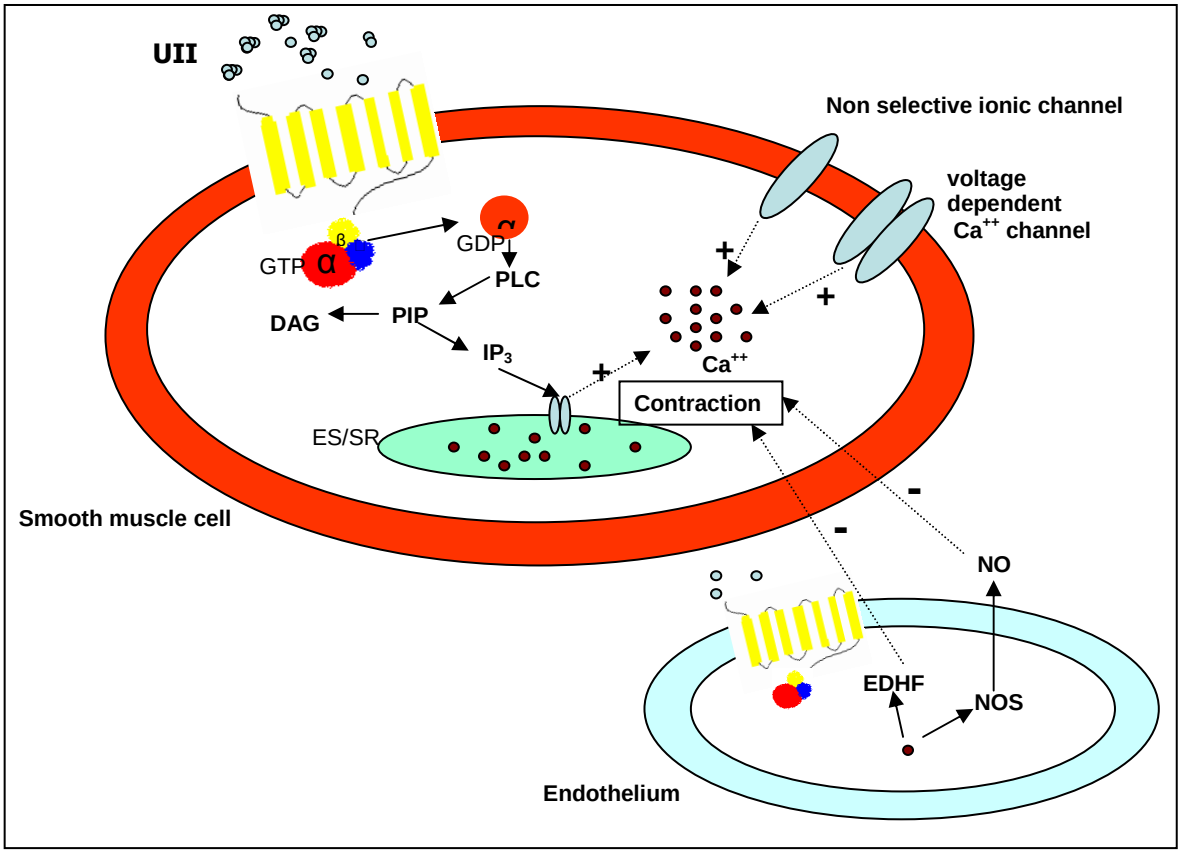



**Figure 22: UT receptor signalling.**

UT receptor is coupled with $G_{\alpha q/11}$ subtype of heterometrimeric G proteins. Activation of $G_{\alpha q/11}$ on smooth muscle cell leads an increase in inositol triphosphate and mobilization of intracellular $Ca^{2+}$ with following contraction. On endothelium the activation of UT receptor may cause a release of EDHF and eNOS activation following in vasodilation.

## 3.2 Materials and methods

### 3.2.1 Investigation of UII/UT receptor pathway involvement in the penile erection

#### *3.2.1.1 Peptide synthesis*

The human U-II was synthesized and purified at the Department of Pharmaceutical and Toxicological Chemistry of the University of Naples, Federico II. The peptide was obtained by solid-phase peptide synthesis (Grieco *et al*., 2000;45:4391–4). Purification was achieved using a semi-preparative reversed phase high-performance liquid chromatography (HPLC) C18 bonded silica column. The purified peptide was 99% pure as determined by analytical reversed-phase HPLC. The correct molecular weight was confirmed by mass spectrometry and amino acid analysis.

#### *3.2.1.2 Human Corpus Cavernosum*

The human corporus cavernosum (HCC) was excised from the penis with male-to-female transsexual surgical procedures. All patients were informed of all procedures and gave their written consent. The protocol was approved by the Ethics Committee of the Medical School of the University of Naples Federico II. Patients undergo appropriate hormonal pre-treatment with antiandrogens and estrogens to adapt to female appearance, and the therapy is discontinued 2 months before surgery. After amputation tissue was immediately placed in ice-cold oxygenated Krebs' solution (Mirone *et al*., 2000). Krebs' solution had the following composition (mM): 115.3 NaCl; 4.9 KCl; 1.46 CaCl2; 1.2 MgSO4; 1.2 KH2PO4; 25.0 NaHCO3; 11.1 glucose.

### *3.2.1.3 Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction* (RT-PCR) for UII receptor (GPR14)

The presence of GPR14 was determined by quantitative PCR. After isolation mRNA from HCC and human colon tissue and subsequent preparation of cDNA, reverse transcription was performed and 100 ng of the RNA samples described earlier were used for quantitative PCR. Samples were run in triplicate in 50 µL reactions by using an ABI PRISM 7500 sequence detector system. Amplification was done using Hs01027998_s1 Taq Man Gene Expression assay amplification set up and cycling were performed according to manufacturer's recommendations.

### *3.2.1.4 Western blot analysis for UII receptor (GPR14)*

Human or rat corpus cavernosum were homogenized in modified RIPA buffer (Tris-HCl 50 mM, pH 7.4, Triton 1%, sodium deoxycholate 0.25%, NaCl 150 mM, ethylenediaminetetraacetic acid 1 mM, phenylmethylsulphonyl fluoride 1 mM, aprotinin 10 mg/mL, leupeptin 20 mM, NaF 1 mM, sodium orthovanadate 1 mM) by liquid nitrogen. Protein concentrations of cell lysates were estimated by the Bio-Rad protein assay using BSA as standard. Equal amounts of protein (40 µg) of the cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). Western blotting was performed by transferring proteins from gel to a sheet of polyvinylidene fluoride membrane at 250 mA for 60 min at room temperature. The filters were then blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk (PM) for 45 min at room temperature and incubated with rabbit polyclonal antibody for GPR14 (H-90) (1:1000) in 1x PBS, 5% nonfat dried milk and 0.1% Tween 20 (PMT) overnight at 4°C. Than the membranes were extensively

washed in PBS containing 0.1% v/v Tween 20, before incubation for 2 hours with horseradish peroxidase-conjugate anti mouse secondary antibody (1:5,000). Membranes were then washed and developed using enhanced chemiluminescence substrate. . To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the mouse β-actin antibody. The protein bands were densitometrically analysed with a model GS-800 imaging densitometer.

#### *3.2.1.5 Immunofluorescence on HCC*

HCC samples were fixed in 4% formalin buffer and paraffin-embedded. Cross-sections were cut (6 mm) and used for the detection of specific proteins by immunofluorescence. Paraffin sections, after being de-waxed and rehydrated, were boiled for 30 minutes in citrate buffer for antigen retrieval. The sections were incubated in blocking buffer (PBS with 1% casein) for 1 hour at room temperature in a humidified chamber. Endogenous biotin was blocked with the Avidin/Biotin Blocking Kit. The sections were incubated (4°C) overnight with anti-GPR14 goat polyclonal antibody (1:100) diluted in 1% blocking reagent 0.3% Triton X-100 in PBS. Sections incubated with 1% goat serum were used as negative controls. Subsequently, sections were incubated with donkey anti-goat secondary antibody Texas Red conjugated (1:100) for 30 minutes. After washing, sections were incubated for 1 hour with mouse monoclonal anti-a-smooth muscle actin (a-SMA) antibody fluorescein isothiocyanate (FITC) conjugated (1:250), or for 2 hours with rabbit polyclonal anti-von Willebrand factor (vWF; 1:100) and donkey anti-rabbit secondary antibody FITC conjugated (1:100) for 30 minutes. 4′,6-Diamidino-2-phenylindole was used to stain the nuclei. Images were taken with the aid of a Leica DFC340 FX video-camera connected to a Leica DMRB microscope using Leica Application Suite software V2.4.0 and processed with Adobe Photoshop 7.0 software.

### *3.2.1.6 Human corpus cavernosum preparation and functional studies*

HCC was dissected in longitudinal strips (2cm) and used for functional studies in isolated organ bath filled with oxygenated (95% $O_2$ and 5% $CO_2$) Krebs' solution at 37°C. Strips were stretched until a resting tension of 2g and allowed equilibrated for at least 90 minutes during which tension was adjusted, when necessary, to 2g and bathing solution was periodically changed. After equilibration, tissues were standardized by performing repeated phenylephrine (PE; 1 mM; Sigma) contractions until three equal responses were obtained. In orther, to verify endothelium integrity, acetylcoline (Ach) cumulative concentration-response curve (0.01–10 mM ) was performed on PE precontracted strips. Strips without a functional endothelium (non responsive to Ach) were obtained by incubating in distilled water for 15 seconds. In the first set of experiments a concentration-response curve to U-II (0.1 nM to 10 mM) was obtained using HCC strips under resting condition. In the second step UII cumulative concentration-response curve (0.1 nM - 10 µM) was obtained in the presence or absence of endothelium, using HCC strips pre-contracted with PE (1 mM). To assess the involvement of NO, we incubated the strips for 30 minutes with N (G)-nitro-L-arginine methyl ester (L-NAME; 100 mM), NOS inhibitors, before U-II challenge. Data were calculated as % of relaxation to PE tone and expressed as SEM from eight separate specimens from four different tissues. The results were analyzed by using analysis of variance (ANOVA) followed by Bonferroni post hoc test.

### *3.2.1.7 Measurement of nitrite and nitrate (NOx) in HCC strips*

HCC strips were incubated with UII (0.1 µM) or vehicle (water) for 30 minutes at 37°C. The reaction was stopped in liquid nitrogen. Homogenate of HCC strips and a standard curve of sodium nitrate were incubated in a microplate with cadmium (50 mg/well) for 1 h to convert $NO_3^-$ to $NO_2^-$ (Thomsen *et al*., 1990). After centrifugation

at 14,000 rpm for 15 min, total nitrite (NOx) content was determined fluorometrically in microtiter plates using a standard curve of sodium nitrite (Misko et al., 1993 *Anal. Biochem.* 214 pp. 11–16). NOx content was calculated by using the internal standard curve.

#### *3.2.1.8 Intracavernous Administration of UII in Anesthetized Rats in vivo*

The present study was performed in accordance with the guidelines of Italian law (N.16/1992) and European Council law (N.86/609/CEE) for animal care. The experimental procedures were approved by the Animal Ethics Committee of the University of Naples. Male Wistar rats (200–250 g) were obtained from Harlan and kept under laboratory conditions (temperature 23±2°C, humidity range 40–70%, 12-hour light/dark cycle and food and water *ad libitum*). In anesthetized rats (with an intraperitoneal injection of urethane- 1 g/kg), an heparinized (5 I.U./mL) polyethylene catheter was introduced into the carotid artery and connected to a pressure transducer (BLPR-2) to measure systemic blood pressure. After a midline perineal incision, followed by blunt dissection of the overlying striated muscles, a 26G-gauge needle attached to an heparinized (10 I.U./mL) polyethylene catheter was inserted into the corpus cavernosum and the intracavernous pressure (ICP) was monitored with a pressure transducer (BLPR-2),. These parameters were recorded and data acquisition and calculations were performed using a computer system (Biopac), (Figure 23).

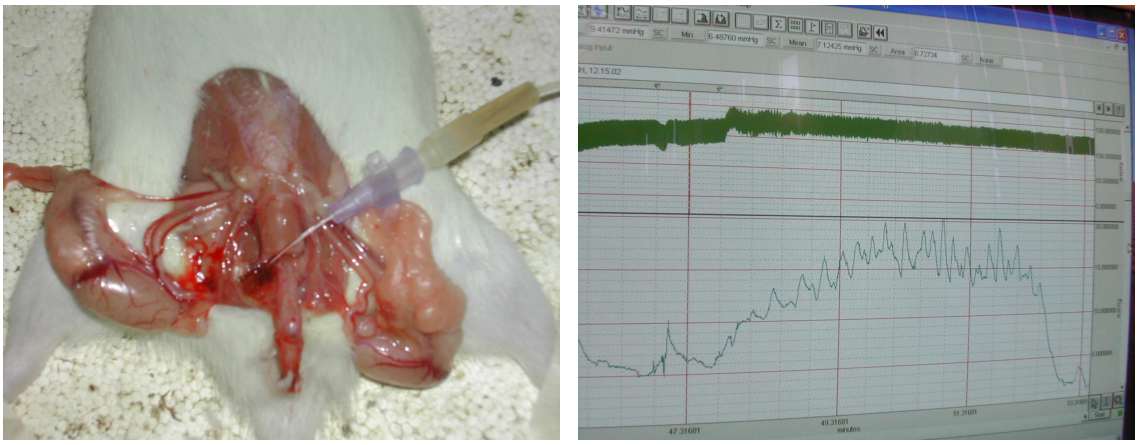


**Figure 23: (A)** Incanulation of rat intracavernous root to evaluate the intracavernous pressure. **(B)** representative tracing of an intracavernous pressure increase after UII intracavernous administration.

For pharmacological evaluation via the intracavernous roote, a 26G-gauge needle was placed at the other crus for drug injection. U-II was given at doses of 0.1, 0.3, 1.0 nmol/rat dissolved in 50 µL saline. To validate the experimental model we used Ach (2.5, 5.0, 25.0 nmol/rat). Saline served as the control vehicle (50 µL). Data were calculated as delta (mm Hg) of ICP and as area under the curve (mm Hg x min) and expressed as mean±SEM from five separate experiments. The changes in systemic blood pressure were calculated as differences from basal values following intra-cavernous drug injection (delta mm Hg) and expressed as mean±SEM. Data were analyzed using ANOVA followed by Bonferroni.

## 3.2.2 Relationship between UII/UT receptor pathway and L-arginine/NO pathway

We have investigate on the mechanism of eNOS activation to evaluate how UII/UT receptor pathway promotes NO production.

### *3.2.2.1 Western blot analysis*

Preliminary time course study was performed incubating HCC strips for 5, 15, 30 or 60 minutes with either UII (10 µM) or vehicle (water). The experimental time selected was 30 minutes. At this time HCC strips were incubated with water, UII, geldanamicin (Hsp90 inhibitor) (1 µM), wortmannin (Akt inhibitor) (1 µM and 100 mM) and with DMSO (wortmannin and geldanamicin vehicle). The reaction was stopped by liquid nitrogen. Tissues are homogenised as previously described.

Western blot analysis was performed using 50 µg of denaturated proteins, separated on 8% sodium dodecyl sulphate polyacrylamide gels and transferred to a PDF membranes. After 45 minutes of blocking in PM containing NaF (50 mM), membranes were incubated overnight at 4°C with rabbit polyclonal anti p-eNOS antibody (1:1000). The filters were extensively washed in PMT before incubation for two hours with anti-rabbit antibody. Membranes were then washed and developed using ECL. The same membranes were incubated overnight at 4°C with mouse monoclonal eNOS antibody. Filters were exposed to ECL after 2 hours of incubation with horseradish peroxidise-conjugate antimouse antibody. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the mouse β-actin antibody. The protein bands were densitometrically analysed with a model GS-800 imaging densitometer.

### *3.2.2.2 Functional studies*

HCC strips were equilibrated in isolated organ bath as previously described. After standardization, on a resting condition, tissues were incubated with Hsp90 inhibitor (geldanamicin, 1 µM), or with Akt inhibitor (wortmannin 1 and 0.1 µM) or with vehicle

(DMSO). After 30’ of incubation UII cumulative concentration-response curve (0.1 nM - 10 µM) was performed on HCC strips pre-contracted with PE (1 mM).

## 3.3 Results

### *3.3.1 UII/UT receptor pathway is involved in penile erection in rats*

Western blot analysis performed on rat corpusu cavernosum showed that UT receptor is expressed in rat penile tissue (Figure 24).

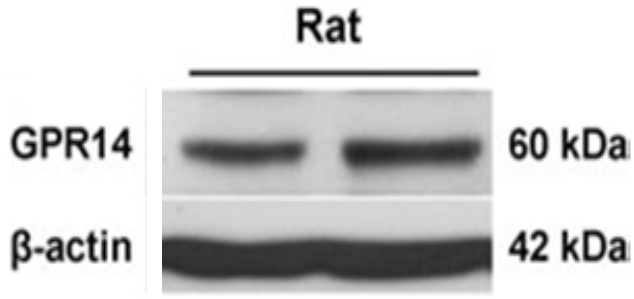



**Figure 24: Representative western blot for urotensin II receptor (GPR14) in rat corpus cavernosum. β-actin was used as loading control.**

In order to evaluate if UII could cause penile erection *in vivo*, we have practised an intracavernous administration of peptide. During these experiments we have monitored both systemic and intracavernous pressure. Administration of U-II (0.1; 0.3; 1.0 nmol/rat) induced a dose-dependent increase in ICP (Figure 25 A and B), thus penile erection, without modify significantly the mean arterial blood pressure (Figure 25 D). The data are expressed as delta of increase in ICP (Figure 25 A, ***P < 0.001) as well as area under the curve (Figure 25 B, ***P < 0.001). To validate the experimental procedure we used Ach (2.5, 5.0, 25.0 nmol/rat), which caused a dose dependent increase in ICP (Figure 25 C; *P<0.05, **P<0.01) and a reduction of blood pressure in a dose-dependent manner (data not shown).

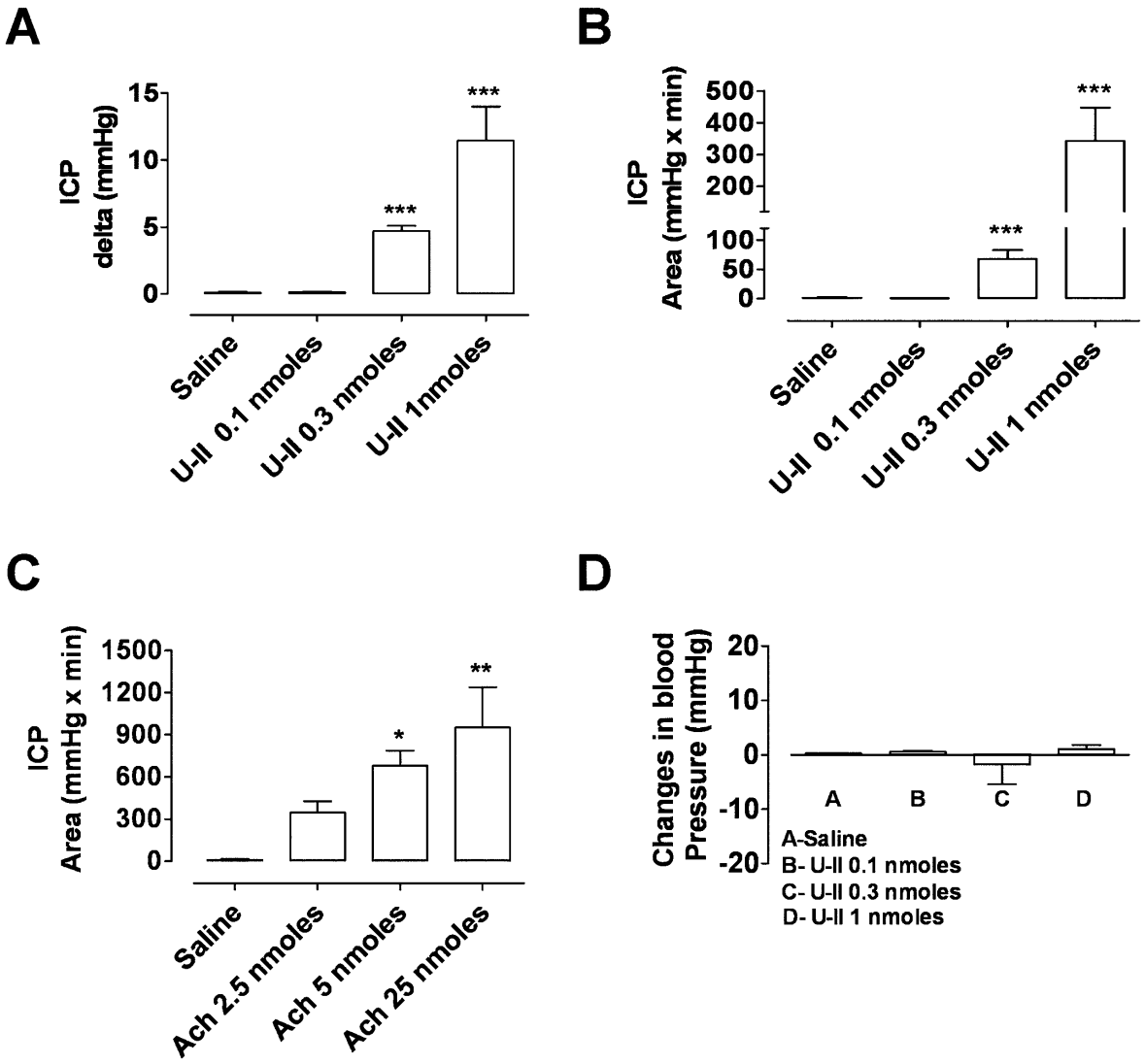



**Figure 25: Effect of urotensin II (U-II) on intracavernous and systemic pressure in anesthetized rats.** U-II (0.1, 0.1, 1 nmol/rat) caused significant dose-dependent increases in intracavernous pressure (ICP) compared with saline. The data are expressed in (A), as delta in mm Hg (***P < 0.001; N = 5) and in (B), as area under the curve (***P < 0.001; N = 5). (C) Ach (2.5, 5, 25 nmol/rat) caused a dose-dependent increase in intracavernous pressure (*P < 0.05, **P < 0.01, N = 5). (D) U-II did not cause any change in arterial blood pressure. Data expressed as mean±SEM were analyzed by one-way ANOVA.

### *3.3.2 HCC distribution of UII receptor*

UT receptor is expressed in human corpus cavernusum either as protein or as mRNA; and are reported in Figure 26 A and B respectively.

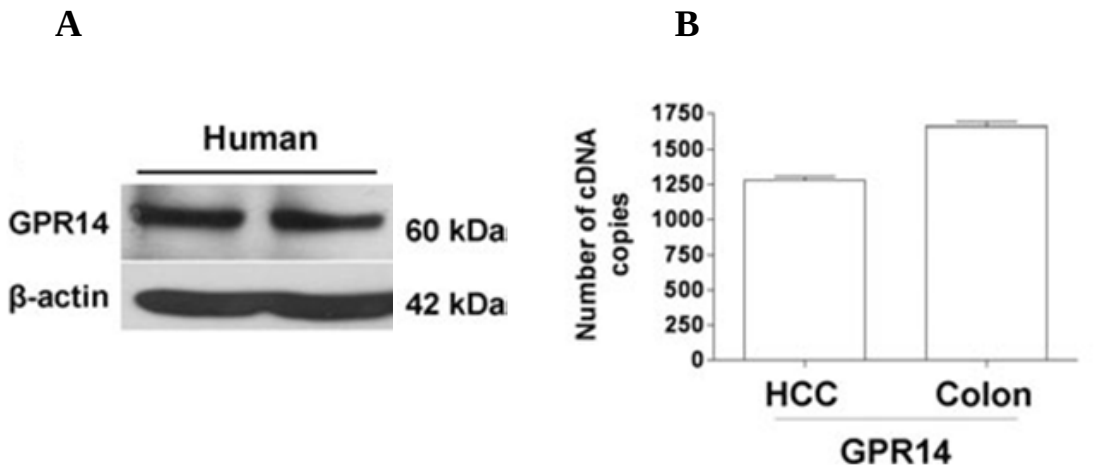



**Figure 26: (A)** Representative western blot for GPR14 in human corpus cavernosum. β-actin was used as loading control. **(B)** Quantitative reverse transcriptase polymerase chain reaction for GPR14 in human corpus cavernosum; as control we used human colon tissue. The data represent the mean ±standard error of the mean of three different human and rat specimens.

In addition, the immunofluorescence analysis, performed to locate UT receptor expression, demonstrated a robust signal for GPR14 on HCC (Figure 27-2B, C). Negative control slides showed no signal (Figure 27 A). The co-localization of UT receptor with endothelial cell marker, vWF, clearly showed a strictly overlapping of the signals indicating that UT receptor was expressed by endothelial cells of the vessels (Figure 27 B). Instead, a positive signal for UT receptor was not showed on smooth muscle cells, marked by α-SMA (Figure 27 C). A signal for GPR14, even if less evident, was also visible in the endothelium of sinusoids (data not shown).

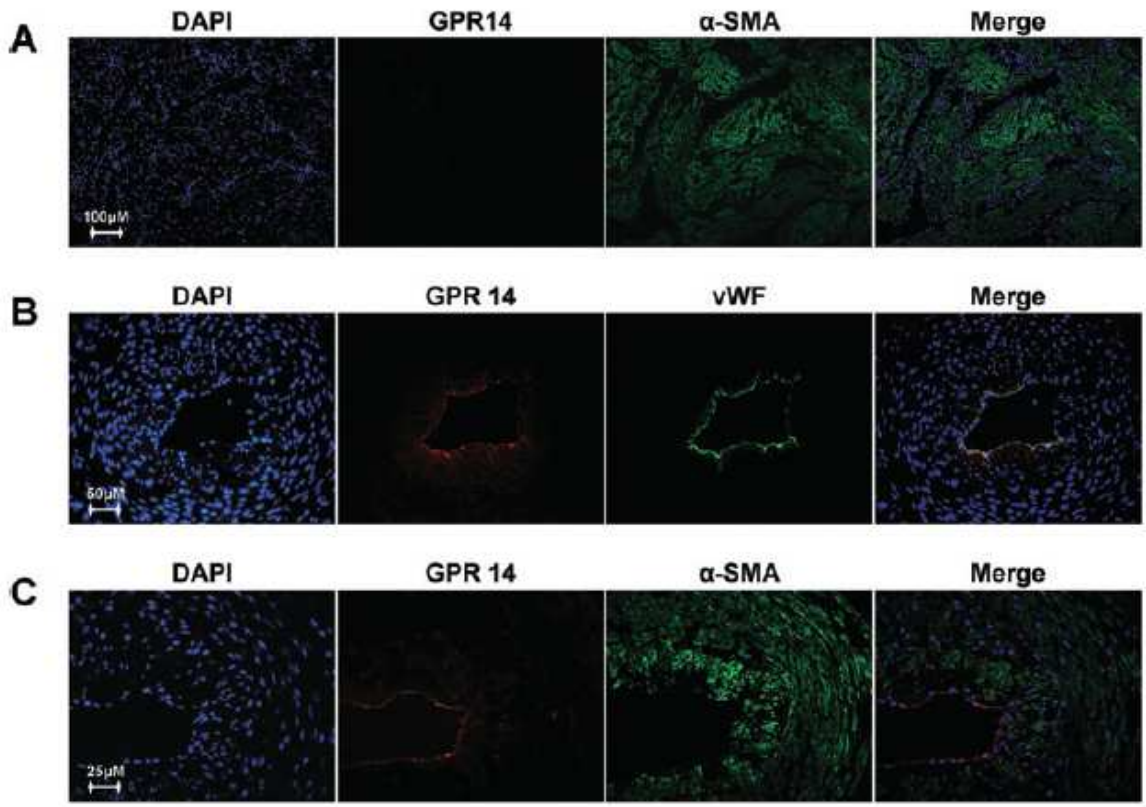



**Figure 27: Localization of UT receptor (GPR14) in human corpus cavernosum.**
Photomicrographs show cellular localization of the GPR14 protein (red) with immunofluorescence. Panel A: negative control. **Panel B and C**: Sections were doubled stained with an endothelial cell marker (von Willebrand factor [vWF]; green) **(B)**, or with a smooth muscle cell marker (a-SMA; green) **(A, C)**. 4i,6-Diamidino-2-phenylindole (blue) was used to stain the nuclei. The merges showed the GPR14 co-localization (yellow). Results illustrated are from a single experiment and are representative of three different specimens.

### *3.3.3 Effect of UII on HCC strips*

U-II (0.1 nM–10 mM) administration on HCC strips, under resting condition, did not cause vasoconstrictor effect (Figure 28 A). Instead the peptide induced relaxation in endothelium and concentration-dependent manner when HCC strips were precontracted with PE 1 µM (Figure 28 B; ***$P < 0.001$). In fact the absence of endothelium abrogated the response to UII (Figure 28 B).

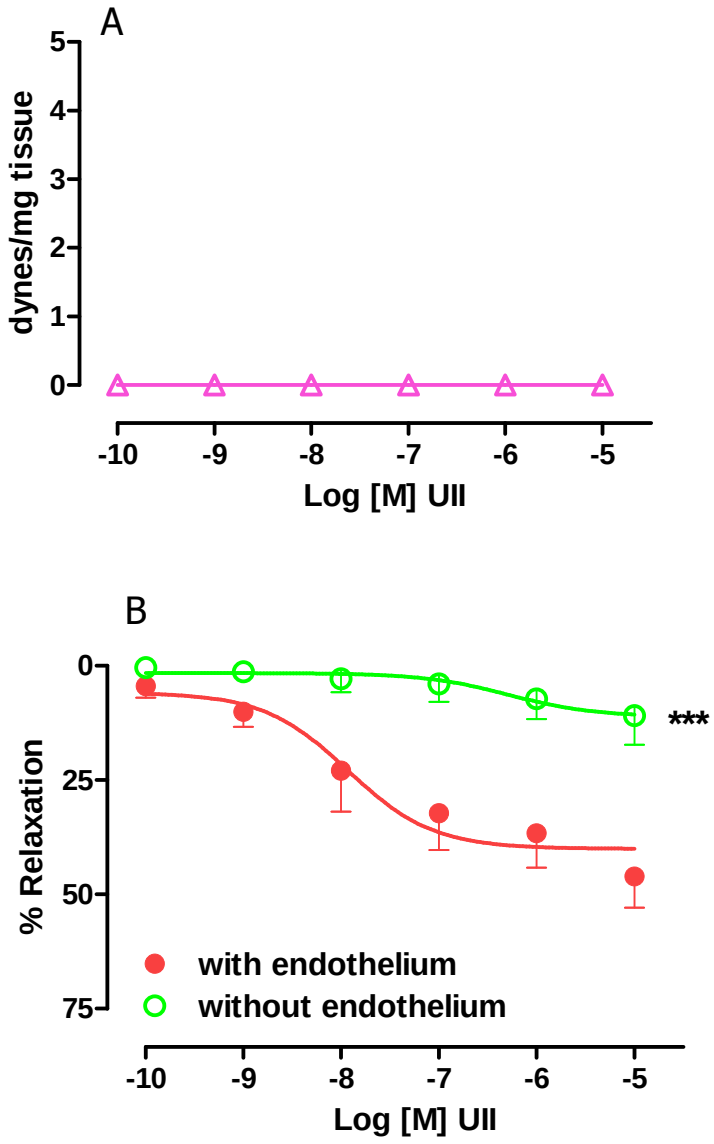



**Figure 28: Urotensin II (U-II) effect on human corpus cavernosum (HCC) strips.**
**(A)** UII did not contract HCC strips in restin condition. **(B)** U-II relaxed HCC strips precontracted with PE (3 mM) in presence of endothelium (***$P < 0.001$). Data expressed as mean±standard error of the mean were analyzed by analysis of variance and by Bonferroni as post-test.

### *3.3.4 Involvement of L-arginine/NO pathway in UII-induced relaxation*

In order to evaluate a possible involvement of NO/eNOS pathway, we have tested the effect of L-NAME, an eNOS inhibitor, on UII-induced vasorelaxation. The incubation with L-NAME, at the concentration of 100 µM for 30 minutes under resting condition, abrogated the effect of vasorelaxation induced by UII on precontracted HCC strips (Figure 29).

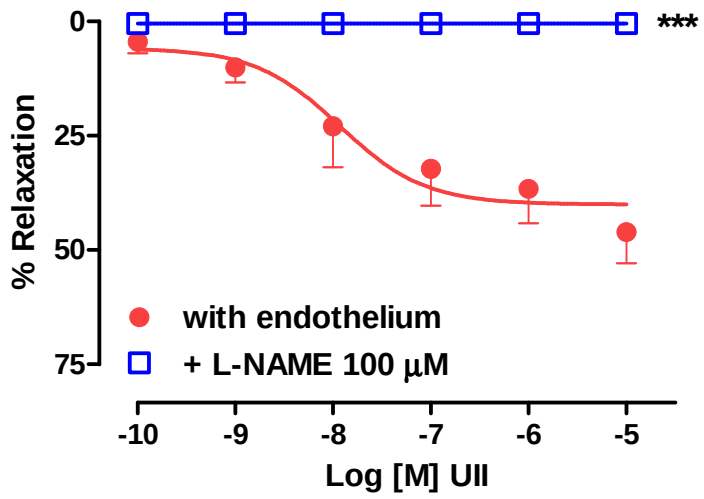



**Figure 29**: **Eeffect of L-NAME on UII-induced relaxation.**
eNOS inhibitor (100 µM, 30 minutes) abolished the vasodilatant response induce by UII on HCC strips precontracted with PE (1 µM). Data expressed as mean±standard error of the mean (N = 8) were analyzed by analysis of variance and by Bonferroni as post-test. CTR = control.

### *3.3.5 UII increase NOx production in HCC strips*

In order to evaluate the involvement of eNOS, we have assessed if there was a modulation of eNOS post-translational activation. To address this specific issue we have monitored NOx production. In basal conditions HCC strips produce an amount of NO, measurable as total nitrite. When HCC strips were stimulated with UII (10 µM for 30 minutes), NO production significantly increases (Figure 30; *p<0.05). Thus, pre-treatment with UII could modulates eNOS activity.

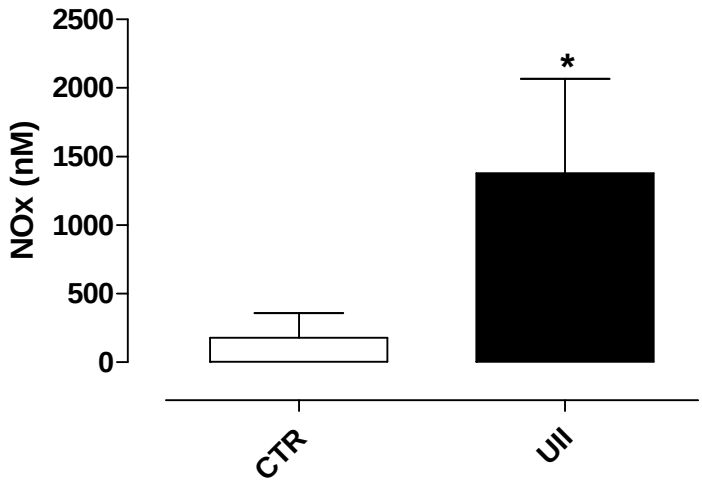



**Figure 30: Effect of UII on NOx production in HCC strips.**
Total nitrite (NOx) content was determined fluorometrically using a standard curve of sodium nitrite. Data are expressed as mean±S.E.M, n=3, *p<0.05.

### *3.3.6 UII phosphorylates eNOS*

Preliminary western blot analysis performed on HCC strips incubated with vehicle or with UII (10 µM) for 5, 15, 30 and 60 minutes showed that peptide induced phosphorylation of eNOS in time-dependent manner. The densitometric analysis is expressed as p-eNOS/eNOS ratio. The incubation with UII caused a time-dependent increase of p-eNOS/eNOS ratio. This effect was significant when UII was incubated for 30 minutes compared with vehicle (CTR) (Figure31).

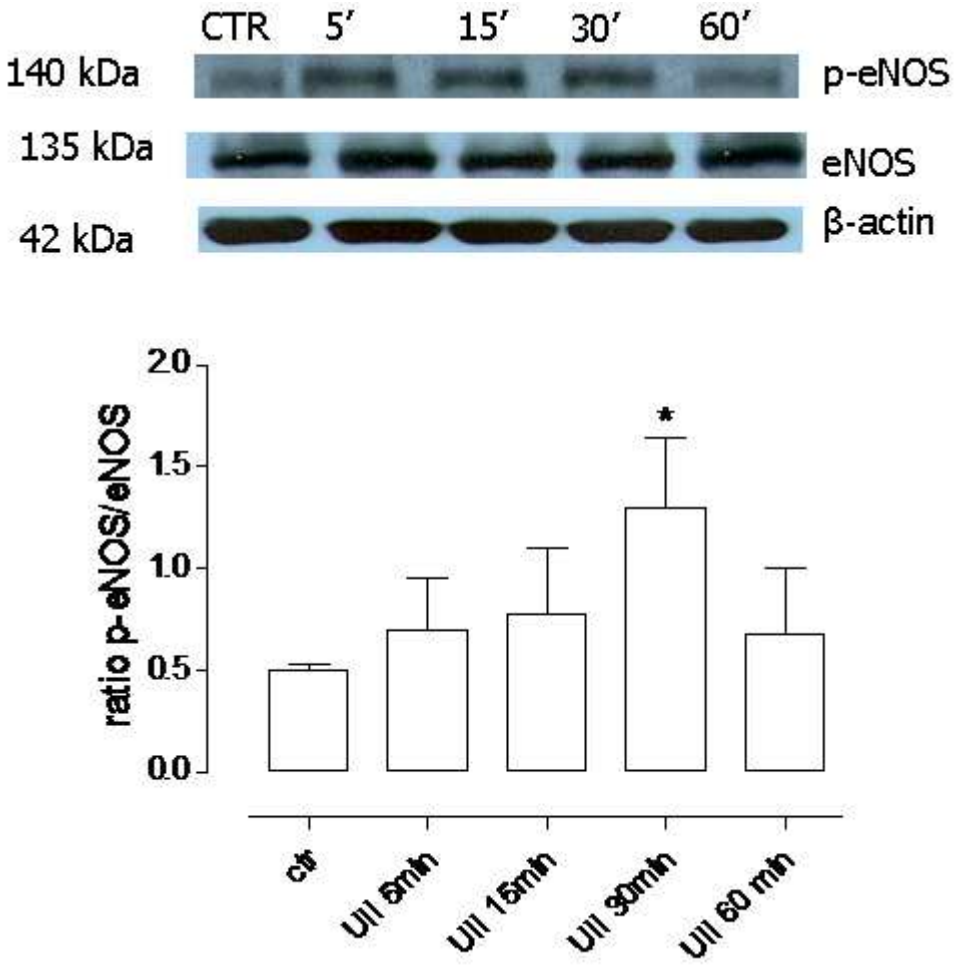



**Figure31: eNOS and phosphorylated (p)-eNOS evaluation in HCC strips.**
Representative western blot for eNOS and phosphorylated eNOS (p-eNOS) in HCC strips stimulated with UII (0.1 µM) at different time, or with vehicle (ctr). Densitometric analysis shows a significant (* $p < 0.05$) change in the eNOS–p-eNOS ratio following 30 minutes of treatment with UII *vs* ctr.

From these results 30 minutes was selected as time of incubation to evaluate the effect of Akt and Hsp90 inhibitors on p-eNOS phosphorylation induced by UII.

At first western blot analysis showed that DMSO, the vehicle of wortmannin and geldanamicin, did not alter the basal level of the ratio p-eNOS/eNOS (figure 32). The incubation with wortmannin, at various concentration (1 and 0.1 µM), for 30 minutes reverted the eNOS phosphorylation induced by UII (Figure 32, °p<0.01 *vs* UII). Instead, pre-treatment with geldanamicin (1 µM), did not alter the UII induced eNOS phosphorylation (**p<0.001 *vs* ctr and DMSO).

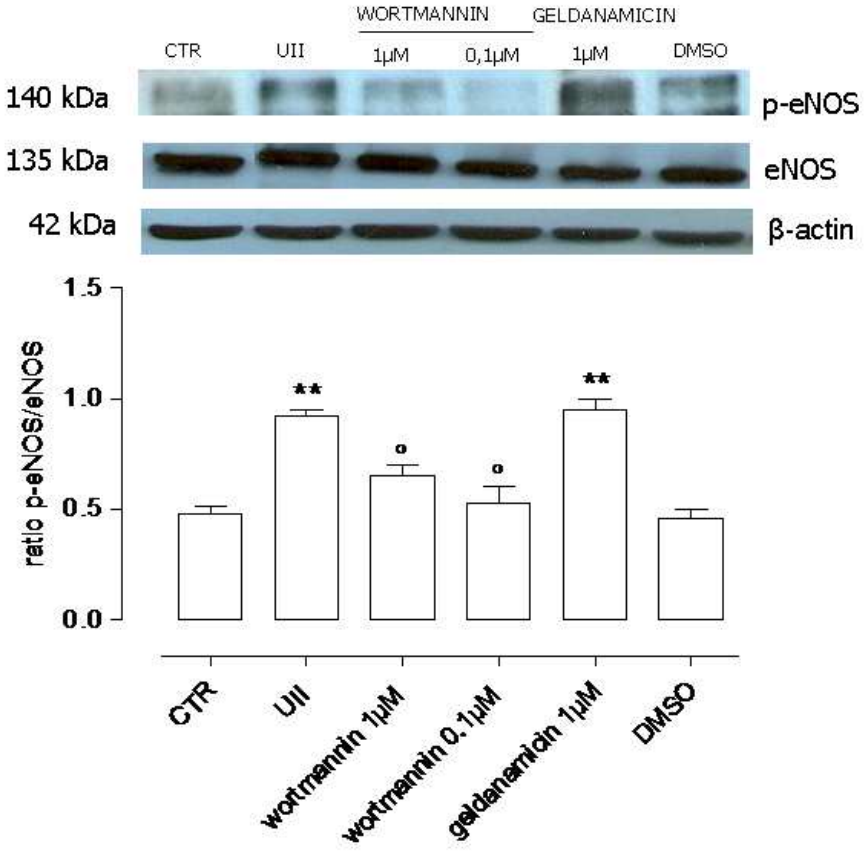



**Figure 32: Evaluation of p-eNOS phosphorylation.**
Representative western blot for eNOS and phosphorylated eNOS (p-eNOS) in HCC strips incubated with wortmannin (1 and 0.1 µM), geldanamicin (1 µ), or DMSO before the treatment with UII (10 µM). Densitometric analysis showes that wartmannin, at both doses, but not geldanamicin, inhibits UII induced phosphorylation in HCC strps (°p< 0.01). .

### *3.3.7 UII activates eNOS via Akt and Hsp90*

Functional studies *in vitro* showed that pre-treatment with both wortmannin (1 and 0.1 µM) and geldanamicin (1 µM), Akt and Hsp90 inhibitors respectively, inhibited the relaxation induced by UII on HCC strips (Figure 33).

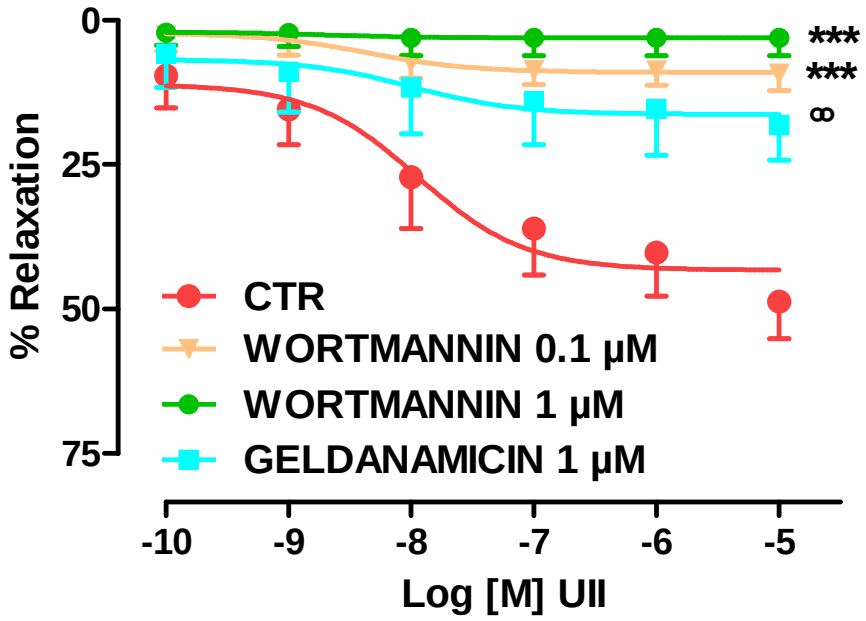



**Figure 33: Effect of Akt and Hsp90 inhibitors on UII-induced relaxation.**
Pre-tratment with either, wortmannin (1 and 0.1 µM) and geldanamicin (1 µM) for 30 minutes, inhibited the vasodilatant response induce by UII on HCC strips precontracted with PE (1 µM). Data expressed as mean±standard error of the mean were analyzed by analysis of variance and by Bonferroni as post-test.

## 3.4 Discussion

UII is a vasoactive 'somatostatine-like' cyclic peptide that was originally isolated from fish spinal cors (Bern *et al*., 1985; Conlon *et al*., 1996), and that has recently been cloned from human (Coulouarn *et al*., 1999). The UII sequence appears well conserved in the evolution of vertebrates (Conlon *et al*., 1997). This might suggest important physiological roles for this peptide. In fact the peptide and its receptor are abundantly distributed in human heart , brain, pancreas, skeletal muscle, vascular smooth muscle and endothelial cells, spinal cord, and endocrine tissue (Ames 1999; Maguire 2000). The vasoactive effects of U-II seem to be dependent upon the caliber of the vessels and the species (Camarda *et al*., 2002; Douglas *et al*., 2000; MacLean *et al*., 2000). For instance, in the rat, UII contracts many arteries such as thoracic aorta, carotid, pulmonary and coronary arteries (Douglas *et al*., 2000; MacLean *et al*., 2000). Its vasoconstrictor effect on the rat thoracic aorta is mediated by phospholipase C and protein kinase C-dependent pathway (Rossowsky *et al*., 2002). Conversely, it has been suggested that U-II-effect in resistance vessels in rat and human, causes vasodilation involving NO pathway (Bottrill *et al*., 2000.;Stirrat *et al*., 2001; Zhang *et al*., 2003). Thus, the cardiovascular actions of this peptide can be influenced by regional factors such as receptor distribution and the species considered. To date it is unclear whether the predominant action of U-II in human disease will be deleterious or protective. At these regards, also in vivo studies are controversial. In fact in conscious rats UII at high doses (3000 $pmol.kg^{-1}$) causes an overall depressor effect, causing dilatation of vascular beds, resulting in reduced men arterial blood pressure and tachycardia (Gardiner *et al*., 2001). Instead in cynomolgus monkeys the intra venous administration of the peptide at low doses result in an increase in cardiac output and regional vasodilation, while high doses lead to a fatal combination of increased vascular resistance and only moderate reduction in blood pressure, coupled with a decrease in cardiac output (Ames *et al*., 1999).

In addition, recently, it has been demonstrated that the endothelium may be determinant for changes in vasomotor tone to U-II *in vivo*. In fact an alteration of U-II/UT receptor pathway contributes to the development of several cardiovascular disorders associated to endothelial dysfunction. Indeed, elevated plasma levels of U-II have been detected in patients with various cardiovascular disease states including heart failure, hypertension, renal dysfunction, and diabetes (Gruson *et al*., 2006; Suguro *et al*., 2007; Totsune et al., 2004; Totsune *et al*., 2003). On the other hand it has been showed that intracelebral injection of UII causes hypertension, bradycardia, increase in locomotor activity without alter the penile function (Do-rego *et al*., 2005). In penile erection there is a strong involvement of the vascular system and the L-arginine/NO pathway plays a major role (Ignarro *et al*., 1990). To evaluate the possible link between UII and NO in penile tissue, we have investigated if UII could act as an endogenous mediator in HCC and thus be involved in penile erection. First we address the question if the penile tissue express the receptor. Our data showed that GPR14 is expressed in rat corpus cavernosum. Thus, by in vivo experimental model, we have monitored the intracavernous pressure after UII local administration. The results demonstrated that intracavernous administration of peptide in anesthetized rats induced a dose- dependent increase in ICP implying that UII is involved in rat penile erection. In addition UII did not cause any significant change in systemic blood pressure, at all doses tested. Next we address the question if the human tissue expresses the receptor and where it is localized. HCC was obtained from patients undergoing sex change. The human tissue possesses the UT receptor as protein as well as mRNA. Particularly, the immunofluorescence highlighted a strongly stained for UT receptor exclusively on the endothelial cells of the HCC tissue, implying that UII might play a role in the HCC vasodilatory response. These data are confirmed by other results obtained by using *in vitro* functional studies. In fact we have proved that UII did not have a

vasoconstrictor effect on HCC strips in resting condition. Conversely, on HCC strips precontracted by PE, the peptide induced relaxation in a concentration- and endothelium-dependent manner. Thus we have evaluated the possible link between UII vasorelaxant effect and L-arginine/NO pathway. To analyze this possibility we assessed the effect of UII after pre-treatment with L-NAME. The inhibition of eNOS abrogated U-II-induced relaxation with a profile overlapping the inhibition operated by the removal of endothelium supporting our working hypothesis. These data are consistent with the analysis of NOx production in HCC strips. In fact, in HCC strips stimulated with UII, NO production was significantly increased up than basal condition, implying the involvement of NO pathway in UII effect. Our results are consistent with several evidence indicating that stimulation of UT receptors on animal vascular endothelial cells can trigger the release of NO (Bottrill *et al*., 2000; Zhang *et al*., 2003; Katano *et al*., 2000; Ishihata *et al*., 2005; Gardiner *et al*., 2006). In addition, U-II binding sites have been localized to vascular endothelial cells, confirming that U-II stimulates NO-dependent vascular relaxation (Douglas *et al*., 2000). In the last step of this study we have investigated on the molecular mechanism of UII eNOS activation. In the last several years, many protein partners that interact with eNOS have been described, including caveolin-1, calmodilin (CaM), heat shock protein 90 (Hsp90) and certain kinases including Akt (Bernier *et al*., 2000; Michell *et al*., 1999). The caveolin-1 binding to eNOS renders it less active. Several studies demonstrated that the actions of CaM are thought to be facilitated in cells by the recruitment of Hsp90 to eNOS and from the dissociation of eNOS from Cav-1 (Brouet *et al*., 2001). Our analysis showed that the incubation with UII induced phosphorylation of eNOS in HCC. This effect was inhibited by pre-tratment with wortmannin, Akt inhibitor, but not with geldanamicin, Hsp90 inhibitor. In addition, in vitro functional studies on HCC strips proved that UII induced relaxation was inhibited by pre-tretment with either wortmannin and

geldanamicin. Thus, based on these data, we propose that UII, by an increase of intracellular calcium, cause an activation of eNOS *via* Akt and Hsp90, following in NO release.

In conclusion U-II can be added to the other vasoactive factors (noradrenaline, endothelins, angiotensins, NO, VIP, and related peptides, prostanoids, $H_2S$) in the physiology of erectile function.

# *CHAPTER 4*

## Platelet cGMP:

## a new biological marker

## of PDE5 inhibitors efficacy

## 4.1 Erectile dysfunction: diagnosis and therapy

Erectile dysfunction, as mentioned before, is the consistent or recurrent inability of a man to attain and/or maintain a penile erection sufficient for sexual activity (2nd International Consultation on Sexual Dysfunction-Paris, June 28th–July 1st, 2003). Male ED represents a worldwide and multi-cultural condition with prevalence rates between 19 and 74% in the age groups 40 - 80 years (Koskimaki *et al*., 2000; Chew *et al*. 2000; Johannes *et al*., 2000). It is well documented the negative impact of ED on the quality of life (Willke *et al*., 1998; Wagner *et al*., 1996). Through history, impotence has influenced the society in two major ways. First, there has often been an immense associated sense of humiliation; second it has a major impact on determining the validity of marriage in low. These aspects have a negative impact on the quality of life, particularly on sexual relationships with the partner and in relations with relatives and friends (National Institute of Health Consensus Development Panel on Impotence. 1993). Thus, the importance of a well-timed diagnosis is crucial event. Actually ED is evaluated by medical questionnaire scores. In 1997 Rosen and co-workers developed the International Index of Erectile Function (IIEF), a new method to diagnose erectile dysfunction. In this method were identified five factors or response domains: (1) erectile function (IIEF-EF); (2) orgasmic function (IIEF-OF); (3) sexual desire (IIEF-SD); (4) intercourse satisfaction (IIEF-IS); (5) overall satisfaction (IIEF-OS) (Rosen *et al*., 1997). In particular, IIEF-EF, a 15-item questionnaire, has been developed and validated as a brief and reliable self-administered scale for assessing erectile function (Figure 34).

**International Index of Erectile Function (IIEF):**

1. Over the past 4 weeks, how often were you able to get an erection during sexual activity?
2. Over the past 4 weeks, when you had erections with sexual stimulation, how often were your erections hard enough for penetration?
3. Over the past 4 weeks, when you attempted sexual intercourse, how often were you able to penetrate (enter) your partner?
4. Over the past 4 weeks, during sexual intercourse, how often were you able to maintain your erection after you had penetrated (entered)
your partner?
5. Over the past 4 weeks, during sexual intercourse, how difficult was it to maintain your erection to completion of intercourse?
6. Over the past 4 weeks, how many times have you attempted sexual intercourse?
7. Over the past 4 weeks, when you attempted sexual intercourse how often was it satisfactory for you?
8. Over the past 4 weeks, how much have you enjoyed sexual intercourse?
9. Over the past 4 weeks, when you had sexual stimulation or intercourse how often did you ejaculate?
10. Over the past 4 weeks, when you had sexual stimulation or intercourse how often did you have the feeling of orgasm or climax (with
or without ejaculation)?
11. Over the past 4 weeks, how often have you felt sexual desire?
12. Over the past 4 weeks, how would you rate your level of sexual desire?
13. Over the past 4 weeks, how satisfied have you been with your overall sex life?
14. Over the past 4 weeks, how satisfied have you been with your sexual relationship with your partner?
15. Over the past 4 weeks, how do you rate your confidence that you can get and keep an erection?

**IIEF score system:**

*Questions 1,2,3,4,7,9:*
0= No sexual activity; 1= Almost never or never; 2= A few times (much less than half the time); 3= Sometimes (about half the time);
4= Most times (much more than half the time); 5= Almost always or always.

*Question 5:*
0=Did not attempt intercourse; 1= Not difficult; 2=Slightly difficult; 3= Difficult; 4= Very difficult; 5= Extremely difficult

*Question 6:*
0=No attempts; 1= One to two attempts, 2= Three to four attempts; 3= Five to six attempts; 4= Seven to ten attempts;
5= Eleven or more attempts

*Question 8:*
0=No intercourse; 1=Not enjoyable; 2= Not very enjoyable; 3= Fairly enjoyable; 4= Highly enjoyable; 5= Very highly enjoyable

*Question 11:*
1= Almost never or never; 2= A few times; 3= Sometimes; 4= Most times; 5= almost always or always

*Question 12,15:*
1= Very dissatisfied; 2= Moderately dissatisfied; 3= About equally satisfied and dissatisfied; 4= Moderately satisfied; 5= Very satisfied

**Figure 34: The International Index of Erectile Function (IIEF) Questionnaire.**

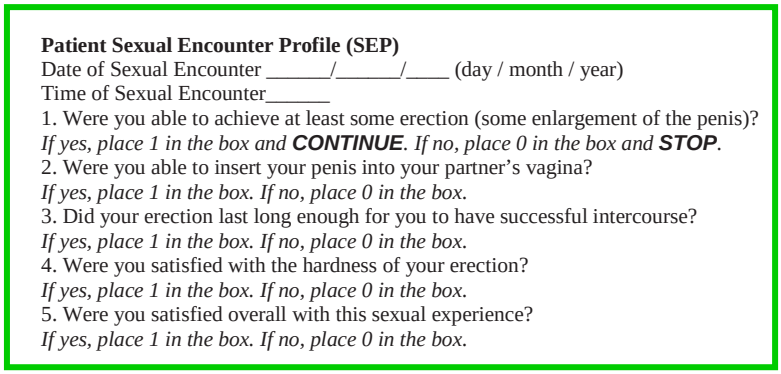
This instrument is psychometrically sound and clinical setting. In addition, IIEF-EF is available in 10 languages for use in multinational clinical trials, and it demonstrate adequate sensitivity and specificity for detecting treatment-related changes in erectile function in patient with ED (Rosen *et al*., 1997).

The reliability as well as consistency of IIEF was originally tested on a large number of patients taking sildenafil. However, about 2.5 years after the launch of sildenafil, clinical practice surveys demonstrated that 38–51% of patients discontinued sildenafil therapy for lack of efficacy (Souverein *et al*., 2002). Following the launch on the market of vardenafil and tadalafil, patients who were non-responders to sildenafil were scheduled successfully for treatment with another PDE5 inhibitor, although they all share the same mechanism of action (Stroberg et al., 2003; Rubio-Aurioles et al., 2006).

PDE5 inhibitors, in fact, decrease the degradation of cGMP. The central role of cGMP signalling in erectogenic response of penile tissue to sexual stimuli is now well established (Burnett. 2006). In particular, NO activates guanylyl cyclase causing an increase in cGMP, which activates cGMP-dependent protein kinase (PKG), resulting in smooth muscle relaxation, dilation of the sinusoids, increase in blood flow and enhancing erection (Hofmann, 2005; Lincoln *et al*., 2001; Sausbier *et al*,. 2000). PDE5 break down cGMP to the inactive 5'-GMP (Gopal *et al*., 2001).The mentioned PDE5 inhibitors, known as competitive inhibitors, have high affinity for the PDE5 catalytic site thereby blocking cGMP access to the site and fostering cGMP accumulation (Blount *et al*., 2004; Ballard *et al*., 1998; Turko *et al*., 1999).

Several clinical studies showed that IIEF can provide information mainly on sexual functioning but is not helpful in clarifying differences among the inhibitors used in clinical studies (Stroberg *et al*., 2003; Rubio-Aurioles *et al*., 2006). Indeed, it mainly measures sexual functioning (Rosen *et al*., 1997), is sensitive to placebo, and does not permit

addressing the potential difference in mechanism among drugs. However, various clinical trials are developed to a careful evaluation of ED. The most used tests, with IIEF, are Sexual Encounter Profile **(**SEP**)** and Rigiscan. SEP is a diary consisting of yes/no-type questions that enables both the patient and his partner to evaluate sexual encounters (Figure 35).



**Patient Sexual Encounter Profile (SEP)**
Date of Sexual Encounter _____/_____/____ (day / month / year)
Time of Sexual Encounter_____
1. Were you able to achieve at least some erection (some enlargement of the penis)?
*If yes, place 1 in the box and* ***CONTINUE****. If no, place 0 in the box and* ***STOP****.*
2. Were you able to insert your penis into your partner's vagina?
*If yes, place 1 in the box. If no, place 0 in the box.*
3. Did your erection last long enough for you to have successful intercourse?
*If yes, place 1 in the box. If no, place 0 in the box.*
4. Were you satisfied with the hardness of your erection?
*If yes, place 1 in the box. If no, place 0 in the box.*
5. Were you satisfied overall with this sexual experience?
*If yes, place 1 in the box. If no, place 0 in the box.*

**Figure 35: The Sexual Encounter Profile (SEP) diary.**

It has been indicated a high degree of correlation between erection and intercourse satisfaction rating between SEP and IIEF measure in patients with mild to moderate degrees of ED (Rosen *et al*., "The sexual encounter profile (SEP): validation of a simple event log for sexual function assessment". Unpublished). The Rigiscan, instead, represents an objective method to evaluate penile tumescence and rigidity. Since each of these measure has certain in advantages and limitation, these three tests are frequently used all together to have an accurate evaluation of both ED and treatment efficacy.

Another important issue, is that patients often report facilitated erections also when plasma concentrations of PDE5 inhibitors drop well below the therapeutic level (Moncada *et al*., 2004; Young *et al*., 2005; Shabsigh *et al*., 2006)

The drugs differ substantially in the rate of clearance from plasma: $t_{½}$ of plasma clearance is 4 hours for sildefail or vardenafil compared to 18 hours for tadalafil. However studies indicate that men still have facilitated erections 12 hours after sildenafil or vardenafil dosing, likewise 36 hours after tadalafil dosing (Moncada *et al*., 2004; Valiquette *et al*., 2005).

Many hypothesis are reported in literature but no one mechanism has been demonstrated to explain the prolonged effect of PDE5 inhibitors (Francis *et al*., 2008).

PDE5, purified in 1980 by Francis and colleagues from rat lungs (Francis and Corbin 1988), a cGMP-specific enzyme that hydrolyzes cGMP, is one of the targets for the action of NO. The tissue distribution of PDE5 generally coincides with that of PKG, probably because both enzymes are the principal intracellular receptors of cGMP. Moreover, PKG is an excellent in vitro catalyst for the phosphorylation of PDE5 (Thomas *et al*., 1990). PDE5 has a typical cytosolic localization with high levels in smooth muscle, platelets, kidney and in some zones of the central nervous system (Francis and Corbin 1999; Kotera *et al*., 2000; van Staveren *et al*., 2004; Dolci *et al*., 2006).

On these basis we have evaluated if platelet cGMP could represent an objective measure of PDE5-inhibitor efficacy and duration.

In our studies we have evaluated cGMP level in a population of 46 men with ED and we have correlated the values obtained with the clinical variables (IIEF, SEP and Rigiscan). 46 ED patients were enrolled and randomly divided into two groups, one treated with vardenafil and one with placebo. Clinical analysis and evaluation of platelet cGMP were performed before and after 6 weeks of treatment.

## 4.2 Materials and methods

### *4.2.1 Washed human platelet preparation*

Fresh blood (20 ml) from human donors anticoagulated with citrate (final concentration 3.8 %, w/v) was centrifuged at 800 rpm for 10 min to obtain the so-called platelet-rich plasma (PRP) as a supernatant. A 1/10 volume of ACD solution (85 mM Na3-citrate, 111 mM d-glucose, 71 mM citric acid, pH 4.4) was added to 9/10 volume of PRP. After centrifugation (2000 rpm for 12 min) the cell pellet was resuspended in Ca2+/Mg2+-free HEPES–Tyrode buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.36 mM NaH2PO4, 5 mM HEPES, 5 mM glucose, 0.5% (w/v) bovine serum albumin, pH 7.4) and the cell number was determined and adjusted to final concentration required (Dunkern *et al*., 2005).

### *4.2.2 Preliminary studies*

Preliminary studies were performed on washed platelets harvested from healthy volunteers. A preliminary time course study was performed incubating different numbers of washed platelets (3x $10^4$ to 5x$10^5$/µl) for 5, 15, or 30 min with either sodium nitroprusside (10, 100, 1000 µM) or diethylamine NONOate (DEA-NONOate, 1, 10, 100 µM) to select the experimental condition for the concentration of platelets, the stimulus and the time of incubation. From these data, the experimental condition were: (1) platelet concentration 5x$10^5$ µl, (2) incubation time 30 min, and (3) DEA-NONOate as stimulus. Indeed, DEA-NONOate gave more reproducible and reliable results because it spontaneously dissociates in a pH-dependent, first-order process with a half-life of 2 min at 37°C, pH 7.4, to liberate 1.5 mol of NO per mole of parent compound (Keefer *et al*., 1996). cGMP content was measured by using a cGMP enzyme immunoassay kit.

### *4.2.3 Platelet assay standardization in healthy volunteers*

In a preliminary study, a group of six normal voluntary subjects (24–30 yr of age) were enrolled to assess the feasibility of the assay. Volunteers gave a blood sample (20 ml) before and 1 h after taking vardenafil (20 mg) (eg, under the peak of the area under the curve [AUC]). Platelets were used to select a suitable NO donor and the optimal incubation time for the ex vivo platelet assay.

### *4.2.4 Evaluation of vasodilator-stimulated phosphoprotein (VASP) in human platelets*

We have investigated on the mechanism of the prolonged effect of PDE5 inhibitors. Francis et al hypothized that the prolonged effect of PDE5 inhibitors could be caused by their permanence in the cell. From this hypothesis, since cGMP signalling cascades relaxes smooth muscle and inhibit platelet activation, it was speculated that VASP mediates these effects by modulating actin filament dynamics and integrin activation. The phosphorylation of VASP at serine 239 is cGMP-dependent protein kinase (PKG). Therefore, VASP phosphorylation level are considered to reflect levels of cGMP in platelets (Aszodi *et al*., 1999; Hauser *et al*., 1999). On these basis we have evaluated the phosphorylation of VASP in human platelets after 6 weeks of treatment with either placebo or vardenafil.

### *4.2.5 Western blot analysis*

Human platelets, resuspended in Ca2+/Mg2+-free HEPES–Tyrode buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.36 mM NaH2PO4, 5 mM HEPES, 5 mM glucose, 0.5% (w/v) bovine serum albumin, pH 7.4) and adjusted to $5 \times 10^5$ platelets/µl, were homogenized in modified RIPA buffer (Tris-HCl 50 mM, pH 7.4, Triton 1%, sodium

deoxycholate 0.25%, NaCl 150 mM, ethylenediaminetetraacetic acid 1 mM, phenylmethylsulphonyl fluoride 1 mM, aprotinin 10 mg/mL, leupeptin 20 mM, NaF 1 mM, sodium orthovanadate 1 mM) by liquid nitrogen. Protein concentrations of cell lysates were estimated by the Bio-Rad protein assay using BSA as standard. Equal amounts of protein (30 µg) of the cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (8% polyacrylamide). Western blotting was performed by transferring proteins from gel to a sheet of polyvinylidene fluoride membrane at 250 mA for 60 min at room temperature. The filters were then blocked with phosphate-buffered saline (PBS) containing 5% non-fat dry milk (PM) and NaF (50mM) for 45 min at room temperature and incubated with mouse monoclonal antibody to VASP (phosphorylated) ($pSer^{239}$) (16C2) (1:3000) in 1x PBS, 5% non-fat dried milk and 0.1% Tween 20 (PMT) overnight at 4°C. Than the membranes were extensively washed by in PBS containing 0.1% v/v Tween 20, before incubation for 2 hours with horseradish peroxidase-conjugate anti mouse secondary antibody (1:5,000). Membranes were then washed and developed using enhanced chemiluminescence substrate. The same membranes were incubated overnight at 4°C with mouse monoclonal anti VASP antibody (IE273) (1:4000). Filters were exposed to ECL after 2 hours of incubation with horseradish peroxidise-conjugate antimouse antibody (1:5000). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the mouse β-actin antibody. The protein bands were densitometrically analysed with a model GS-800 imaging densitometer.

### *4.2.6 Study design and population*

Patients were selected following an interview at the time of their visit. All patients gave written informed consent before random assignment to one of the two study arms. The

protocol was approved by the ethical committee of Federico II University of Napoli. Table 3 lists the patient inclusion and exclusion criteria.

| | Inclusion criteria |
|---|---|
| 1 | Patients affected by ED for at least 3 mo and >18 yr of age |
| 2 | Naive for PDE5-Is; willing not to assume other ED treatment during the study |
| 3 | IIEF-EF score <26 |
| 4 | Written informed consent |
| | Exclusion criteria |
| 1 | ED secondary to endocrine disorders or premature ejaculation |
| 2 | Non–nerve-sparing pelvic surgery |
| 3 | Penis anatomic abnormalities |
| 4 | Moderate to severe hepatic and/or kidney failure |
| 5 | Coronary artery disease |

ED = erectile dysfunction; IIEF-EF = International Index of Erectile Function-Erectile Function domain; PDE5-Is = phosphodiesterase type 5 inhibitors.

**Table 3: Inclusion and exclusion criteria**

To perform the study, 46 PDE5-I–naive patients with ED were enrolled and were scheduled either on placebo (n = 23) or on vardenafil, 5mg (n = 23) at bedtime for 6 wk daily (Montorsi *et al.*, 2008; De Young *et al.*,2008; Carson *et al.*, 2004). Study protocol assumed a run-in period of 1–6 wk during which patients were enrolled, and, if eligible, the IIEF-EF and SEP questionnaires were administered. Thereafter a provocative real-time VSS-Rigiscan section was performed. All patients donated 20 ml of venous blood for

platelet cGMP and VASP determination. The clinical evaluations as well as the sample blood were repeated after 6wk of treatment in both study arms within 12 h from the last drug dose. Table 4 lists the baseline characteristics of the patients who participated in the study. The patient groups were balanced, and the baseline IIEF-EF values were not different among the groups considered. All 46 of the patients enrolled completed the study. The sample size was computed considering an a error equal to 0.05, a β-error equal to 0.20 (power equal to 80%), and an increase from 13 to 18 points of the IIEF with a standard deviation of 6. The 5-point increase of the effect size was selected because it is generally considered the minimum clinically significant response to the drug.

| | 5-mg vardenafil group | Placebo group |
|---|---|---|
| No. | 23 | 23 |
| Mean age, yr (range) | 50 (27–71) | 48.8 (20–68) |
| Etiology of ED | | |
| Psychogenic, % (*n*) | 56.5 (13) | 69.6 (16) |
| Mixed, % (*n*) | 26 (6) | 21.7 (5) |
| Organic, % (*n*) | 17.5 (4) | 8.7 (2) |
| Yes to SEP 3 question (*n*) | 34.8 (8) | 8.7 (2) |
| IIEF-EF score mean ± SD | 17 ± 4.4 | 17 ± 4.7 |

ED = erectile dysfunction; IIEF-EF = International Index of Erectile Function-Erectile Function domain; SD = standard deviation; SEP = sexual encounter profile.

**Table 4: Characteristics of patients at baseline**

### *4. 2.7 Real-time visual sexual stimulation-Rigiscan*

The provocative real-time Rigiscan-coupled VSS was used (Mizuno et al., 2004). Patients were asked to watch an erotic movie during the Rigiscan recording (30 min).

### *4.2.8 Ethical permission*

The protocol will be submitted to the Ethics Committee of the Medical School of the University of Naples. Healthy volunteers and ED asked to sign an informed consent.

## 4.3 Resultes

### *4.3.1 Healthy volunteers*

Platelet cGMP levels were evaluated in 6 healthy volunteers before and 1 hour after treatment with vardenafil, 20 mg. The results, reported in Figure 36, showed a dose-dependent challenged with diethylamine (DEA)-NONOate (1, 10, and 100 mM) in both, before and after treatment. In addition 1 hour after treatment with vardenafil 20 mg there was a significantly increase in cGMP than values observed at baseline.

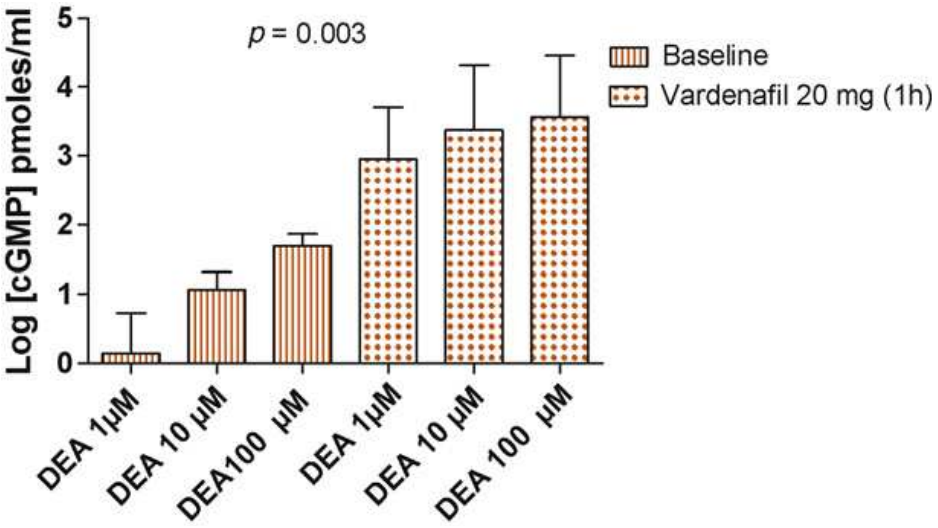



**Figure 36: Effect of vardenafil on platelet cGMP in healthy volunteers**.
Platelet harvested from healthy volunteers prior to or 1 h following the administration of vardenafil, 20 mg, were challenged with diethylamine (DEA)-NONOate (1, 10, and 100 mM). Platelet cGMP significantly increased in the vardenafil group (n = 6; p = 0.003).

### *4.3.2 Effect of Vardenafil on platelet cGMP in patients with ED*

Platelet cGMP levels were evaluated following *in vitr*o stimulation with diethylamine (DEA)-NONOate (1, 10, and 100 mM) prior to and after 6 wk following the treatment with either placebo or vardenafil**.** Data reported in figure 37 showed that in placebo group (Figure 37 a) there was not challenge in cGMP level at all doses of DEA-NONOate. On the contrary, after 6 weeks of vardenafil treatment (Figure 37 b) there was an increase of cGMP level at all doses of DEA-NONOate. To better analyzed GMP release, the AUC variation from baseline, in each dose-response curve, was used for both placebo and vardenafil. The treatment produced a significant increase in cGMP ($p<0.05$; Fig. 37 c).

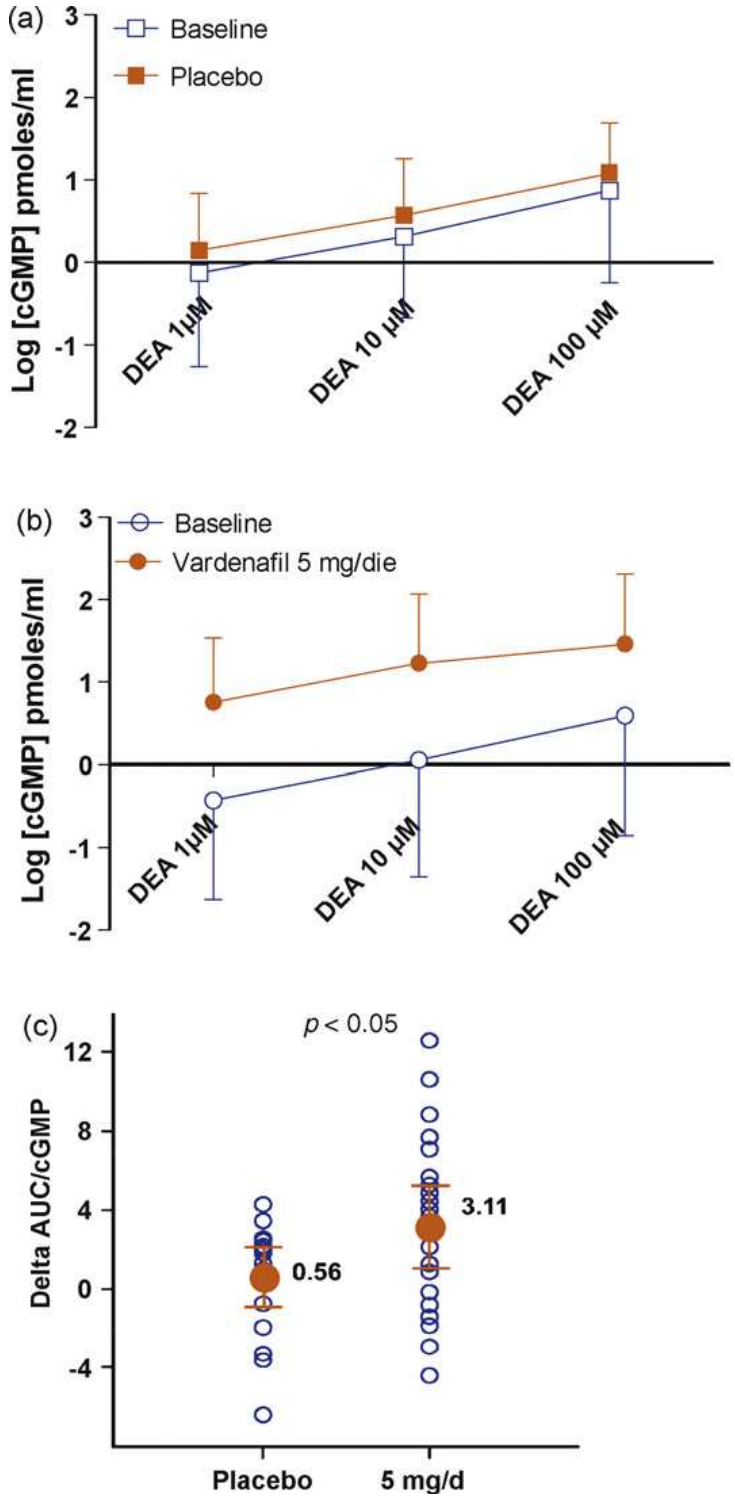



**Figure 37: Effect of vardenafil, 5 mg, versus placebo**.
The production of cyclic guanosine monophosphate (cGMP) following stimulation in vitro with diethylamine (DEA)-NONOate (1, 10, and 100 mM) was evaluated prior to or after 6 wk following the treatment with either (a) placebo or (b) vardenafil. (c) The same data were analyzed as area under the curve (AUC) and expressed as changes in AUC. The analysis demonstrates a significant increase ( $p < 0.05$) in platelet cGMP in the vardenafil group versus placebo.

### *4.3.3 Effect of vardenafil in clinical parameters*

The IIEF-EF (Figure 38 a), the SEP (Figure 38 b) and the VSS-Rigiscan (Figure 39) score were analyzed by comparing within each group, the response prior to and after the treatment. Analyzing absolute data of IIEF-EF and SEP prior and after treatment (Figures 38 a and b), was observed a significant challenge in both group, placebo and vardenafil. However, when the δ increase in both IIEF-EF (Figure 38 c) and SEP (Figure 38 d) were compared, they did not reach statistical significance between placebo and vardenafil group.

In contrast the VSS-Rigiscan score clearly showed a significant amelioration in the vardenafil but not in placebo group as absolute data (Figure 39a; $p < 0.05$). When data were evaluated as δ increases, calculated as the difference between the effect prior to and after the treatment, it has been showed a significant difference between placebo and vardenafil group.

Thus it seems that although IIEF-EF and SEP are sensitive to the placebo effect, platelet cGMP and VSS-Rigiscan are not.

To summarize the clinical response to treatment (placebo or drug), a factorial analysis was performed on IIEF-EF, SEP, and VSS-Rigiscan. This analysis was necessary to compare parametric with nonparametric data. The first component was strictly correlated with both the IIEF-EF and SEP scores; the second accounted for the VSS-Rigiscan measurement. The multiple linear regressions between these two components and the AUC variation were used to assess the degree of association between the clinical and the cGMP response. IIEF-EF and SEP did not significantly correlate with cGMP as opposed to the Rigiscan measure that reached statistical significance ( $p = 0.037$).

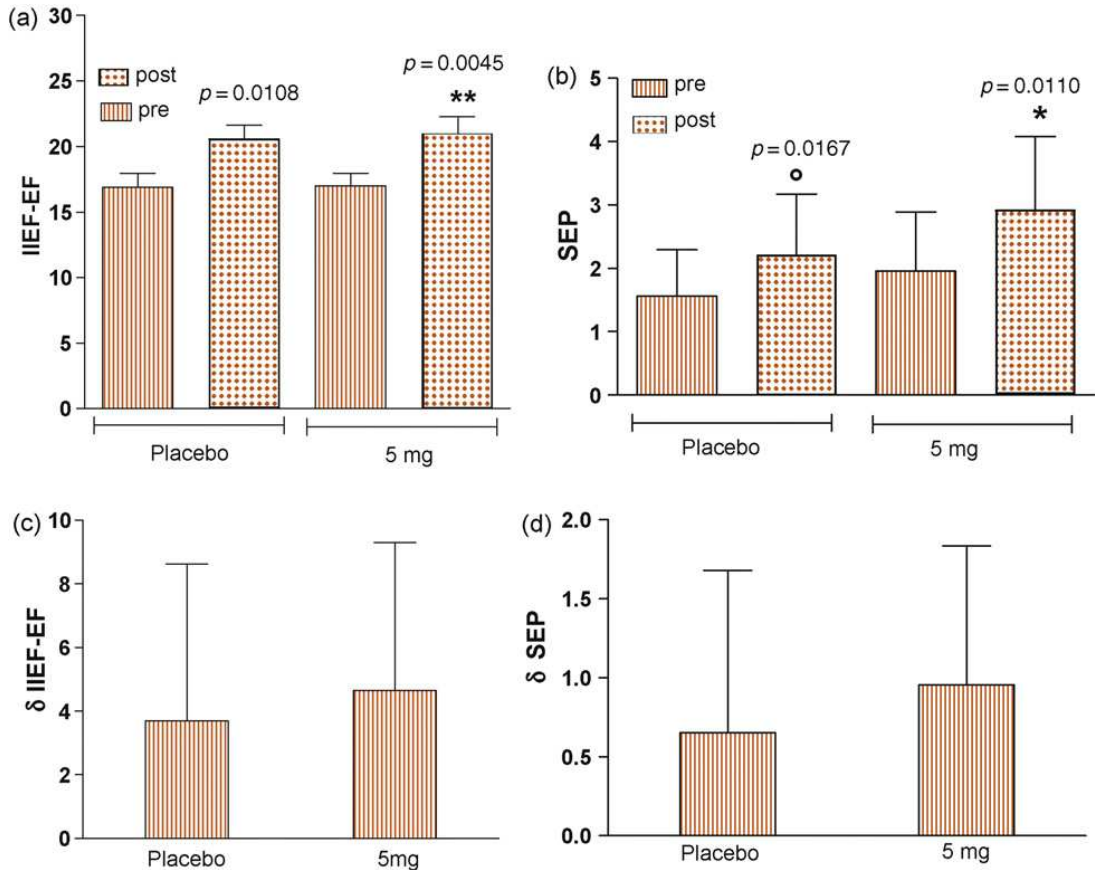



**Figure 38: International Index of Erectile Function (IEF), sexual encounter profile (SEP).**
(a) IIEF and (b) SEP were significantly increased after 6 wk of treatment with either placebo or vardenafil, 5mg. However, the d increase, calculated as the difference between the effect prior to and after the treatment, in (c) IIEF and (d) SEP were not significantly different between placebo and vardenafil.

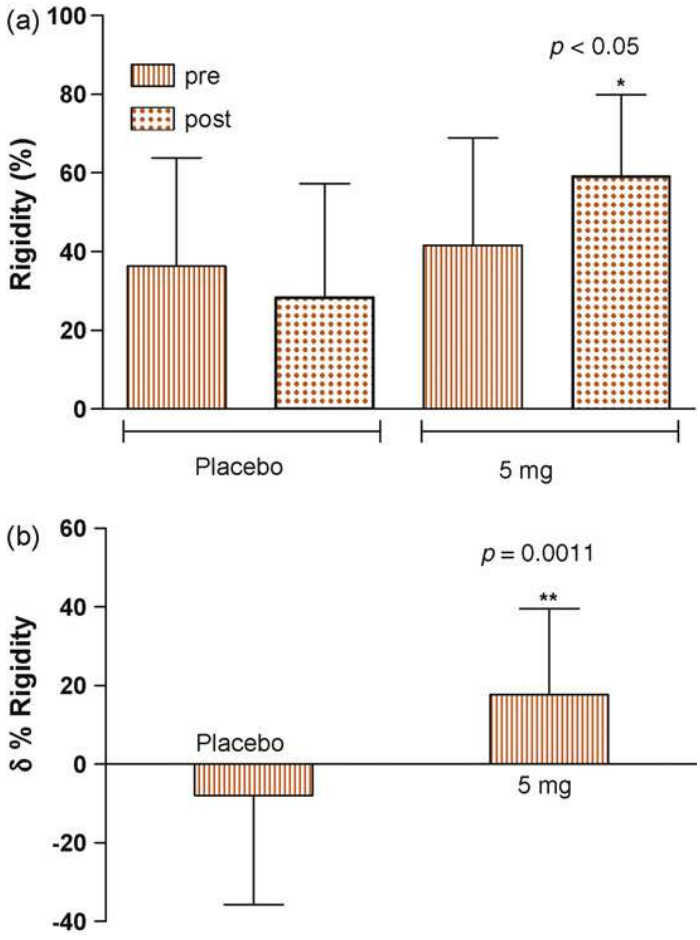



**Figure 39:visual sexual stimulation (VSS)-Rigiscan.**

VSS-Rigiscan data were calculated as the maximum rigidity achieved and expressed as a percentage. (a) Placebo did not cause any significant increase in VSSRigiscan response after 6 wk of treatment. Conversely, (a) vardenafil treatment caused a significant increase in the response. (b) The δ increase in VSS-Rigiscan response, calculated as the difference between the effect prior to and after the treatment, was significantly different between the placebo and the vardenafil group.

### *4.3.4 Protein kinase G activation*

Determination of p-VASP/VASP protein expression was assumed as a measure of the persistent activation of the protein kinase G (PKG) (Oelze M. Circ Res 2000;87:999–1005), and as such, a marker of the cGMP platelet accumulation beyond the half-life of vardenafil. Western blot analysis was performed on washed human platelets from either healthy volunteers prior and after treatment and from placebo and vardenafil group after 6 weeks of treatment (Figure 40 a).

The densitometric analysis, expressed as p-VASP/VASP ratio, showed that there was a significantly ( $p < 0.05$) increase in patients taking vardenafil compared with the placebo group (Figure 40 b).

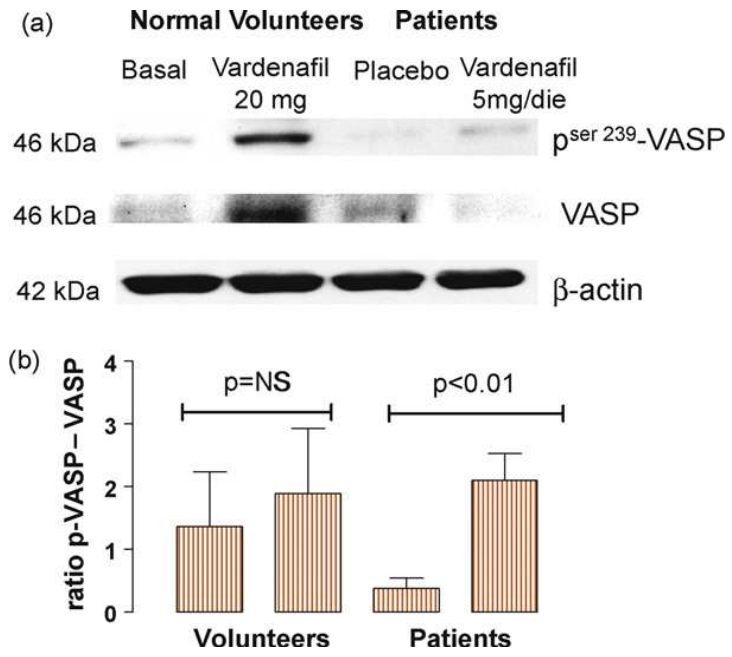



**Figure 40: Vasodilator-stimulated phosphoprotein (VASP) and phosphorylated (p)-VASP evaluation in platelets.** (a) A representative western blot for VASP and p-VASP. (b) Densitometric analysis shows a significant ( $p < 0.01$) change in the p-VASP/VASP ratio following the 6 wk of treatment with vardenafil versus placebo as opposed to the acute treatment in healthy volunteers. NS = not significant.

## 4.4 Discussion

PDE5 inhibitors are the mainstay in the treatment of ED, pathological condition characterized by the inability to achieve or maintain an erection sufficient for satisfactory sexual performance. Sildenafil (Viagra©) was the first drug to be marked for oral treatment of ED. By inhibiting PDE5, sildenafil increases the intracellular concentration of cGMP, causing amplification of the endogenous NO-cGMP pathway (Gopal *et al*., 2001). In the clinical studies on PDE5 inhibitors the primary effect measure is the IIEF, a validated questionnaire that measures the degree of ED and its improvement following therapeutic treatment (Rosen *et al*., 1997). In addition, also Sexual Encounter Profile (SEP), another widely questionnaire was diffused to detect treatment related changes in patient with ED (Rosen *et al*., "The sexual encounter profile (SEP): validation of a simple event log for sexual function assessment". Unpublished). While from one side the introduction of these simple and widely accepted questionnaires has given the possibility to quantify the degree of ED, the introduction in the market of tadalafil (Cialis©) and vardenafil (Levitra©) has revealed some weak point and raised data interpretation problems. The major problems of these tests are represented by their non objective measure of evaluation  To have an accurate evaluation of both ED and treatment efficacy also Rigiscan is used in association with questionnaire tests. However in a several studies IIEF does not well correlate with rigiscan test (Yang *et al*., 2006;  Melman *et al*., 2006).

The main goal of this study was to define if platelet cGMP could be used as a reliable marker of PDE5 activity. In preliminary study performed on healthy volunteers we have demonstrated that, following an acute administration of vardenafil (20 mg), platelets harvested under the AUC pharmacokinetic peak (Klotz *et al*,. 2001; Gupta 2005)  showed a twofold increase in cGMP, respect basal condition, ensuring a high sensitivity to the assay. To achieve a prolonged stimulation of the cGMP/PKG pathway (Giuliano 2009), patients

took 5 mg vardenafil each day for 6 weeks at bedtime versus placebo. The bedtime positioning of the therapy was selected to achieve the best possible patient compliance. In addition all patients completed the IIEF-EF and SEP questionnaires and performed the provocative rigiscan.

Our data, obtained by platelet cGMP evaluation, highlighted a statistical significance increase of cGMP levels in patients treated with chronic administration of a low dose of vardenafil *versus* placebo group. These results didn't fit with IIEF-EF and SEP scores. In fact, even though the questionnaire tests showed a significantly amelioration in both the placebo and the vardenafil group after 6 wk of treatment, the increase in both IIEF-EF and SEP were not significantly different between placebo and vardenafil. The discrepancies showed between cGMP evaluation and both IIEF-EF and SEP implies that the placebo effect influenced the questionnaires. Conversely, the rigiscan data analysis demonstrated that there was a significant amelioration in the patients treated with vardenafil *versus* placebo group. The evaluation of a relation with the variables, by using a factorial analysis, showed that platelet cGMP displayed a weak association with IIEF-EF and SEP, whereas a significant correlation was found with rigiscan. Since platelet cGMP data fit with a clinical objective measurement (eg, VSS-Rigiscan but not with either IIEF-EF or SEP), we can define platelet cGMP as a possible biomarker of PDE5 inhibitor efficacy in the treatment of ED.

Plasma levels of vardenafil could not explain the significant effect observed because the platelet cGMP measurement and VSS-rigiscan were performed 12 h after dosing. In fact several studies reported that $t_{½}$ of plasma clearance is 4 hours for vardenafil. Thus our results compared to plasma levels of vardenafil, could not explain the significant effect observed because the platelet cGMP measurement and VSS-rigiscan were performed 12 h after dosing. However, our data fit well with clinical studies reporting that men still have

facilitated erections when the levels of PDE5-I are well below that is considered the therapeutic plasmatic concentration (Moncada *et al*., 2004; Young *et al*., 2005; Shabsigh, *et al*,. 2006). On these basis, another crucial point of this study is the evaluation of the mechanism of the prolonged effect of PDE5 inhibitors. Many hypothesis are reported in literature but no one mechanism has been demonstrated. Francis *et al*. sustained that the prolonged effect of PDE5 inhibitors could be explained by a prolonged exposure of PDE5 to inhibitors (Francis *et al*., 2008 ). On this hypothesis, if drug is not cleared in parallel with clearance from plasma, PDE5 activity would continue to be blocked and high level of cGMP resulting from earlier sexual stimulation would be preserved since PDE5 is the main cGMP-hydrolyzing PDE in cells. Continued activation of PKG would sustain phpsphorylation events mediated by this enzyme including phosphorylation of PDE5 and PKG autophosphorylation (Figure 41). Evaluation of PKG phosphorylation in platelets is normally carried out by evaluating as surrogate marker a vasodilator-stimulated phosphoprotein (VASP) that is the major substrate for cGMP-dependent PKG in human platelets (Oelze *et al*., 2000). Our western blot analysis, performed on VASP activation, clearly show that the prolonged therapy with vardenafil but not placebo leads to a significant change in the p-VASP/VASP ratio and thus of PKG phosphorylation. Interestingly, the p-VASP/VASP ratio change in healthy volunteers following an acute administration of vardenafil was not significant.

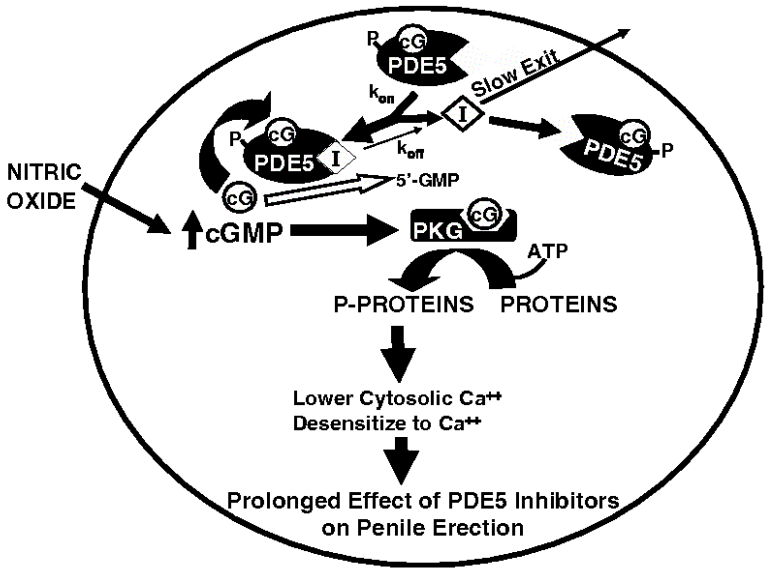



**Figure 41: Potential process that could contribute to persistence of the effect of activation of the cGMP signalling pathway in vascular smooth muscle cells in presence of PDE5 inhibitors (Francis et al. 1997).**

On the basis of the hypothesis reported by Francis et al., our data strongly suggest that most likely a prolonged exposure to vardenafil coupled with a sexual stimulus is necessary to trigger the cascade of events just described. In addition, it is feasible to assume that what we have seen in platelets reflects what is happening in competent cells in the human penile tissue (eg, endothelial and smooth muscle cells).

In conclusion platelet cGMP level could represent a relative simple assay to provide information on the activity and duration of PDE5 inhibition within and over plasma levels. Since its correlation with a clinical objective measure, it could be helpful in interpreting and/or correcting the data obtained by IIEF-EF and SEP. This characteristic makes the platelet cGMP assay a useful tool to be used in future ED clinical studies, for example on patients who are nonresponders to a PDE5-I scheduled as an alternative or in comorbidity conditions (diabetes, lower urinary tract symptoms, etc).

# *CHAPTER 5*

## Conclusions

## Conclusions

Erectile dysfunction can be due to a number of different pathophysiological events. On these basis ED may be classified as psychogenic, organic (neurogenic, hormonal, vasculogenic and drug-induced), and mixed. Vasculogenic (arterial and cavernosal) ED is characterized by impaired veno-occlusive mechanism and inadequate arterial inflow. Peyronie's disease could represent one of this pathological process. In fact Peyronie's disease (PD) is a connective tissue disorder involving the growth of fibrous plaques of tunica albuginea of the penis with curvature and distortion, usually during erection, penile pain and erectile dysfunction (Gelbard *et al*., 1990). However, PD is a poorly understood disorder on molecular and physiopathological basis also due to the lack of appropriate animal model. We have given a contribute in the understanding of this pathology by discovery a new animal model of Peyronie Disease naturally occurring: Thight skin (*Tsk*) mouse. In fact *Tsk* mouse replicates conditions that are similar enough to human disease. In particular, (i) *Tsk* mice spontaneously develop the disease with age, (ii) several mediators that have been implicated as the major players in the human disease are involved and (iii) this animal model shares many morphological, histological and biochemical features with the human disease. For all these characteristics *Tsk* mouse represents the first example of naturally occurring model of PD in laboratory animals.

Since PD represents a particular case of organic ED which is generally caused by the breaking of the balance vasorelaxant and vasoconstrictor factors, in the second part of these studies we have looked for a pathway involved in this balance. Our attention has been focused on urotensin II (UII), an endogenous, vasoactive peptide and its receptor (GPR14 or UT receptor). At first we have showed that GPR14 is expressed in the rat corpus cavernosum and in anesthetized rats, intracavernous administration of UII causes an increase in ICP. Also HCC possesses U-II receptor that is located predominantly on the

endothelium. The receptor is functional and mediates an endothelium-dependent relaxation that involves the L-arginine/NO pathway. In particular, UII activates eNOS via Akt and via Hsp90, following in NO release and thus relaxation. These observations may help to unravel the complex mechanisms underlying the pathophysiology of human penile erection and may lead to the development of novel therapeutic approaches in the treatment of ED and sexual arousal disorders.

Since the difficulty in ED diagnosis and evaluation of ED treatment efficacy, in the last part of these studies we have looked for a new, objective, non invasive and reliable methods. In our clinical study it has been showed that platelet cGMP could represent a valuable marker for PDE5 activity on ED. Indeed this measure is probably less sensitive to the placebo effect. Thus it represents an important objective measure that helps to interpreter and/or correct the data obtained by IIEF and SEP. In addition, by measuring cGMP it could be possible to better define those patients that are non-responders. Finally we show evidence for a possible biochemical mechanism of action that can justify the prolonged activity of PDE5 inhibitors beyond their half life.

# *CHAPTER 6*

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