EPFL

Thèse n° 7326

Multidisciplinary Approach to Treat Erectile Dysfunction

Présentée le 16 octobre 2020

à la Faculté des sciences et techniques de l'ingénieur
Laboratoire d'hémodynamique et de technologie cardiovasculaire
Programme doctoral en biotechnologie et génie biologique

pour l'obtention du grade de Docteur ès Sciences

par

Mikaël Nils STURNY

Acceptée sur proposition du jury

Prof. D. Pioletti, président du jury
Prof. N. Stergiopulos, directeur de thèse
Prof. A. L. Burnett, rapporteur
Prof. I. Adamakis, rapporteur
Prof. S. Micera, rapporteur

École polytechnique fédérale de Lausanne

2020

"*Men explores the universe around him and calls the adventure Science.*"

Edwin Powell Hubble

"*Science is not only a disciple of reason but, also, one of romance and passion.*"

Stephen Hawking

"*The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny…'.*"

Isaac Asimov

To my familly

Line-France, Daniel, Grégory and Anaïs

Acknowledgements

Firstly, I would like to express my extreme gratitude to Professor Nikos Stergiopulos, without who none of this work would have been possible. Thank you for believing in me and for giving me the opportunity to conduct my PhD (and several other projects before that…) in your lab. It has been an honor to conduct this work under your supervision and there is nothing more rewarding than hearing one of your now famous "fantastic". Although "*Education isn't something you can finish*" as quoted by Isaac Asimov, physician and one of my favorite author, I greatly appreciate all the knowledge and experience I have learned throughout the years thanks to you. I also owe my deepest thanks to Dr. Rodrigo Araujo Fraga da Silva, my direct supervisor, colleague and friend during all my projects conducted over the years. I wish to acknowledge here your positive attitude, sense of humor and never ending patience. This thesis would not have been possible without your unconditional help, advices and support.

I am very fortunate to have met Professor Arthur L. Burnett, a constant source of inspiration and advices and most certainly one of the foremost leader of the sexual medicine field. Thank you for welcoming me to your lab in Baltimore for a while in the frame of this thesis. Your humbleness, passion for science and availability have been inspirational and marked me forever. Thank you also to Professor Ioannis Adamakis, one of the most skilled surgeon I have ever met, for the help and support with the conduction of part of this thesis in Greece. Your kindness, enthusiasm and patience during the intraoperative experimentation have been tremendous and greatly appreciated. Thank you also to the jury committee members, Professor Micera and Professor Pioletti, for taking the time to read and discuss this dissertation. I highly appreciate your valuable comments and feedback.

To my dear past and present labmates, thank you for the incredible ambiance inside the LHTC lab all these last years. I will remember forever all the ski days, BBQ and after-work beers. A warm thank you to Fabiana, Bram, Michel, Mauro, Lydia, Natàlia, David, Seb, Stephanie, Valeria, Augusto, François, Stephan, Adan, Sylvain, Constantinos, Matina, Vicky, George and Quentin. Also a special thanks to Sylvia for your constant support with administrative tasks and in organizing everything around us and to Maiia for sharing an office and listening to my complaints the last 5 years. You have been the best officemate and friend I could have expected. To all the members of our neighbor labs, LBO and MICROB, thank you also for the great atmosphere in the MED 3rd floor, our traditional annual raclette and the countless discussions and laughs in the corridor.

To my closest friends Matthias, Jérémie, Marc, Pascal, Jean, Alain and all members of the "chapeaux de paille" crew as well as all my friends collected around the years at EPFL, Lilly, Ophélie, Julie, Letty, Linda, Théo, Nadir, Theodoros and Serkan, thank you for being there for me when I needed, for your un-discontinued and unconditional support and for all the memorable moments we have shared together. I value your friendship more than anything.

A very special thank you also for my godfather, Marcel-Louis, for your financial, logistical and emotional support all these years and for my very first valuable and complete suit.

At last but not least, I would like to thanks my parents, Line and Daniel and my siblings, Anaïs and Grégory for their support, help and motivation throughout all these years. This thesis was possible only thanks to you. I love you from the bottom of my heart.

Mikaël Sturny

Lausanne, September 2020

Abstract

Erectile dysfunction (ED) is a widespread disease affecting approximately 20% of men. With a rising incidence due to the global ageing of the world population, it is estimated that over 322 million patients will be affected in 2025. While not being a life-threatening disease, ED has a strong detrimental impact on the patient's quality of life, commonly leading to a reduction of work productivity, anxiety, chronic stress, depression or loss of self-esteem. Despite all the recent advances in understanding the molecular mechanisms and pathophysiology of ED, and the discovery of effective oral treatments that are the PDE5 inhibitors (such as Viagra®), there is still an important portion of the population that does not respond to this therapy. Therefore, they unfortunately resort on problematic, uncomfortable, and invasive last-resort solutions due to the lack of better alternatives. In addition, current treatments are inducing assisted erection rather than handling the root cause of the disease. Considering the limited number of strategies currently existing for the treatment of ED and their drawbacks, there is an urgent need for alternative therapies. Due to the fact that both vascular and neural components are required to induce and maintain a satisfactory erectile response, as well as the multifactorial origins of ED, new therapies able to combine multidisciplinary approaches would increase the chances of new treatment success. Therefore, this thesis focuses on different multidisciplinary strategies attempting to provide novel therapies to alleviate ED or its causes. In the first part of this thesis, we have investigated the anti-fibrotic capabilities of a vasoactive endogenous peptide called Apelin-13 on a mouse model of ED, providing promising results and pointing out Apelin as a candidate for the development of fibrosis-associated ED treatments.

Secondly, we have developed a novel concept enabling the neurostimulation of the cavernous nerves (CN) to induce penile erection. This thesis shows the first intraoperative proof of principle in humans, opening the way for the development of an innovative medical device for the neuromodulation of erectile function to treat neurogenic ED.

Finally, we have studied further the effects of CN low-intensity electrostimulation (LIES), another application of nerve electrical stimulation, with the potential of promoting CN regeneration. We observed a significant protective effect of LIES on penile tissue structure and functions following nerve trauma in a rat model of cavernous nerve injury.

Altogether, the work underwent in this thesis add to the development of more effective and comfortable treatment options to alleviate ED, which could not only improve the quality of life of patients but could also reduce the burden and cost this disease places on medical and public health systems.

Keywords

Erectile dysfunction (ED), Fibrosis, Hypercholesterolemia, Apelin, Vascular ED, Neurogenic ED, Cavernous nerves, Peripheral nerve stimulation, Neurostimulation, Low-intensity electrostimulation (LIES).

Résumé

Les troubles de l'érection sont une affection mondialement répandue affectant près de 20% des hommes. Son incidence est en hausse dû au vieillissement constant de la population mondiale. En effet, il est estimé que le nombre de patients atteints de troubles de l'érection va surpasser les 322 millions dès 2025. Bien que cette maladie ne mette pas la vie des patients en danger, les troubles de l'érection ont une impacte importante sur la qualité de vie des malades et entraine fréquemment une baisse de la productivité professionnelle, de l'anxiété, un stress chronique, un état de dépression ou une perte d'estime de soi chez ces personnes. Malgré les récentes avancées dans la compréhension des mécanismes moléculaires à l'origine de la dysfonction érectile et la pathophysiologie associée à cette maladie, et malgré la découverte de traitements oraux tels que les inhibiteurs de l'enzyme PDE5 (comme le Viagra®), une portion importante de la population ne répond pas à ces traitements. En conséquence, dû au manque d'alternatives, ces patients dépendent de solutions de dernier recours qui sont malheureusement problématiques, peu confortables et invasives. De plus, les traitements actuels permettent d'induire une érection mais ne traitent pas la cause à l'origine des troubles de l'érection. De ce fait, en vue du nombre limité d'options à disposition et des effets indésirables de ces traitements, il y a un besoin urgent pour de nouvelles thérapies contre les troubles de l'érection. Dès lors, en raison du fait que la combinaison et la coordination d'une réponse du systèmes nerveux ainsi que du système vasculaire soit requise pour l'induction et le maintien d'une érection satisfaisante, et en raison des nombreuses causes et pathologies sous-jacentes à l'origine des troubles de l'érection, de nouvelles thérapies capables de combiner des approches multidisciplinaires dans le but d'offrir de nouvelles thérapies contre la dysfonction érectile ont plus de chances d'être efficaces. Cette thèse se concentre donc sur plusieurs stratégies multidisciplinaires dans le but de développer de nouvelles thérapies contre les troubles de l'érection et de traiter les causes à l'origine de cette maladie.

Dans la première partie de cette thèse, nous avons étudié les effets anti-fibrotiques d'une protéine endogène aux effets vasoactifs appelée Apelin-13 sur un modèle animal de la dysfonction érectile. Les résultats promettants obtenus mettent en évidence cette protéine, Apelin, comme candidat intéressant pour le développent d'un nouveau traitement contre les troubles de l'érection associés à une fibrose des tissus nécessaires à l'érection.

Deuxièmement, nous avons développé un nouveau concept permettant de stimuler électriquement les nerfs caverneux et d'induire ainsi une érection. Cette thèse présente les résultats de la première étude clinique chez l'homme démontrant l'efficacité de ce concept, ouvrant ainsi la voie au développement futur d'un nouvel implant médical innovant pour le traitement des troubles de l'érection d'ordre neurologiques.

Finalement, nous avons également étudié plus en profondeur les effets d'une stimulation électrique de faible intensité sur les nerfs caverneux, une autre application possible de la neurostimulation, qui pourrait potentiellement améliorer la régénération nerveuse. Dans cette étude nous avons observé un effet protecteur de ce type de stimulation sur la structure et le fonctionnement des tissus du pénis suivant un dommage des nerfs caverneux chez le rat.

En définitif, le travail effectué durant cette thèse a permis d'ajouter une contribution au développement de traitements plus efficaces et plus confortables contre les troubles de l'érection, ce qui pourrais non seulement permettre d'améliorer la qualité de vie des patients, mais aussi diminuer le poids et le coût financier que cette maladie porte sur le système médical et les assurances maladies.

Mots-clés

Dysfonction érectile (DE), Troubles de l'érection, Fibrose, Hypercholestérolémie, Apelin, DE vasculaire, DE neurogénique, Nerfs caverneux, Stimulation des nerfs périphériques, Neurostimulation.

Contents

List of Figures

Chapter 1 Introduction

1.1 Motivation

Erectile dysfunction (ED) is defined by the National Institutes of Health (NIH) as the persistent inability to achieve or maintain penile erection sufficient for satisfactory sexual intercourse[1]. ED is a widespread sexual disorder, affecting over 150 million men worldwide[2]. Provided the rapid aging of the world's population, this number is expected to substantially rise within the next 10 years and exceed 322 million[2]. While not being a life-threatening disease, ED often has a strong detrimental impact, not only on the personal life of the patient, but also on the social, familial, and occupational aspects of life[3]. In particular, reduction of work productivity, anxiety, chronic stress, depression and loss of self-esteem are documented complications of patients with ED[3,4]. Despite the great success of the first-line oral therapy (e.g. sildenafil, Viagra®) in ED treatment, a substantial portion of ED patients either cannot use it due to adverse effects, or have low levels of response to none[5]. Non-responsive patients to oral pharmacotherapy have to resort to other treatments such as intrapenial injections of vasodilators[6] or penile prosthesis, which present numerous side effects and have been shown to be uncomfortable solutions in many cases[7]. These alternative therapies, although non-optimal and chronically problematic, are largely used due to the lack of better alternatives. In addition, current treatments combatting ED are providing relief to the patient by restoring an assisted penile erection, while only limited therapies are focusing on preventing the cause of ED itself, or restoring physiological erectile function[8]. Consequently, there is an urgent need for novel ED treatment solutions for patients who are non-responsive to oral drugs and new effective medications to protect or restore the natural erectile function.

1.2 Anatomy and Physiology of Penile Erection

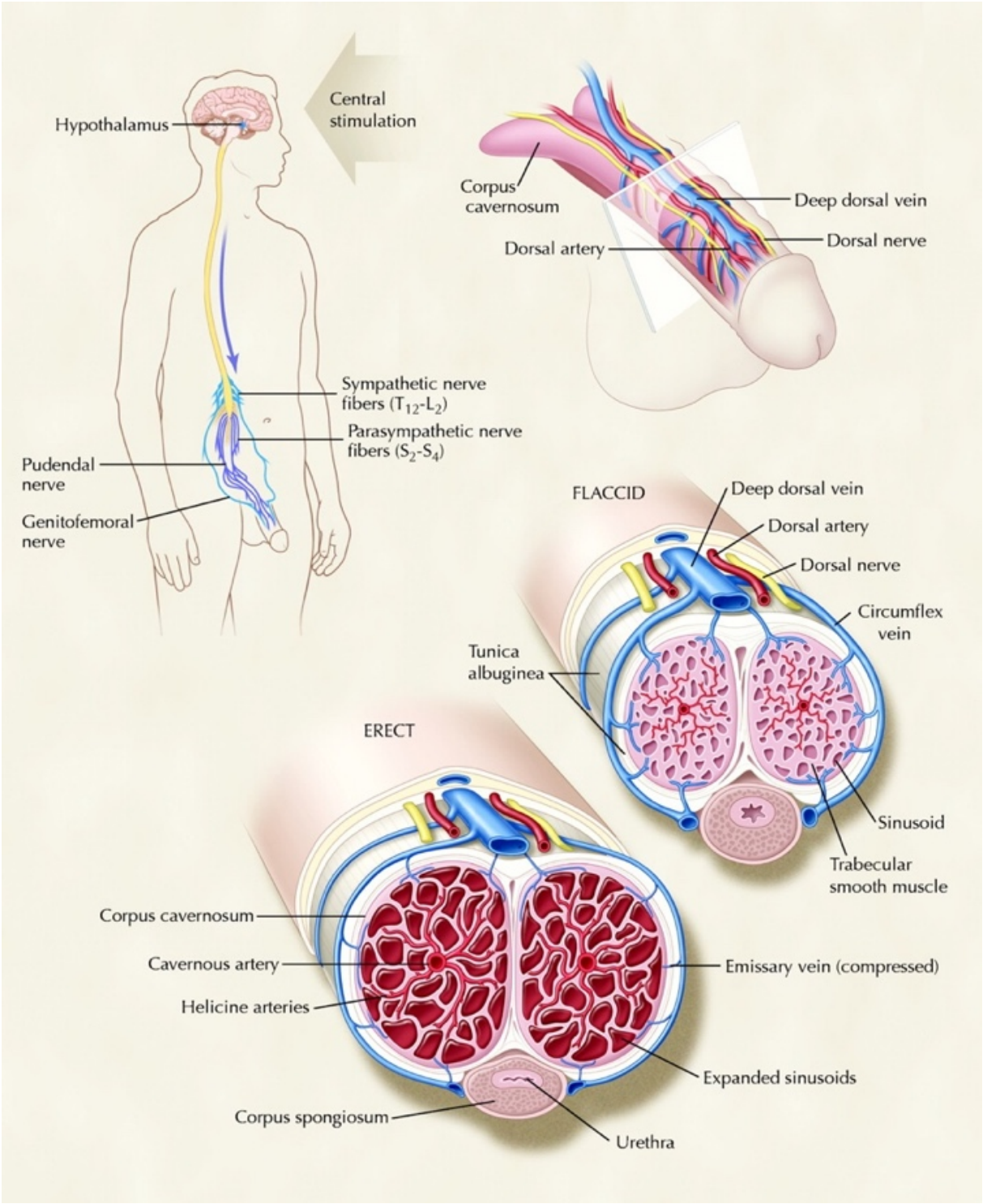

Figure 1:1 Schematic representation of the veno-occlusive mechanism.

(Reproduced from Fazio and Brock[9])

Penile erection is a coordinated neurovascular response[10,11]. In the flaccid state, the penile smooth muscles are tonically contracted, allowing only a small amount of blood flow for nutritional purposes. Penile erection occurs when sexual stimulation triggers the release of neurotransmitters from the parasympathetic cavernous nerve terminals within the penile vascular structures[12], inducing

relaxation of cavernosal smooth muscles. The erectile tissue in the penis contains large interspaces capable of being distended with blood. The neurotransmitters cause relaxation of the smooth muscle cells in cavernosal arterioles and sinuses, resulting in increased blood flow into the penis. The blood inflow fills the cavernous sinuses, which expand against the tunica albuginea, an external fibrous tissue surrounding the corpora cavernosa, occluding the venous outflow[10]. This vein-obstruction process is commonly known as corporal veno-occlusive mechanism. The consequent entrapment of blood and increase in pressure leads to penile erection[13] (Figure 1.1). When the neurotransmitters available to maintain erection are depleted or following detumescence signalling, the corporal smooth muscles return to the basal contracted state, decreasing the blood inflow and pressure in the corpora cavernosa, allowing the venous channels to slowly open until the return of basal arterial flow and penile flaccid state[10].

Erectile function is controlled by a complex balance between pro- and anti-erectile signalling regulated by the central nervous system (CNS), the peripheral nervous system (PNS), as well as extra-, intra- and inter-cellular mechanisms[14].

Central Neural Regulation

The central nervous regulation is responsible for the integration of sexual stimuli, resulting in the initiation and maintenance of penile erection. Different brain areas have been identified to play an important role in controlling and influencing the erectile response and sexual behaviour, while different neurotransmitters and hormones affect the erectile function in opposing ways (Figure 1.2). For example, dopamine and norepinephrin promote sexual function, while serotonin, also called 5-hydroxytryptamin (5-HT), is believed to be inhibitory[14]. Patients taking serotonin reuptake inhibitors (SRI), an antidepressant drug that increases the intersynaptic levels of 5-HT, were found to often have a suppressed libido, while those taking a 5-HT neuron suppressor had an increased libido. On the other hand, data suggest that increased central norepinephrin activity exerts positive effects on sexual function[15]. In addition, oxytocin and melanocortin (α-, β-, γ-melanocyte-stimulating hormone) have been shown to induce erections[16,17], while γ-aminobutyric acid (GABA), and opioids have an inhibitory effect[18]. Finally, prolactin, a hormone released by the pituitary gland as a sexual gratification, counteracts the effect of dopamine and is thought to cause the sexual refractory period[19].

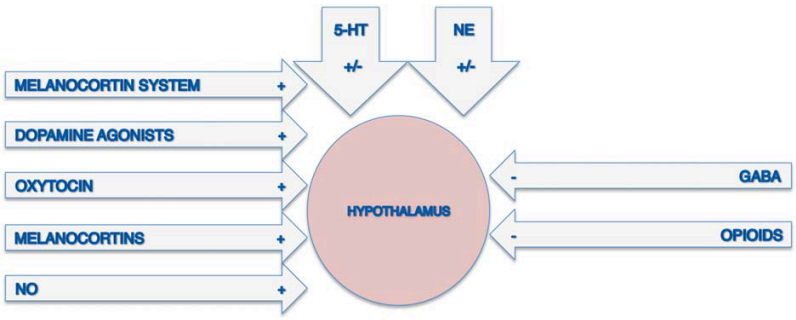

Figure 1:2 Central modulation of erection.
NO, nitric oxide; 5-HT, 5-hydroxytryptamin (serotonin); NE, norepinephrine; GABA, γ-aminobutyric acid. (Modified from Hawksworth and Burnett[14])

Peripheral Neural Regulation

Penile erection is also controlled by the peripheral nervous system through sympathetic, parasympathetic, somatic, and sensory innervation. The nerves involve specific neurotransmitters and have been categorized as adrenergic, cholinergic, and nonadrenergic noncholinergic (NANC). Together, the nerves and vascular endothelium produce and release transmitters and modulators, which in turn interact and control the contractile state of the penile smooth muscles[14]. The sympathetic nervous system, originating from the T12th to the L2th segments of the spine, controls the corporal smooth muscle contraction and induces penile flaccidity, while the parasympathetic division, originating from the S2 to the S4 sacral segments, is responsible for the smooth muscle relaxation and penile erection (Figure 1.1)[9].

Norepinephrine (NE) represents the principal sympathetic neurotransmitter controlling penile flaccidity. It is released from the local adrenergic nerve terminals and stimulates postsynaptic α-adrenergic receptors in the penile vasculature and in the corpus cavernosum, resulting in the contraction of the helicine vessels and trabecular smooth muscle, respectively (Figure 1.3 - A)[20]. On the other hand, nitric oxide (NO) is considered the principal neurotransmitter in mediating penile erection[21]. NO is synthesized as a by-product of the catalytic conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS). Constitutive NOS enzymes are found in endothelial cells (eNOS) and NANC neurons (nNOS), and are activated in the presence of calcium, calcium-binding calmodulin, oxygen, and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Upon stimulation, intracellular calcium concentration increases

and binds to calmodulin, forming a calcium–calmodulin complex that binds to and activates NOS. NO is then produced and released in small amounts until calcium levels decrease. Following release of NO from the neurons and endothelial cells, NO activates guanylyl cyclase, and this enzyme catalyses the production of cyclic guanosine monophosphate (cGMP), which in turn relaxes the cavernous smooth muscle (Figure 1.3 - B)[20]. NO derived from nNOS is responsible for the initiation and the majority of the smooth muscle relaxation, while NO originating from eNOS contributes to the maintainability of the erection[22].

Acetylcholine is also released from cholinergic presynaptic nerve endings and indirectly contributes to penile erection by the presynaptic inhibition of adrenergic neurons and the stimulation of nitric oxide (NO) release from endothelial cells[23].

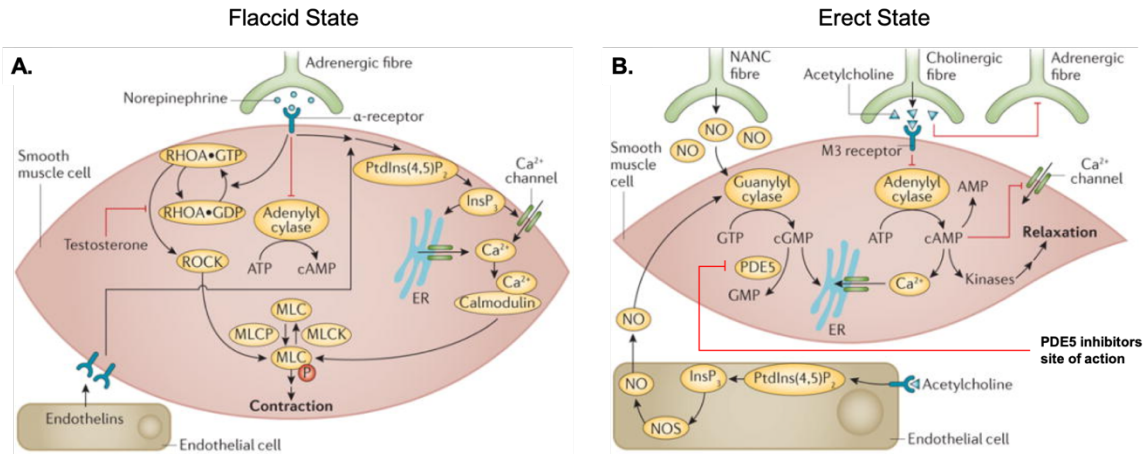

Figure 1:3 Peripheral and cellular modulation of erection.
A) Flaccid state. Adrenergic stimulation by norepinephrine induces the contraction of smooth muscle cells maintaining the penis in a flaccid state. B) Erect state. Cholinergic and NO stimulation induce the relaxation of smooth muscle cells and the induction and maintenance of penile erection. (Modified figure from Yafi et al.[20])

Cellular Control Mechanisms

The regulation of penile erections, in addition to the intricate interplay of the multiple neurotransmitters and hormones originating from the CNS and PNS, also depends on molecular mechanisms operating at the level of the corporal smooth muscle cell[10]. Mechanisms involved in the intracellular calcium ion (Ca^{2+}) concentration play a significant role in the regulation of corporal smooth muscle contraction and relaxation. Increased levels of intracellular Ca^{2+} result in its binding to calmodulin. Following a conformational change, the Ca^{2+}-calmodulin complex binds to and activates the myosin light-chain kinase (MLCK) by phosphorylation. Phosphorylated myosin light-chain subunits activate myosin adenosine triphosphatase (ATPase), which hydrolyses ATP to generate energy for myosin crossbridging along actin filaments, thus resulting in the smooth muscle contraction (Figure 1.3 - A). Following the contraction, levels of intracellular Ca^{2+} decrease and once calmodulin dissociates from the complex, MLCK is dephosphorylated by myosin light-chain phosphatase (MLCP), detaching from the actin filament and reverting back to its inactive state, resulting in smooth muscle relaxation[14]. RhoA/ROCK pathway is also required for penile detumescence and flaccidity. The Rho-associated protein Kinase (ROCK) phosphorylates MLCP, which renders it inactive. This process sensitizes the myosin-actin contraction to lower levels of cytosolic calcium in smooth muscles, which facilitates tonic contraction and the penile flaccid state[24].

The mechanism of smooth muscle relaxation responsible to initiate and maintain an erection occurs via the second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). They activate cAMP- and cGMP-dependent protein kinases, which in turn phosphorylate certain proteins and ion channels, resulting in the opening of potassium channels and subsequent hyperpolarization, sequestration of intracellular Ca^{2+} by the sarcoplasmic reticulum, and inhibition of voltage-dependent calcium channels, blocking calcium influx. This all results in a significant drop in the intracellular free calcium and furthers smooth muscle relaxation (Figure 1.3 – B)[10]. The termination of the signals evoked by cAMP and cGMP is mainly executed by phosphodiesterase enzymes (PDEs), which catalyse the hydrolysis of cAMP and cGMP to AMP and GMP, respectively. The superfamily of PDEs consists of 11 families, with PDE5 considered as the primary enzyme responsible for the termination of cavernous cGMP signalling[25]. The PDE5 enzyme is the target of the PDE5-inhibitors (PDE5-Is), which is the initial and most common treatment against erectile dysfunction. By inhibiting the hydrolysis of cAMP and cGMP, PDE5-Is promotes the accumulation of cGMP, resulting in increased smooth muscle relaxation, facilitating erection induction and maintenance[14]. In addition to the mechanisms described above,

communication among individual smooth muscle cells and thus coordination of their synchronized relaxation or contraction is achieved via gap junctions. These intercellular channels allow the interchanging of ions as calcium and second-messenger molecules[26].

1.3 Pathophysiology of Erectile Dysfunction

Erectile dysfunction is the most common sexual dysfunction in men[27]. It is estimated that approximately 5-20% of men have moderate-to-severe ED, and currently, up to 70% of men with ED are not treated[28]. In a survey carried out in France, Germany, Italy, Spain, and the UK among 28,511 adult men (47.18±16.07 years old), 17% of the participants self-reported cases of ED over the past 6 months[29]. By extrapolation of those results on the whole population, an estimated 21.7 million men in these countries may experience problems with initiating or maintaining a satisfactory erection[29], which is in accordance with the results obtained in a similar study in the US, where the prevalence of erectile dysfunction was estimated as 18.4% for men aged ≥20 years old[30].

The incidence of ED is highly related to age[30]. According to the Massachusetts Male Aging Study, men at 60 to 69 years old have a four-times higher risk of ED than those aged 40 to 49 years old[27]. Taking into account the rapid ageing of the population, the prevalence of ED is expected to rise to approximately 322 million worldwide by the year 2025, which corresponds to an increase by nearly 170 million in comparison to the year 1995[2].

Table 1:1 Etiology and risk factors of erectile dysfunction (ED) (adapted from Porst et al.[31])

Psychogenic factors
Psychosexual development in childhood/adolescence (special negative experiences, etc.)
Sexual orientation problems?
Partnership problems?
Lifestyle factors and individual health conditions
Permanent stress factors? (private/business life)
Sedentary lifestyle?
Nicotine?
Alcohol abuse?
Drug addictions?
Cardiovascular risk factors
Hypertension (including antihypertensive medications)
Dyslipidemia
Coronary artery disease
Peripheral arterial occlusive disease
Diabetes mellitus (types 1 and 2)
Urological disorders
Benign prostate hyperplasia (BPH) and lower urinary tract symptoms (LUTS)
Chronic prostatitis
Penile/cavernous factors
Cavernous myopathy and fibrosis (veno-occlusive dysfunction)
Peyronie's disease or penile fracture
Medical/metabolic disorders
Renal insufficiency
Hepatic insufficiency
Dyslipidemia

Endocrine factors
Hypogonadism (primary/secondary)
Hyperprolactinemia (microprolactinoma/macroprolactinoma or drug induced)
Thyroid disorders (hypothyroidism/hyperthyroidism)
Neurologic disorders
Cerebral, spinal, and peripheral affections: central (brain) diseases such as multiple sclerosis, cerebrovascular atherosclerosis,
Parkinson's disease, dementia, etc.
Somatic and autonomic polyneuropathy
Pudendal nerve lesions ("vulnerable perineum")
Iatrogenic ED
Drug induced (drugs with a negative impact on central and peripheral erectile functions)
Postoperative (pelvic, perineal, urethral, and penile surgery)
Post-radiation (prostate cancer, bladder cancer, and rectum cancer)
Posttraumatic ED
Brain/spinal cord injuries
Pelvic fractures, urethral ruptures, straddle traumas, and penile injuries
Respiratory disorders
Chronic obstructive pulmonary disease
Sleep apnea

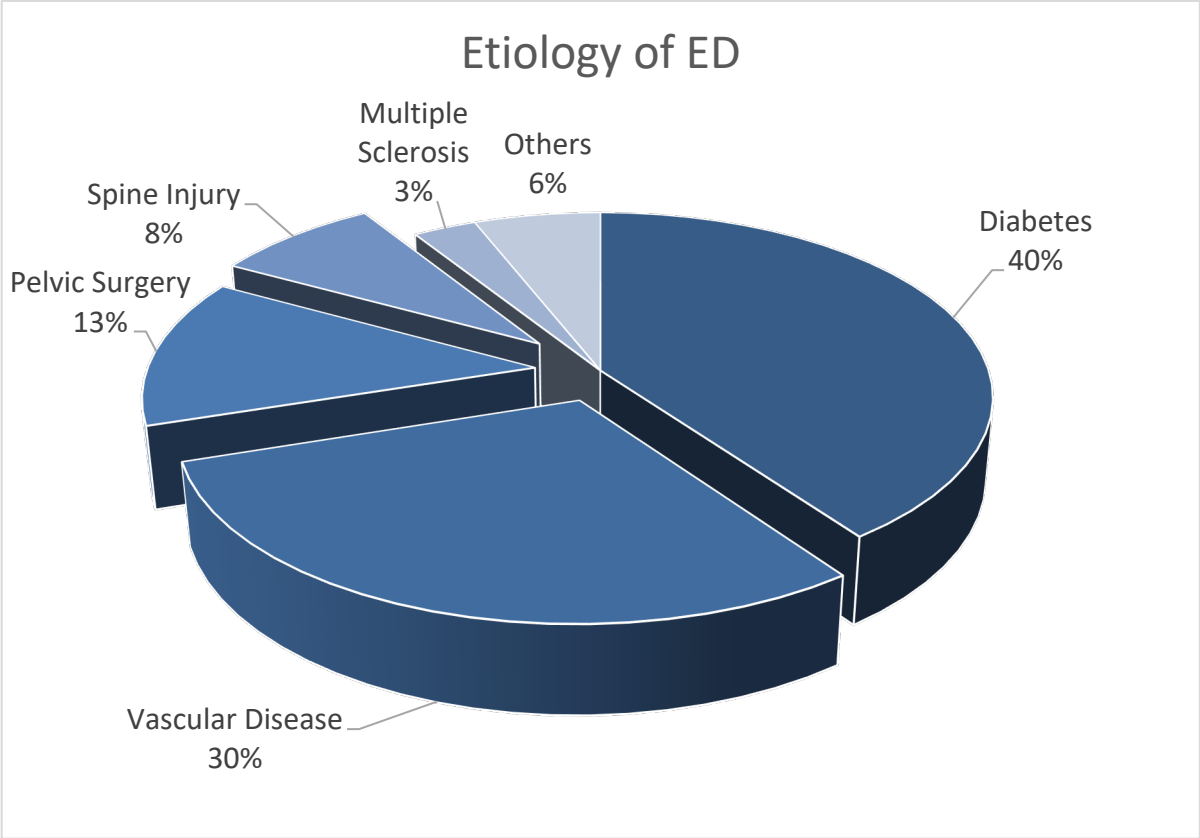

Figure 1:4 Demographics of erectile dysfunction.

In addition to age, erectile dysfunction has a number of organic and psychogenic etiology (Table 1:1 and Figure 1.4). Additionally, for many patients, the disorder may be multifactorial[32]. Indeed, the risk of ED development is higher in men with one or more cardiovascular or metabolic risk factors, such as hypertension, diabetes, hypercholesterolemia, obesity, unhealthy diet, lack of physical activity, smoking or alcohol abuse[27,30,33]. As an example, the incidence of ED among men with diagnosed diabetes mellitus is above 50%[34]. ED is often classified as psychogenic, vascular, or neurogenic, depending on the root cause of the disease. As the penile erection mechanism involves central, peripheral, and cellular regulation, numerous origins of ED exist in the case of impairment of any of the regulatory pathways or direct tissue properties. An increased fibrosis of the corpora cavernosa and the media of penile arteries is a highly prevalent process that is the underlying factor in most cases of erectile dysfunction (ED). The excess of collagen deposition of stiff fibres and decrease of elastic fibre content impairs the elastic properties and expandability of the corpus cavernosum[35,36], which are required to insure tissue compliance and accommodation of blood within the cavernosal sinuses during erection, and is essential for proper compression of subtunical venules, guaranteeing the pro-erectile veno-occlusive mechanism[10]. In addition to organic etiologies, patients undergoing pelvic surgery often suffer from ED due to nerve injury during the surgical procedure. Amongst them, radical prostatectomy surgery is one of the most prevalent causes of erectile dysfunction[37].

Although the research presented in this thesis may benefit multiple etiologies of ED, this work focuses primarily on hypercholesterolemia-induced ED and post-prostatectomy ED.

Hypercholesterolemia-induced ED

Hypercholesterolemia resulting in atherosclerosis of the penile vasculature is one of the leading cause of vasculogenic ED[38,39]. One of the major mechanisms involved has been attributed to oxidative stress within the corpus cavernosum, which triggers a higher vasoconstrictor tonus and a reduction of nitric oxide (NO) bioavailability[40,41]. In the long-term, prolonged oxidative stress within the corpus cavernosum leads to hypoxia and inflammatory response[42], triggering the release of mitogenic and pro-fibrotic cytokines[43] inducing fibrosis. Therefore, in the long-term, hypercholesterolemia may lead to severe ED by promoting fibrosis and exaggerated collagen deposition[36,39].

Fibrosis

The fibrosis process is characterized by an increased collagen fiber deposition, following fibroblast activation and differentiation into myofibroblast (Figure 1.5). The activation and differentiation of fibroblasts to myofibroblasts, which are major cells responsible for the collagen production, represent a key event in the fibrosis establishment and progression[43,44]. Myofibroblast differentiation is known to be initiated by multiple inflammatory factors, especially by transforming growth factor beta (TGF-β1), leading to a phenotype modification characterized by the acquirement of a stellate shape, expression of alpha smooth muscle actin (α-SMA), and augmented secretion of collagen type-I fibers[45]. Activated myofibroblasts are then able to secrete TGF-β1 themselves, amplifying the pro-fibrotic process[43]. TGF-β1 is known to mediate its fibrotic effects by activating the receptor-associated Smads, including Smad2 and Smad3. TGF-β1 binds to a type I receptor known as activin receptor-like kinase (ALK). The phosphorylation of serine/threonine residues in ALK5 subsequently phosphorylates the major downstream signaling molecules Smad2 and Smad3, that form a heteromeric complex with Smad4, which then translocate into the nucleus and regulates the transcription of TGF-β1-responsive genes, thereby inducing fibrosis-related changes[46] (Figure 1.5). In the other hand, Smad7 has been identified as inhibitory of Smad2/3 signaling pathways, while, TGF-β-Smad2/3 signaling pathways are known to mediate smooth muscle cell apoptosis and to inhibit endothelial cell proliferation[47].

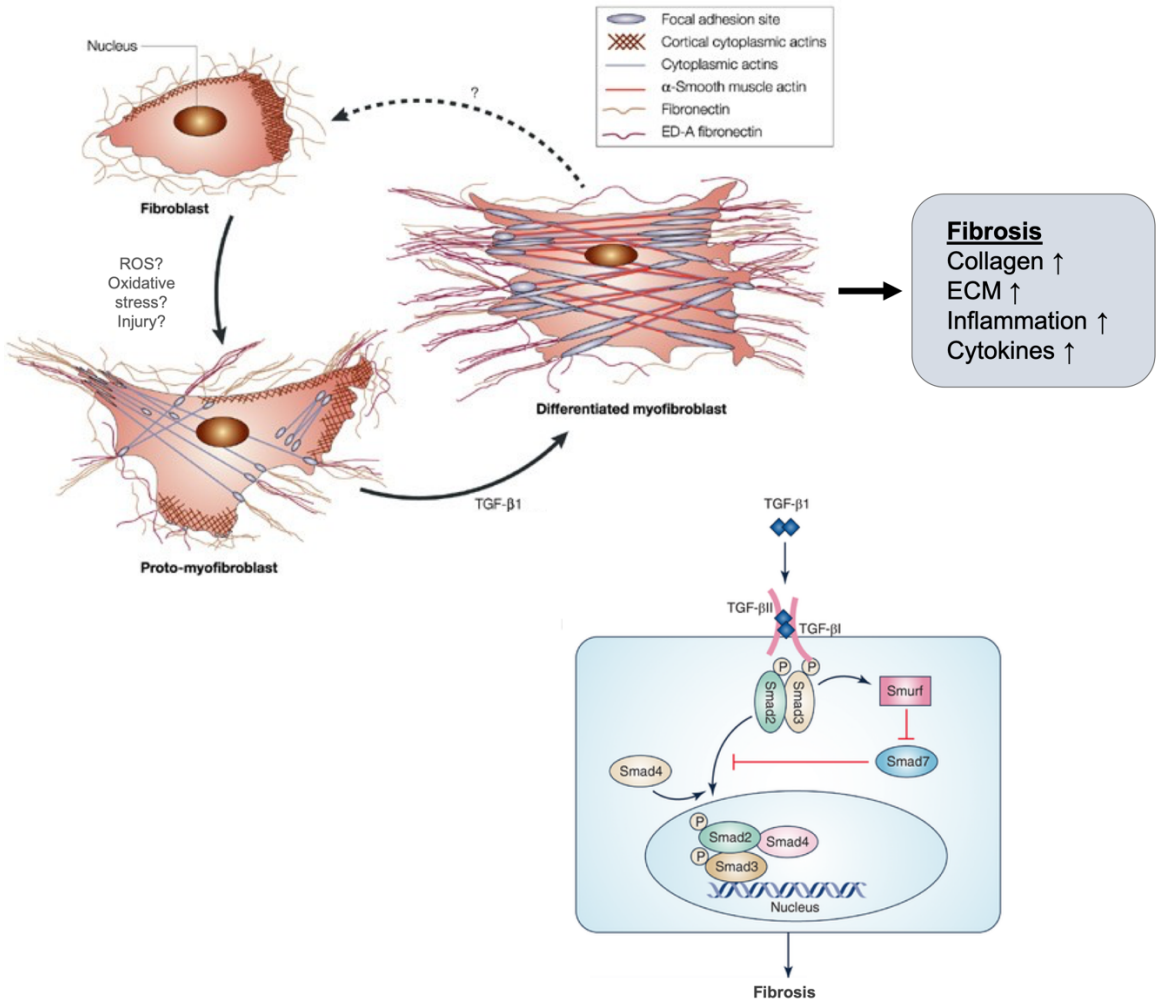

Figure 1:5 Fibrosis process. (figure modified and adapted from Tomasek et al.[48] and Tang et al.[49])

Post-prostatectomy ED

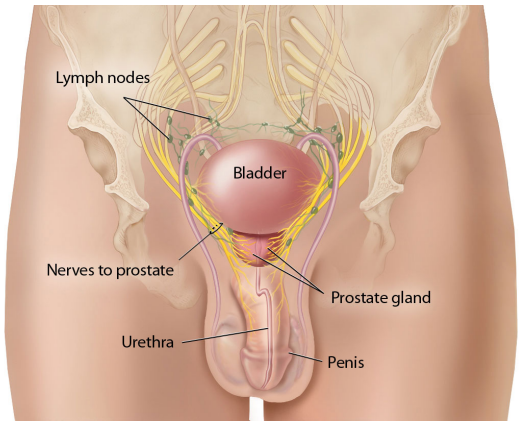

Figure 1:6 Schematic representation of the male pelvic anatomy showing the bladder, the prostate, the penis and the nerve plexus.
(Modified Image from National Cancer Institute (NCI), NIH Medical Arts, 2007)

The prostate is a walnut-sized gland located between the bladder and the penis (Figure 1.6). The urethra runs through the centre of the gland, from the bladder to the penis. The prostate secretes fluid that nourishes and protects sperm cells, expelled during ejaculation as semen. Prostate cancer is the most common cancer found in men[50], and despite the fact that prostate cancer remains the second leading cause of cancer deaths[51,52], it is the cancer with highest rate of survival, mostly due to the radical prostatectomy surgery, which is a gold-standard treatment. Over 450'000 radical prostatectomies are performed annually in the EU and US[37,53,54]. This surgical procedure leads to ED due to the inevitable damage to the neural pathway triggering penile erection[55]. The human cavernous nerves travel from the pelvic-plexus, a highly interconnected and twisted network with microscopic nerves[56], to the penile tissue through a complex anastomosis. Moreover, the fine nerve fibres responsible for penile erection are intimately located around the prostate[56–58] (Figure 1.7), and may consequently become damaged during surgery. A significant anatomic variability in the location of the cavernous nerve, as well as each patient's specific anatomy, disease stage, and cancer location, are factors increasing the risk of nerve damage during the surgery. The neural injury is thought to be caused by the mechanical stretching of the nerves that may occur during prostate retraction, thermal damage due to electrocautery, ischemia of the nerves as a result of the disruption of blood supply while attempting to control surgical bleeding, and local inflammatory effects associated with surgical trauma[59–61]. Surgeons often attempt to perform nerve-sparing surgery, however, even with meticulous dissection, some degree of nerve damage is inevitable because of the close proximity of the nerves with the prostate gland[62]. As a result, up to 70% of patients undergoing prostatectomy will develop ED[37,63,64]. Assisted-robotic surgery emerged as superior method for prostatectomy. Indeed, this method delivers several beneficial outcomes, yet despite the industry claim of improved cavernosal nerve preservation, studies point out minor to no improvement of erectile function following this method when compared to open surgery[65].

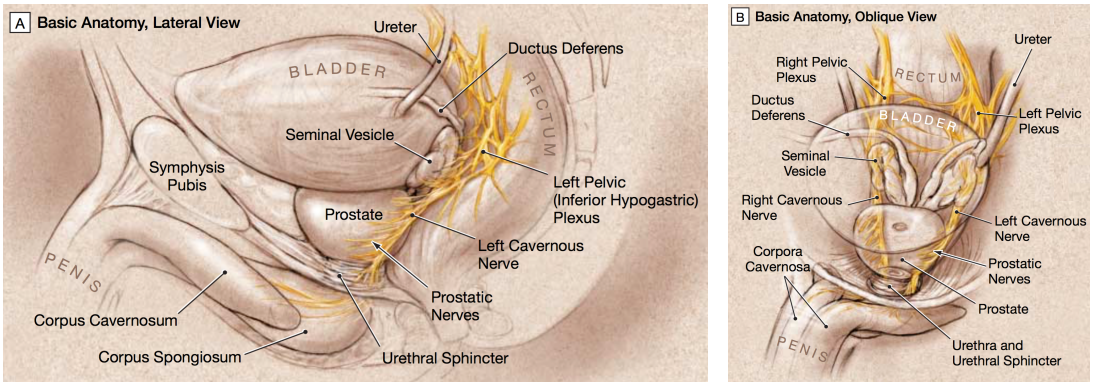

Figure 1:7 Illustration of the neuronal path for erection in the pelvic region.
Left image (A) shows the lateral view of the course and distribution of the left cavernous nerve fibres. Right image (B) shows the antero-superior oblique view of the same anatomical structures. In both images the nerves and pelvic plexus are shown in yellow colour.
(Figure adapted from Burnett et al [66])

Due to peripheral nerve regeneration and regrowth, erectile functions (EF) can be naturally recovered in some patients after prostatectomy[67]. However, long periods of EF loss, which may last a few months to years[61], can lead to irreversible vascular ED condition. Prolonged absence of penile erection leads to chronic penile hypoxia and consequent fibrogenic cytokine production. This local intrapenial environment can result in apoptosis and increased collagen production, altering the cavernosal structures[68]. As nocturnal autonomous or voluntary erection is important to allow blood flow renewal and appropriate oxygenation of the erectile tissues[69,70], prolonged inability to achieve an erection makes EF recovery to previous baseline difficult, and requires the need for faster nerve regeneration and penile rehabilitation therapy during this time-lapse[61,71].

Erection rehabilitation (sometimes named penile rehabilitation) emerged recently as an approach to protect erectile structures after prostatectomy[67]. Its concept is defined as the use of any medical intervention or combination, at the time of or after prostatectomy, with the goal to increase in penile blood flow and improve the intracorporal oxygenation to avoid or minimize fibrosis until the natural erectile function is recovered[61]. This concept has not been clearly proven to be a viable option as, collectively, clinical trials reported minor to no improvement using current tools[67,72–76]. Nevertheless, the scientific community strongly supports the penile rehabilitation concept, pointing out the need for better tools and strategies to provide efficient penile rehabilitation, neural regeneration, and prevent penile structure alteration following pelvic surgery.

1.4 Current Treatments of Erectile Dysfunction

Until the mid-1970s, psychological factors were considered to be the main etiology for ED[7]. Later on, with the development of novel diagnostic strategies and research expansion, it has become clear that most cases of ED are organogenic[77]. Nowadays, it is well accepted that the main mechanisms responsible for ED include failure in the neuronal response/transmission, increased tone/contractility of the smooth muscle, and structural abnormalities of the erectile tissues[13,78].

Three lines of treatment with varying degrees of success are currently available (Figure 1.8). The first and preferred treatments are oral pharmacological therapies. The most prominent of them being isozyme-selective inhibitors of phosphodiesterase 5 (e.g. sildenafil, Viagra®; tadalafil, Cialis® or vardenafil, Levitra®)[7] which are efficient for the majority of ED patients. However, they show low effectiveness for severe ED, or ED resulting from prostatectomy or others causes associated with failure in the neuronal response[63]. Patients, who cannot receive sildenafil therapy due to the adverse effect or contraindications, as well as non-responsive patients, often resort to the second and third-line of treatment.

1st Therapeutic Line

In the late 90's, a pioneering drug class for the treatment of male ED emerged, the phosphodiesterase-5 (PDE5) inhibitors (e.g.: sildenafil, Viagra®). These oral drugs showed high effectiveness and safety, and rapidly expanded into the clinic, revealing the size of the clinical problem than ED represented, which were masked by social conventions. PDE5 inhibitor administration was proven to be effective in up to 70% of the patients with ischemic heart disease and 52% in men with diabetes[5]. At the same time, more than 50% of ED patients taking PDE-5 inhibitors reported some level of treatment dissatisfaction[29]. They required, at least, an hour to exert effects after administration, and may have produced significant side effects including abnormal vision, flushing, headache, and diarrhea[79]. Such drugs potentiated the actions of NO[5] by inhibiting the enzyme phosphodiesterase type 5. PDE-5 is responsible for breaking down the intracellular second messenger cGMP generated by NO stimulus, which in turn regulates protein-dependent kinases responsible for smooth muscle cell relaxation[5]. Due to the dependency of PDE-5 inhibitors for the initial NO release by the cavernosal nerve terminals, patients with neurogenic ED were poorly-responsive to this therapy[6,13]. At the same time sildenafil and its analogs provided few benefits for men suffering from severe impotence, associated with strong deterioration of endothelial/smooth muscles function and/or pronounced structural abnormalities within the tissue[80,81].

In addition to oral therapies, several topical therapies have been explored as a potential treatment of ED, as this delivery system is a simple, safe, reversible, and relatively non-invasive alternative to the 2nd and 3rd lines of treatment, and would be considered for patients who are nonresponsive to oral PDE5 inhibitors or who have contraindications to their use. The most promising candidate for this type of administration have been prostaglandin E1 (alprostadil) combined with percutaneous absorption enhancer or permeation enhancer respectively (Topiglan®, MacroChem or Alprox-TD®, NexMed). When studied in cats and humans, topical alprostadil has been shown to induce a sustainable erection, presenting only minimal adverse systemic effects, such as transient penile oedema, genital pain, penile burning, and erythema[82,83]. The formulation, however, does not seem to be an effective treatment option as a

single agent, but rather may be of benefit when used in combination with other medications such as PDE5 inhibitors. Further clinical trials are needed to determine its importance in ED treatment and its approval worldwide[14].

2nd and 3rd Therapeutic Lines

The alternative therapies for patient's non-responsive to oral or topical treatments are unfortunately more invasive and less comfortable. The second most common therapeutic line is intrapenial injection of vasodilators, which produce direct smooth muscle relaxation and consequent erection, independently of the neural pathway[63,84]. Alprostadil (Prostaglandin E1, PGE1) is the most common vasodilator used for penile injection[85,86]. This drug binds to a specific receptor on smooth muscle cells and activates intracellular adenylate cyclase to produce cAMP, which in turn induces smooth muscle relaxation. When administrated directly into the erectile tissues, it is effective in over 80% of patients[85]. However, despite the high efficacy of this second-line treatment, its usage is often associated with high discontinuation rate caused by the necessity of the drug injection directly into the penis[6]. Local administration of the vasodilators into corpus cavernosum leads to adverse effects such as penile pain, prolonged erection (priapism), infection, subcutaneous haemorrhage, emergence of fibrotic nodules, scarring and penile fibrosis, which contributes to further deterioration of the erectile function[6,7,84,87,88]. In addition, this therapy is contraindicated in men with psychologic instability, history of priapism, history of unstable cardiac disease, coagulopathy, and use of monoamine oxidase inhibitors[14].

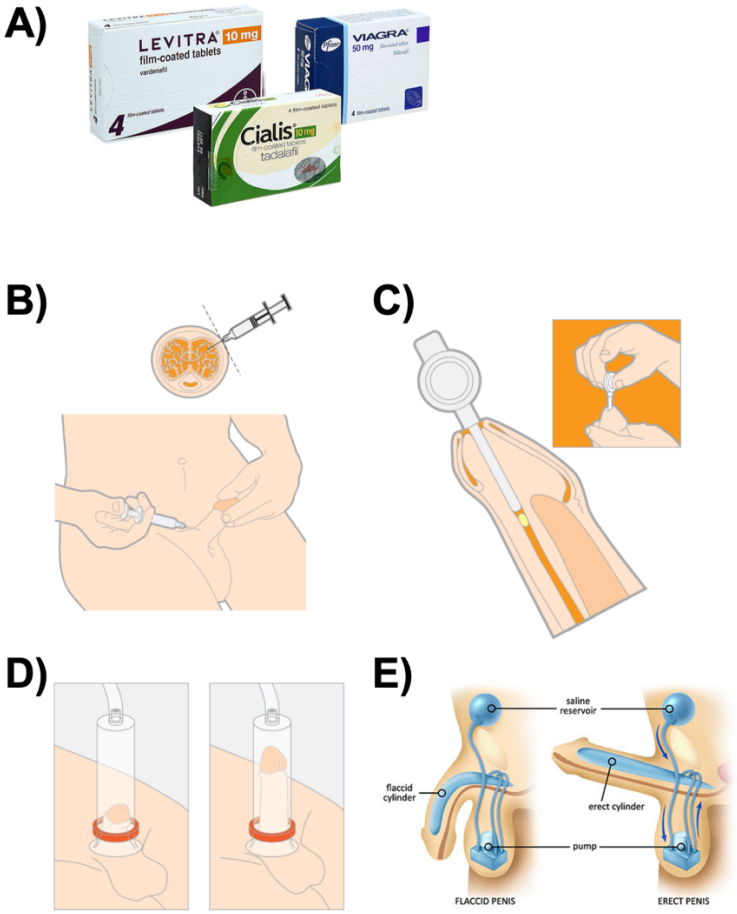

Figure 1:8 Therapeutic lines for ED. A) Oral treatment: PDE5 inhibitors. B) Intracavernosal injection. C) Intraurethral suppositories. D) Vacuum erection device. E) Inflatable Penile implant. (Obtained and modified from https://orchid-cancer.org.uk, https://www.zavamed.com and https://www.universityurology.com)

An alternative to intracavernosal injection is the use of intraurethral suppositories of alprostadil. Introduced in 1994 and currently known as MUSE® (Medicated Urethral System for Erection, Vivus), this system relies on absorption of the medication through the mucosa into the corpus spongiosum and then further diffusion into the corpus cavernosum[14]. Although the transurethral application

seems to be preferable to intracavernosal injection therapy, it provides limited efficacy, with about 50% erection response rate, and with limited successful responses resulting in sexual intercourse. Moreover, the treatment has frequent side effects such as pain, burning sensation or urethral bleeding[14]. Because of its inferiority with regard to PDE5 inhibitors and intracavernosal injection therapy, it is mostly offered as combination therapy in patients who are poor responders to single-agent therapy.

Patients who are non-responsive to pharmacological treatments may resort on the third line of therapies which consist of medical devices such as the vacuum erection devices (VED). VED promotes erection through creating a negative pressure (vacuum) around the penis, facilitating penile engorgement and subsequent erection. A constriction band is then placed at the base of the penis to prevent blood drainage and maintain the erection. The use of VED is safe and only minor adverse effects are reported, including hematomas or pain, while penis numbness or anejaculation is often reported due to the obstruction of the venous outflow and compression of the penis base by the constriction band[89]. Although the satisfaction rate of VED is high, patients often report erections that are not firm enough for sexual intercourse. This therapy is usually used in combination with oral treatments and/or as an erection rehabilitation tool in the attempt to increase the penile bloodflow and maintain a proper tissue oxygenation to avoid irreversible tissue remodelling following post-surgery ED[90]. As a last option, patients with severe erectile dysfunction currently resort to penile prosthesis[91]. This consists of a pair of malleable or inflatable rods surgically implanted within the erection chambers of the penis[92]. There are different types of penile prosthesis (rigid, semi-rigid or inflatable), all of them requiring an irreversible and destructive surgery with the risk of intra- and post-operative complications, such as infections[93], hematoma[94], penile shortening[7], device erosion and mechanical failure[7], frequently requiring surgical revision[95]. Nevertheless, implantation of penile prosthesis is a common procedure, as about 25'000 prostheses are implanted every year in the United-States and Europe, due to the lack of better alternatives[96,97].

1.5 Limitations of Current Treatments and Unmet Needs

Despite all the recent advances in understanding the molecular mechanisms and pathophysiology of ED and the discovery of PDE5 inhibitors, which allow the treatment of millions of patients, there is an important demographic of the population that is non responsive to the first line of oral treatment and therefore resort to problematic, uncomfortable, non-optimal and invasive 2nd and 3rd lines of treatment, especially patients with severe or neurogenic ED. Taking into account the limited number of strategies currently existing for the treatment of ED and their drawbacks, there is a clear unmet need for alternative therapies. In addition, current treatments are inducing assisted erection rather than handling the root cause of the disease. Due to the fact that both vascular and neural components are required to induce and maintain a satisfactory erectile response, as well as the multifactorial origins of ED, new therapies able to combine multidisciplinary approaches would increase the chances of treatment success, while focusing on providing more comfortable solutions for patients with neurogenic ED and aiming on alleviating the initial cause of ED.

1.6 Clinical and Pre-clinical Research in the Treatment of ED

Recently, restoring NO bioavailability by rejuvenating or regenerating cavernous endothelial cells, cavernous nerves, or inhibiting tissue fibrosis secondary to tissue hypoxia from neurovascular dysfunction, was found to be a promising therapeutic strategy for ED, especially in men with severe ED that are unresponsive to PDE5 inhibitors[47]. This section reviews the current state of research on ED treatment. Novel technologies and drug research are focusing mainly on the modulation of the central and peripheral control of erection, such as hormonal treatments or neuromodulation of the erectile function; the research on new potential vasoactive and/or anti-fibrotic compounds; or the neuro-regeneration of injured cavernous nerves.

1.6.1 Modulation of the central and peripheral control of erection

As PDE5 inhibitors are not suitable for all ED patients due to contraindications, side effects or limited responsiveness, researchers around the world have investigated different potential therapeutic targets of either the central or peripheral pathways controlling erection. In an attempt to induce or facilitate erection, multiple agonists of the central pro-erectile regulation of erection have been investigated. Among them, melatonin II and bremelanotide (PT-141), two melanocortin receptor agonist and ABT-670, a selective dopamine receptor agonist, have all shown successful induction of penile erection in animals or humans[98,99]. In addition, Clavulanic acid (zoraxel) improved erectile function in rats and humans through the upregulation of serotonin and dopamine[100]. Apomorphine, a central dopamine receptor agonist, produced a significantly durable erection when orally administered[101] and Trazadone, an atypical antidepresive agent that selectively inhibits central 5-HT uptake and increases the brain dopamine turnover, successfully induced penile erection in rats[102]. Phentolamine mesylate, a selective adrenergic receptor antagonist that inhibits corporal smooth muscle

contraction, facilitates penile erection in humans[103]. When administered orally, no severe side effects were shown, and it was concluded that phentolamine is a safe, well-tolerated, and efficacious treatment for mild to moderate ED. However, further comparative clinical trials are needed to determine its ability to be a competitive treatment alternative[14]. Similarly, peripherally acting agents, including guanylate cyclase activators (BAY60-4552), Rho-kinase inhibitors (SAR407899) or calcium-sensitive Maxi-K ion channel complementary DNA injection (hSlo cDNA), which all induce smooth muscle cell relaxation, have been shown to improve erectile function[47]. However, further development of these centrally and peripherally acting compounds have been interrupted due to failure in showing superiority to PDE5 inhibitors or to reproduce a beneficial effect in humans as observed in animal studies, and the sole recommended oral treatment for ED remains PDE5 inhibitors[31,47].

In addition to pharmacological compounds acting on the central and peripheral regulation of erection, scientific reports have shown that electrostimulation (ES) of the cavernous nerves can induce and maintain penile erection in animals and humans[104–108]. Since then, neurostimulation to activate the erectile response has been considered as a marvellous solution for ED, particularly for post-prostatectomy ED. However, despite the recognized potential, this technology has not been further developed. The main obstacle for this is the complex anatomy of human cavernous nerve. Locating the optimal site for stimulation has proven to be rather difficult, since the human cavernous nerve travels from the pelvic-plexus to the penis through a complex anastomosis and is not macroscopically visible, as already stated previously. Therefore, the identification of the cavernosal nerve segments for selective stimulation is fundamentally difficult. An intraoperative tool, called CaverMap™, which applies electrical stimulation to the pelvic nerves while monitoring changes in penile tumescence, was designed to map and identify the cavernous nerves during prostatectomy, allowing surgeons to perform optimal nerve sparing decisions[109,110]. Despite initial reports documenting a better rate of erectile function recovery after prostatectomy[110], CaverMap™ was never integrated by surgeons due to the extensive time added to the surgical procedure and the fact that, even with the nerve path identification, some degree of damage or neuropraxia is inevitable[111,112]. Nevertheless, the technique confirmed that ES of the cavernosal nerve within the pelvic plexus to evoke penile response is feasible. Therefore, in the frame of this thesis, we have investigated whether intra-operative ES of the CN in patients undergoing radical prostatectomy may induce a penile erection, providing a new strategy to avoid the requirement of precise CN identification. This research is presented and discussed intensively in the chapter 3.

1.6.2 Vasoactive and Anti-fibrotic compounds

Erection formation requires sufficient relaxation of the cavernosal vessels and large increase of the blood flow into the organ[13], which indicates that the cardiovascular system plays an important role in the maintenance of male sexual function. Indeed, occurrence of ED might indicate asymptomatic oncoming cardiovascular disorders[113]. A clear link between ED and cardiovascular disease has been uncovered, and both diseases were shown to share the same risk factors, including hypercholesterolemia, hypertension, diabetes mellitus, and smoking, with endothelial cell dysfunction being the common denominator between these two conditions[114]. The vascular and cavernous endothelium have a crucial role in regulating the tone of the underlying smooth muscle and physiologic penile erection[115]. Therefore, in the investigation of new ED treatment, it is reasonable to pay attention to the molecular pathways involved in the cardiovascular homeostasis and vascular structure. In addition, fibrosis from increased collagen synthesis is also an important cause of ED. An increased fibrosis of the penile structure has been associated to vaso-occlusive mechanism failure and subsequent ED[38].

Preclinical studies of angiogenic growth factor therapy, such as vascular endothelial growth factor (VEGF) and angiopoietins, have been extensively studied. Local intracavernous delivery of VEGF gene or protein has been shown to restore erectile function in rat models[116,117] . However, it was reported that VEGF administration by itself cannot promote the formation of mature and functional vessel in adult animals[118], which greatly limits the therapeutic utility of VEGF. Nonetheless, it was recently shown that the delivery of both angiopoietin-1 and VEGF into the corpus cavernosum produces an additive effect on erectile function compared with that of either therapy alone, promoting endothelial cell proliferation, eNOS phosphorylation, increasing cGMP expression and decreasing the production of ROS[119]. Similarly, adenovirus-mediated combined gene therapy of adrenomedullin, a vasoactive peptide that has vasorelaxant and proangiogenic activities, and angiopoietin-1 into the corpus cavernosum of diabetic mice produced an additive effect on erectile function by increasing the expression of vascular endothelial-cadherin and smooth muscle α-actin[120]. Although promising results have been achieved in animal models, difficulty in large-scale production of the recombinant protein and current restrictions on viral-mediated gene therapy limit the clinical application of such treatments[47].

As explained in section 1.3, TGF-β1 is considered the main pro-fibrotic cytokine responsible for activating the cellular pathway inducing the production and accumulation of collagen fibers. An increase in cavernous TGF-β1 expression and collagen synthesis has been reported in patients with ED[121]. Therefore, inhibition of TGF-β1 signaling pathway might be a potential strategy for the development of ED treatments. Smad7, an inhibitory Smad protein that blocks TGF-β1 signaling through a negative feedback loop, has emerged recently as a potential target. Indeed, Smad7 protein binds to TGF-β type I receptor, which prevent the phosphorylation of the Smad2

and Smad3 proteins, inhibiting further down-stream signaling[122] (Figure 1.5). It was recently reported that injection of an adenovirus encoding the Smad7 gene into the corpus cavernosum of a mouse model of cavernous nerve injury significantly decreased the production of fibronectin, collagen I, and collagen IV, decreased endothelial cell apoptosis and induced eNOS phosphorylation, enhancing the erectile function[123]. Alternatively, injection of PT144 peptide, a TGF-β1 antagonist known to have an anti-fibrotic effect in animal models of fibrotic diseases, was reported to decrease cavernous fibrosis and restored erectile function in rats[124]. These pre-clinical results represent a promising strategy to treat one of the major causes of ED, however, the safety and effectiveness of such treatment remains to be demonstrated in humans.

Apelin system is an important regulator of the cardiovascular function present in the heart and vasculature, modulating cardiovascular homeostasis by activating the G-protein-coupled receptor APJ[125–127]. Isoforms of Apelin peptide are potent inotrope effectors, peripheral vasodilators and regulators of vascular NO bioavailability and have therefor emerged as promising targets for the development of ED treatment[128]. Apelin precursor is secreted as a 77 amino acid, which is later cleaved to form several active peptides, denoted by their length as apelin-13, -16, -17, -19 and -36[129–131]. Among the apelin family, apelin-13 and apelin-17 exhibit stronger physiologic responses[129]. The role of Apelin–APJ system in cardiovascular physiology has not been fully elucidated. However, intravenous apelin administration in anesthetized rats evoked a dose-dependent transient drop in systolic and diastolic blood pressure without altering heart rate[132,133]. Such an effect was abrogated by pre-treatment with a nitric oxide synthase inhibitor[133]. In addition, apelin produced venous and arterial dilatation in conscious rodents. These initial experiments suggest that apelin is a peripheral vasodilator whose effects may be mediated by a NO dependent mechanism. Furthermore, Apelin appears to also regulate extracellular matrix content and vascular remodelling. For instance, Apelin was shown to inhibit TGF-β-mediated fibrotic response in cardiac fibroblasts and prevented their phenotypic switching to hyper-secretory myofibroblasts[134,135]. A significant restoration of erectile function was noted 24 hours after injection of apelin protein into the penis of hypercholesterolemic mice. However, erectile function returned to baseline values thereafter[136]. Although numerous observations indicate beneficial effects of apelin-APJ axis on the cardiovascular system, no clear investigation was carried out to determine its chronic role on erectile function and penile structure. Further investigation of Apelin-13 as a potential treatment for ED and its anti-fibrotic effect were therefor studied in a mouse model of hypercholesterolemia-induced vascular ED in the framework of this thesis, and are presented and discussed in more detail in the Chapter 2.

Vasoactive and/or anti-fibrotic compounds have shown promising results as potential treatments against ED and may potentially overcome the limitations of oral PDE5 inhibitors for some patients, however, no compound has yet made it through clinical evaluation and drug regulations into the clinic.

1.6.3 Neuro-Regenerative Strategies

Nerve injury results in a complex set of interactions between different cell types, the extracellular matrix, and a large array of intercellular signaling molecules[137]. After the peripheral nerve is damaged, the axon stumps retract and the distal stump undergoes Wallerian degeneration[138] (Figure 1.9). Following injury, the proximal segment swells and degenerates to the first node of Ranvier and multiple axonal sprouts arise. These axonal sprouts form a growth cone, which is guided by both physical and molecular cues[139]. The Schwann cells act as a guide through which regenerating axons grow towards their target end organ. They are important not only as a physical guide to growth, but also as a source of neurotrophic factors[140]. Neurotrophic factors include cytokines and constitutive growth factors (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and -4/5, ciliary neurotrophic factor, glial-derived neurotrophic factor, and acid and basic fibroblast growth factor) that interact during nerve regeneration. The neurotrophic factor's role is wide, and includes promoting cell survival, dendrite and axonal growth, and enhancement of axonal sprouting[141].

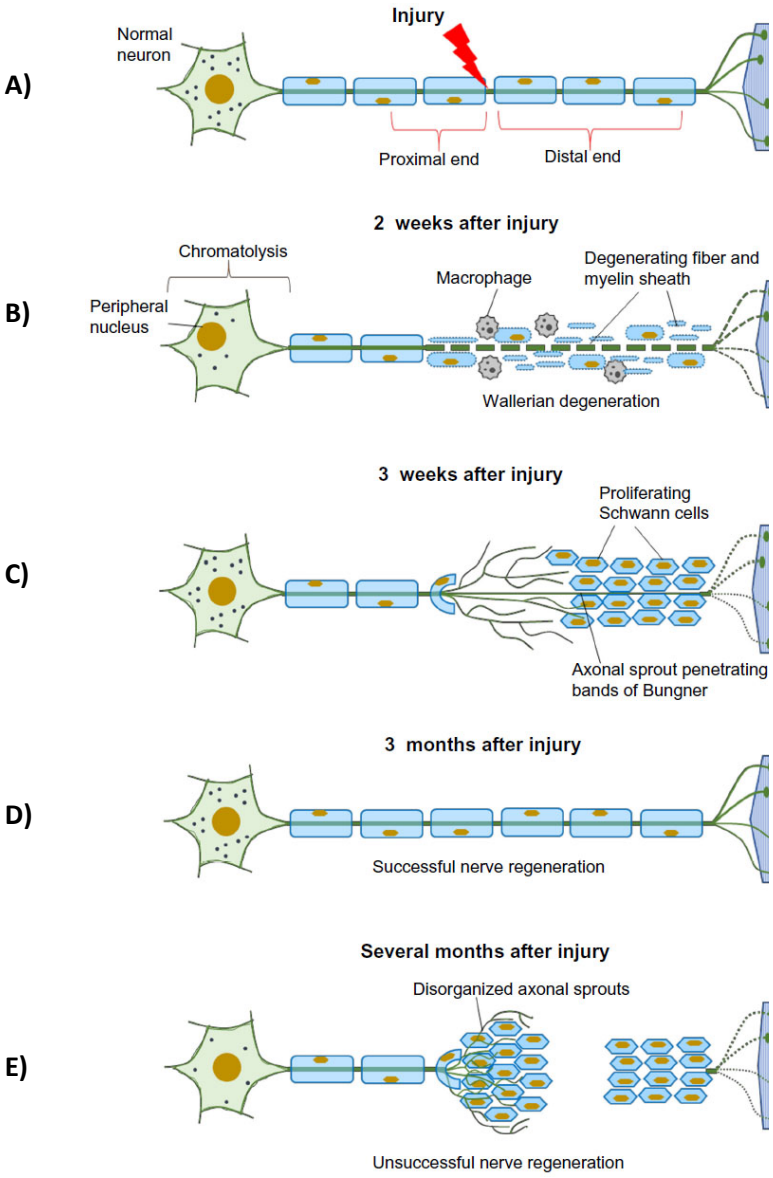

Figure 1:9 Nerge injury and regeneration process. A) Nerve injury. B) Wallerian degeneration of axon. C) New axonal sprouts and growth cone. D) Axonal sprout eventually reaches target organ and successfully regenerate. E) Failed regeneratin due to axonal sprout unable to reach target organ. (Modified from Arslantunali et al.[142])

Neuroregenerative and neuroprotective strategies offer the potential to restore a normal erectile function by restoring NO released from the cavernous nerve within erectile tissue, causing relaxation of the smooth muscles and subsequent penile erection. Several treatment modalities are currently under investigation in animal models, including immunophilin ligands, neurotrophins, sonic hedgehog (SHH), monoclonal antibody to nerve injury-induced protein 1 (Ninjurin-1), neurotrophic tyrosine kinase receptor type 1 (TrkA), and neuregulins growth factors[47]. Neurotrophins are a family of proteins that induce the survival, development, and function of neurons. Brain-derived nerve growth factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), and growth differentiation factor-5 (GDF-5) all demonstrated the ability to restore erectile function in animal models of ED[143]. After CNI, BDNF was shown to facilitate recovery of nNOS-containing nerve fibers and myelination of Schwann cells and thereby preserved erectile function[144]. Likewise, immunophilin ligands have been shown to promote recovery of erectile function in animals[145], however, it failed to induce a similar recovery in men undergoing nerve-sparing radical prostatectomy[143]. Neuregulins are a family of growth factors that play a crucial role in axoglial signaling during the development of the peripheral nervous system[146]. Recently, intracavernous administration of glial growth factor-2 (GGF-2) protein protected axonal integrity and preserved erectile function in a rat model of CNI[147]. Ninjurin-1, on the contrary, is known to influence neuro-inflammatory processes and have a negative impact

on peripheral nerve regeneration[148]. Recently, it was found that local inhibition of Ninjurin-1 by monoclonal Ninj1 antibody (Ninj1-mAb) significantly increased penile neuronal NOS (nNOS) and neurofilament contents, induced cavernous endothelial proliferation, phosphorylation of Akt and eNOS, and decreased endothelial cell apoptosis in mice. These findings suggest that dual neurotrophic and angiogenic effects of Ninjurin-1 blockage may provide a good opportunity for treating neurogenic ED[47]. Finally, erythropoietin (EPO) has also been investigated recently in the treatment of ED following nerve injury. EPO plays an important neuroprotective role by attenuating hypoxic damages, exert anti-apoptotic effects and activate BDNF, which is known to play a protective role in neurogenic ED[149].

Another promising regenerative strategy is the use of gene transfer therapy. It consists of transferring a gene to the target cells usually using a viral vector. A potential clinical advantage of gene therapy for the treatment of neurogenic ED is the opportunity to develop a patient-specific treatment[143]. Many different gene targets, focusing mainly on nerve regeneration, have been studied in animal models, however, due to the difficulties in developing a safe vector, none of these strategies have reached clinical trial in humans yet. While this topic will not be discussed further here, nonetheless, two recent review studies may provide additional information on the use of gene therapy for the treatment of ED[150,151].

Stem cell therapy (SCT) has emerged recently as a treatment option that has the potential to reverse the structural and neuronal causes of ED[152]. SCT is based on the ability of cells to differentiate into endothelial, neural or smooth muscle cells, thereby restoring normal tissue molecular signaling and architecture. In addition to their multipotent ability, SC are also known to secrete a wide range of paracrine factors such as anti-inflammatory and anti-fibrotic factors and to have an immunoregulatory effect on the immune system[153]. Multiple types of SC have been used in animal models of ED. Adipose tissue-derived stem cells (ADSC), bone marrow-derived SC, and mesenchymal SC have all been investigated in animal models of ED[152]. The functional outcomes in the pre-clinical studies consistently demonstrated an improvement in erectile function[152]. The beneficial structural changes observed in the penile tissue following SCT treatment include an increase in endothelial and smooth muscle cell markers, an increase in neural cell markers, a decrease in collagen content, and a decrease in CN cell apoptosis. Importantly, pre-clinical studies have established that treatment with intracavernosal SCT is safe in animal models, while recent advances in SCT research have allowed clinical trials in men following RP, leading to promising preliminary results[154,155]. However, phase II clinical trials with longer follow-up periods and with more patients are needed to further evaluate this exciting application. Combination therapy including SCT, gene therapy, growth factors or central regulation can possibly become a standard ED treatment in the future.

It has been demonstrated recently that low frequency electro-stimulation (LIES), similar to physiological neural mean firing frequency (20Hz), accelerate and enhance peripheral nerve regeneration[156–159]. The underlying mechanisms are still not fully elucidated, but may involve the recruitment and guiding of Schwann cell migration[160], the induction of neurotrophic factors expression and secretion[161], the facilitation of axon residue elimination from the Wallerian degeneration and speed up of the axonal growth[158], and/or provide a direct electrical guidance to the new growing axonal sprouts facilitating and fastening the regeneration of the neural function[162]. Additional to the action of evoking and maintaining penile erection for sexual intercourse and benefits on penile rehabilitation as discussed previously on section 1.6.1, LIES may then assist the cavernous nerve regeneration following nerve injury. This concept has been demonstrated to improve peripheral nerve regeneration in different anatomical areas[157–159,162–166], however, it has never been investigated for the cavernous nerve. Therefore, in the framework of this thesis, the effects of LIES on erectile function and tissue structure have been investigated in a rat model of nerve injury ED. The details and results of this research are presented and discussed in more detail in the chapter 4.

1.7 Animal Models of Erectile Dysfunction

Due to the difficulty in obtaining human penile samples and the multicausal origin of ED, as well as for the initial investigations of novel treatment safety and effectiveness, a number of animal models of ED have been developed. Other than larger animals such as cats, dogs and monkeys, rodent models have become the predominant choice and have been extensively used for obvious economic and practical reasons[167]. Likewise, mice rapid reproduction and life cycle also allowed the development of genetically modified models allowing to implement human-like diseases. In this thesis, two rodent models of ED have been used; a mice model of hypercholesterolemia-induced vascular ED and a bilateral cavernous nerve injury (BCNI) rat model.

1.7.1 Vasculogenic ED Model

Hypercholesterolemia is largely known to induce ED[168]. This condition impairs penile erection mainly due to increased oxidative stress within the corpus cavernosum[115]. Moreover, in the long-term, it may induce remodelling of the corpus cavernosum, impairing the mechanical properties of the penis[36,169]. In chapter 2 of this thesis, a well-established model of hypercholesterolemia inducing ED was used[170]: the Apolipoprotein E gene deleted ($ApoE^{-/-}$) mouse model of atherosclerosis. Experimental studies of hyperglycemia-induced vascular lesions in rodents began in the mid-50's with the feeding of cholesterol and cholate-rich diets in rats[171]. In 1992, Andrew et al.[172] and Zang et al.[173] independently described an Apolipoprotein E deficient mouse ($ApoE^{-/-}$), which developed atheromatous plaques even when fed standard chow diets. Though atherosclerosis still develops when $ApoE^{-/-}$ mice are fed standard foods, its development is accelerated with a Western-type diet. This model has been extensively used for basic research of atherosclerosis and hyper-cholesterolemia-induced ED. Indeed, this mouse model presents erection deficiency, which has been linked to an increased oxidative stress, reduction of NO release and exaggerated collagen deposition within the corpus cavernosum and penile arteries[170,174]. Therefore, this model exhibits a phenotype closely related to the human ED associated with hypercholesterolemia[168,175].

1.7.2 Neurogenic ED Model

To investigate the effects of LIES on the CN regeneration and penile structure, presented in chapter 4 of this thesis, a well-established rat model of nerve-sparing prostatectomy inducing neurogenic ED was used. First developed by Quinlan et al.[176], this model was then optimized by Martinez et al.[177] and Mills et al.[178] by introducing the measurement of the intracevernosal pressure (ICP) and the normalization of the ICP measurement by the mean arterial pressure in order to generate reproducible data, while taking into account animal inter-variability and effects of blood pressure on the EF. Different techniques causing nerve injury have been investigated previously, such as nerve transection, freezing, crush with a bulldog clamp or a fine hemostat, with all leading to a reproducible and consistent damage resulting in ED[179,180]. However, bilateral CN crush injury is considered to best represent nerve-sparing RP, along with inducing neuropraxia and ED, while not being as severe as the nerve transection which better represents a non-nerve-sparing surgery[180]. BCNI has been shown to induce neuropraxia, causing CC hypoxia and inflammation, as well as leading to erectile tissue modifications such as fibrosis and loss of smooth muscle cells in rats[181–184]. CN injury was also seen to induce apoptosis of nNOS positive ganglion in rat MPG[185]. In accordance, similar histological changes were observed in human penis after RP[182], endorsing the reliability of the animal model selected here.

1.8 Aim of the Thesis

As seen in this introduction, ED is a multicausal disease with many different etiologies and associated risk factors. Three main lines of treatment are currently available; however, a significant part of the patient population poorly responds to, or has no response to the first line oral therapies. Resorting on the second and third therapeutic lines is correlated with problematic side effects and is often associated with treatment discontinuation. In addition, none of the therapeutic lines currently treat the root cause of the disease and those that provide an assisted erection will most likely be needed by the patient for their entire lifetime. As the extensive amount of ongoing research in the field points out, there is a clear need for better therapies, especially those that can tackle the root causes of the disease and can provide better solutions for patients not responding to PDE5 inhibitors.

The goal of this thesis is to provide a multidisciplinary approach to manage the problem that ED represents for patients who are non-responsive to PDE5 inhibitors and to investigate or develop novel solutions. ED incidence is closely correlated to aging, yet since little can be done about the increasingly aging population worldwide, this thesis focuses primarily on hypercholesterolemia induced ED and neurogenic ED as a result of pelvic surgery. Nevertheless, the research presented in this thesis could potentially be applied to the multiple causes of ED including ageing, and provide a better understanding of ED and ways to prevent or mitigate its causes. This research also aims to add to the development of more effective and comfortable treatment options which would not only improve quality of life of patients, but would also reduce the burden and cost it places on medical and public health systems[14].

1.9 Summary of Chapters

As pointed out by this introduction, there is a clear need for the development of novel strategies and treatment against ED. Particularly, there is a need for multidisciplinary approaches able to tackle the multiple etiology and multicausal property of this disease. The first chapter of this thesis presents a general introduction of the field and current state of the research, while the following chapters present the research on different novel strategies investigated in this thesis. The second, third and fourth chapters of this

dissertation, summarized below, have been written as manuscripts that have been published in peer-reviewed scientific journals, have been submitted, or are in preparation for publication.

Chapter2: Apelin-13 Protects Corpus Cavernosum against Fibrosis induced by High-Fat Diet in an MMP-dependent mechanism

An increased fibrosis of the corpora cavernosa is a prevalent process that underlies most cases of erectile dysfunction ED. Apelin, an endogenous circulating peptide, has been documented as an important effector on cardiovascular homeostasis, controlling vascular function and reducing fibrosis in multiple pathological conditions. Recently, initial studies have shown that Apelin, acting through the APJ receptor, also modulates penile erection, however, the role of this system on penile structure and intracorporal collagen remodelling has not yet been investigated. In this work, we are investigating the effect of chronic Apelin treatment on the corpus cavernosum structure of hypercholesterolemic mice, using Apolipoprotein gene-deleted ($ApoE^{-/-}$) mice fed with a Western diet that received intraperitoneal injection of Apelin-13 or vehicle during the last 3 weeks to evaluate the potential protective effects against fibrosis of this peptide. Furthermore, the molecular effects of Apelin-13 were evaluated in cultured NIH3T3 mouse fibroblasts stimulated with TGF-β.

Interestingly, three weeks of Apelin-13 treatment strongly reduced intracavernosal collagen content. Additionally, in cultured fibroblast, Apelin-13 inhibited TGF-β-induced fibroblast to myofibroblast differentiation and collagen production, possibly through the activation of ERK1/2 kinase. These results suggest an essential protective role of Apelin, indicating Apelin/APJ system as a promising candidate for the development of fibrosis-associated ED treatments.

Chapter3: Novel Concept Enabling an Old Idea: A Flexible Electrode Array to Treat Neurogenic Erectile Dysfunction

Many studies have shown that electrostimulation of the cavernosal nerve can induce and maintain penile erection. Based on these discoveries, neurostimulation to activate the erectile response has been considered a potential solution to treat erectile dysfunction (ED). However, despite recognized potential, this technology has not been further developed. The barrier with this being the complex anatomy of the human cavernous nerve, which challenges the intraoperative identification of the cavernosal nerves for placement of the electrodes. In this work, to overcome this major barrier, we have developed a practical solution: a 2-dimensional flexible electrode array which can cover the entire plexus area, ensuring that at least one of the electrodes will be in optimal contact with the cavernosal nerve, without the need of intraoperative identification. A significant time of the PhD studies from which the results are presented here has been spent in the development, prototyping, manufacturing and validation of the biocompatible multi-electrode arrays (Figure 1.10) that have consecutively been used to evaluate this concept intraoperatively. The electrode arrays developed have been positioned on the pelvic nerve plexus of twenty-four patients enrolled for open radical prostatectomy and electrical stimulation was applied to induce penile erection.

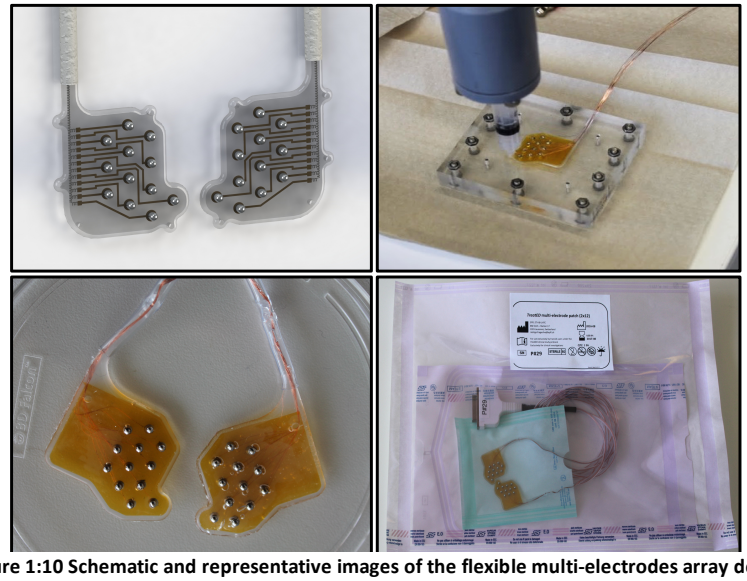

Figure 1:10 Schematic and representative images of the flexible multi-electrodes array developped.
Schematic design of the electrodes array for bilateral stimulation (Top left), injection of the silicone during the manufacturing process (Top right), representative image of the 2x12 electrodes array (Bottom left) and picture of the electrodes array cleaned and packed before sterilization by EtO (Bottom right).

Electrical stimulation produced immediate penile response in all patients with different levels of penile engorgement. These data bring the first proof of concept that the flexible electrode array concept can ensure the electro-stimulation of erectogenic neuronal path in men without the need of extensive nerve localization, and bring hope for the development of a novel biomedical implant for the treatment of ED caused by mechanical nerve injury such as prostatectomy.

Chapter 4: Low-intensity electrostimulation enhances neuroregeneration and improves erectile function in a rat model of cavernous nerve injury

In this last work, following the study on ES to induce on-demand penile erection in men, we have evaluated whether LIES can improve erectile function and prevent penile tissue remodelling induced by CN injury in a rat model of nerve-sparing prostatectomy, and explored the potential underlying molecular mechanisms. Bilateral cavernous nerve injury (BCNI) was induced by crushing the CN on treated and control animals, while nerve bundles of sham animals were exposed but kept intact. A bipolar electrode was then implanted on each CN bundle. LIES was applied daily for 1 hour over 7 days on the treated group. After that, EF was assessed by measuring the continuous intra-cavernosal pressure in response to electrical stimulation. Penises and cavernous nerves were collected for biochemical and histological analysis.

LIES treatments significantly enhanced EF recovery after 7 days compared to the control group, while treated rats showed a reduction in intra-cavernosal oxidative stress, a reduction of inflammation markers and pro-inflammatory cytokines, and a preservation of nitrergic CN fibres and myelination, leading to the protection of intra-cavernosal smooth muscle cell content and reduction of fibrosis compared to the control. This indicates a protective effect of LIES in the preservation of corporal cavernosa and CN function and structure.

This study shows the first evidence of the beneficial effects of LIES on EF recovery, corporal remodelling and CN structure protection following nerve injury, indicating LIES as potential new tool for penile rehabilitation and ED management following radical prostatectomy, minimizing the side effects of this important surgery.

1.10 References

(1) NIH. Erectile Dysfunction https://www.niddk.nih.gov/health-information/health-topics/urologic-disease/erectile-dysfunction/Pages/facts.aspx (accessed Aug 22, 2016).

(2) Aytaç; Mckinlay; Krane. The Likely Worldwide Increase in Erectile Dysfunction between 1995 and 2025 and Some Possible Policy Consequences. BJU Int. 1999, 84 (1), 50–56. https://doi.org/10.1046/j.1464-410x.1999.00142.x.

(3) Kalaitzidou, I.; Venetikou, M. S.; Konstadinidis, K.; Artemiadis, A. K.; Chrousos, G.; Darviri, C. Stress Management and Erectile Dysfunction: A Pilot Comparative Study. Andrologia 2014, 46 (6), 698–702. https://doi.org/10.1111/and.12129.

(4) Cuenca, A. I. C.; Sampietro-Crespo, A.; Virseda-Chamorro, M.; Martín-Espinosa, N. Psychological Impact and Sexual Dysfunction in Men with and without Spinal Cord Injury. J. Sex. Med. 2015, 12 (2), 436–444. https://doi.org/10.1111/jsm.12741.

(5) Rotella, D. P. Phosphodiesterase 5 Inhibitors: Current Status and Potential Applications. Nat. Rev. Drug Discov. 2002, 1 (9), 674–682. https://doi.org/10.1038/nrd893.

(6) Hatzichristou, D. G.; Apostolidis, A.; Tzortzis, V.; Ioannides, E.; Yannakoyorgos, K.; Kalinderis, A. SILDENAFIL VERSUS INTRACAVERNOUS INJECTION THERAPY: EFFICACY AND PREFERENCE IN PATIENTS ON INTRACAVERNOUS INJECTION FOR MORE THAN 1 YEAR. J. Urol. 2000, 164 (4), 1197–1200. https://doi.org/10.1016/S0022-5347(05)67140-6.

(7) Ali, W.; Besarani, D.; Kirby, R. Review: Modern Treatment of Erectile Dysfunction. Br. J. Diabetes Vasc. Dis. 2002, 2 (4), 255–261. https://doi.org/10.1177/14746514020020040301.

(8) Egydio, P. H.; Kuehhas, F. E. Treatments for Fibrosis of the Corpora Cavernosa. Arab J. Urol. 2013, 11 (3), 294–298. https://doi.org/10.1016/j.aju.2013.05.004.

(9) Fazio, L.; Brock, G. Erectile Dysfunction: Management Update. CMAJ 2004, 170 (9), 1429–1437.

https://doi.org/10.1503/cmaj.1020049.

(10) Dean, R. C.; Lue, T. F. Physiology of Penile Erection and Pathophysiology of Erectile Dysfunction. Urol. Clin. North Am. 2005, 32 (4), 379–395.

(11) Gratzke, C.; Angulo, J.; Chitaley, K.; Dai, Y.; Kim, N. N.; Paick, J.-S.; Simonsen, U.; Ückert, S.; Wespes, E.; Andersson, K. E.; others. Anatomy, Physiology, and Pathophysiology of Erectile Dysfunction. J. Sex. Med. 2010, 7 (1pt2), 445–475.

(12) Fa, G.; O, R.; G, B.; A, J. Neural Control of Penile Erection. Urol. Clin. North Am. 1995, 22 (4), 747–766.

(13) Fraga-Silva, R. A.; Montecucco, F.; Mach, F.; Santos, R. A. S.; Stergiopulos, N. Pathophysiological Role of the Renin–Angiotensin System on Erectile Dysfunction. Eur. J. Clin. Invest. 2013, 43 (9), 978–985. https://doi.org/10.1111/eci.12117.

(14) Hawksworth, D. J.; Burnett, A. L. Pharmacotherapeutic Management of Erectile Dysfunction. Clin. Pharmacol. Ther. 2015, 98 (6), 602–610. https://doi.org/10.1002/cpt.261.

(15) Bitran, D.; Hull, E. M. Pharmacological Analysis of Male Rat Sexual Behavior. Neurosci. Biobehav. Rev. 1987, 11 (4), 365–389. https://doi.org/10.1016/s0149-7634(87)80008-8.

(16) Argiolas, A. Oxytocin Stimulation of Penile Erection. Pharmacology, Site, and Mechanism of Action. Ann. N. Y. Acad. Sci. 1992, 652, 194–203. https://doi.org/10.1111/j.1749-6632.1992.tb34355.x.

(17) Bertolini, A.; Gessa, G. L. Behavioral Effects of ACTH and MSH Peptides. J. Endocrinol. Invest. 1981, 4 (2), 241–251. https://doi.org/10.1007/BF03350461.

(18) El-Sakka, A. I.; Lue, T. F. Physiology of Penile Erection https://www.hindawi.com/journals/tswj/2004/491269/ (accessed Aug 2, 2020). https://doi.org/10.1100/tsw.2004.58.

(19) Haake, P.; Exton, M. S.; Haverkamp, J.; Krämer, M.; Leygraf, N.; Hartmann, U.; Schedlowski, M.; Krueger, T. H. C. Absence of Orgasm-Induced Prolactin Secretion in a Healthy Multi-Orgasmic Male Subject. Int. J. Impot. Res. 2002, 14 (2), 133–135. https://doi.org/10.1038/sj.ijir.3900823.

(20) Fa, Y.; L, J.; M, A.; G, C.; Am, I.; S, G.; M, M.; Cj, N.; S, P.; A, S.; R, T.; Jp, M.; Wj, H. Erectile Dysfunction. Nat. Rev. Dis. Primer 2016, 2, 16003–16003. https://doi.org/10.1038/nrdp.2016.3.

(21) Burnett, A. L.; Lowenstein, C. J.; Bredt, D. S.; Chang, T. S.; Snyder, S. H. Nitric Oxide: A Physiologic Mediator of Penile Erection. Science 1992, 257 (5068), 401–403. https://doi.org/10.1126/science.1378650.

(22) Hurt, K. J.; Musicki, B.; Palese, M. A.; Crone, J. K.; Becker, R. E.; Moriarity, J. L.; Snyder, S. H.; Burnett, A. L. Akt-Dependent Phosphorylation of Endothelial Nitric-Oxide Synthase Mediates Penile Erection. Proc. Natl. Acad. Sci. U. S. A. 2002, 99 (6), 4061–4066. https://doi.org/10.1073/pnas.052712499.

(23) Saenz de Tejada, I.; Kim, N.; Lagan, I.; Krane, R. J.; Goldstein, I. Regulation of Adrenergic Activity in Penile Corpus Cavernosum. J. Urol. 1989, 142 (4), 1117–1121. https://doi.org/10.1016/s0022-5347(17)39009-2.

(24) Sopko, N. A.; Hannan, J. L.; Bivalacqua, T. J. Understanding and Targeting the Rho Kinase Pathway in Erectile Dysfunction. Nat. Rev. Urol. 2014, 11 (11), 622–628. https://doi.org/10.1038/nrurol.2014.278.

(25) Küthe, A.; Wiedenroth, A.; Mägert, H.-J.; Ückert, S.; Forssmann, W. G.; Stief, C. G.; Jonas, U. EXPRESSION OF DIFFERENT PHOSPHODIESTERASE GENES IN HUMAN CAVERNOUS SMOOTH MUSCLE. J. Urol. 2001, 165 (1), 280–283. https://doi.org/10.1097/00005392-200101000-00079.

(26) Christ, G. J.; Brink, P. R.; Melman, A.; Spray, D. C. The Role of Gap Junctions and Ion Channels in the Modulation of Electrical and Chemical Signals in Human Corpus Cavernosum Smooth Muscle. Int. J. Impot. Res. 1993, 5 (2), 77–96.

(27) Johannes, C. B.; Araujo, A. B.; Feldman, H. A.; Derby, C. A.; Kleinman, K. P.; McKINLAY, J. B. INCIDENCE OF ERECTILE DYSFUNCTION IN MEN 40 TO 69 YEARS OLD: LONGITUDINAL RESULTS FROM THE MASSACHUSETTS MALE AGING STUDY. J. Urol. 2000, 163 (2), 460–463. https://doi.org/10.1016/S0022-5347(05)67900-1.

(28) Kubin, M.; Wagner, G.; Fugl-Meyer, A. R. Epidemiology of Erectile Dysfunction. Int. J. Impot. Res. 2003, 15 (1), 63–71. https://doi.org/10.1038/sj.ijir.3900949.

(29) Jannini, E. A.; Sternbach, N.; Limoncin, E.; Ciocca, G.; Gravina, G. L.; Tripodi, F.; Petruccelli, I.; Keijzer, S.; Isherwood, G.; Wiedemann, B.; Simonelli, C. Health-Related Characteristics and Unmet Needs of Men with Erectile Dysfunction: A Survey in Five European Countries. J. Sex. Med. 2014, 11 (1), 40–50. https://doi.org/10.1111/jsm.12344.

(30) Selvin, E.; Burnett, A. L.; Platz, E. A. Prevalence and Risk Factors for Erectile Dysfunction in the US. Am. J. Med. 2007, 120 (2), 151–157. https://doi.org/10.1016/j.amjmed.2006.06.010.

(31) Porst, H.; Burnett, A.; Brock, G.; Ghanem, H.; Giuliano, F.; Glina, S.; Hellstrom, W.; Martin-Morales, A.; Salonia, A.; Sharlip, I.; ISSM Standards Committee for Sexual Medicine. SOP Conservative (Medical and Mechanical) Treatment of Erectile Dysfunction. J. Sex. Med. 2013, 10 (1), 130–171. https://doi.org/10.1111/jsm.12023.

(32) Rosen, R. C.; Riley, A.; Wagner, G.; Osterloh, I. H.; Kirkpatrick, J.; Mishra, A. The International Index of Erectile Function (IIEF): A Multidimensional Scale for Assessment of Erectile Dysfunction. Urology 1997, 49 (6), 822–830. https://doi.org/10.1016/S0090-4295(97)00238-0.

(33) Seftel, A. D.; Sun, P.; Swindle, R. The Prevalence of Hypertension, Hyperlipidemia, Diabetes Mellitus and Depression in Men with Erectile Dysfunction. J. Urol. 2004, 171 (6, Part 1), 2341–2345. https://doi.org/10.1097/01.ju.0000125198.32936.38.

(34) Gur, S.; Peak, T.; Kadowitz, P.; Sikka, S.; Hellstrom, W. Review of Erectile Dysfunction in Diabetic Animal Models. Curr. Diabetes Rev. 2014, 10 (1), 61–73. https://doi.org/10.2174/1573399809666131126151024.

(35) Musicki, B.; Liu, T.; Lagoda, G. A.; Strong, T. D.; Sezen, S. F.; Johnson, J. M.; Burnett, A. L. Hypercholesterolemia-Induced Erectile Dysfunction: Endothelial Nitric Oxide Synthase (ENOS) Uncoupling in the Mouse Penis by NAD(P)H Oxidase. J. Sex. Med. 2010, 7 (9), 3023–3032. https://doi.org/10.1111/j.1743-6109.2010.01880.x.

(36) El-Sakka, A. I.; Yassin, A. A. Amelioration of Penile Fibrosis: Myth or Reality. J. Androl. 2010, 31 (4), 324–335. https://doi.org/10.2164/jandrol.109.008730.

(37) PENSON, D. F.; McLERRAN, D.; FENG, Z.; LI, L.; ALBERTSEN, P. C.; GILLILAND, F. D.; HAMILTON, A.; HOFFMAN, R. M.; STEPHENSON, R. A.; POTOSKY, A. L.; STANFORD, J. L. 5-YEAR URINARY AND SEXUAL OUTCOMES AFTER RADICAL PROSTATECTOMY: RESULTS FROM THE PROSTATE CANCER OUTCOMES STUDY. J. Urol. 2005, 173 (5), 1701–1705. https://doi.org/10.1097/01.ju.0000154637.38262.3a.

(38) El-Sakka, A. I. Reversion of Penile Fibrosis: Current Information and a New Horizon. Arab J. Urol. 2011, 9 (1), 49–55. https://doi.org/10.1016/j.aju.2011.03.013.

(39) Gonzalez-Cadavid, N. F. Mechanisms of Penile Fibrosis. J. Sex. Med. 2009, 6 (Supplement 3), 353–362. https://doi.org/10.1111/j.1743-6109.2008.01195.x.

(40) Behr-Roussel, D.; Bernabe, J.; Compagnie, S.; Rupin, A.; Verbeuren, T. J.; Alexandre, L.; Giuliano, F. Distinct Mechanisms Implicated in Atherosclerosis-Induced Erectile Dysfunction in Rabbits. Atherosclerosis 2002, 162 (2), 355–362.

(41) Yesilli, C.; Yaman, Ö.; Anafarta, K. Effect of Experimental Hypercholesterolemia on Cavernosal Structures. Urology 2001, 57, 1184–1188. https://doi.org/10.1016/S0090-4295(01)00974-8.

(42) Grosche, J.; Meißner, J.; Eble, J. A. More than a Syllable in Fib-ROS-Is: The Role of ROS on the Fibrotic Extracellular Matrix and on Cellular Contacts. Mol. Aspects Med. https://doi.org/10.1016/j.mam.2018.03.005.

(43) Kisseleva, T.; Brenner, D. A. Mechanisms of Fibrogenesis. Exp. Biol. Med. Maywood NJ 2008, 233 (2), 109–122. https://doi.org/10.3181/0707-MR-190.

(44) Hinz, B.; Gabbiani, G. Fibrosis: Recent Advances in Myofibroblast Biology and New Therapeutic Perspectives. F1000 Biol. Rep. 2010, 2. https://doi.org/10.3410/B2-78.

(45) Hinz, B.; Phan, S. H.; Thannickal, V. J.; Galli, A.; Bochaton-Piallat, M.-L.; Gabbiani, G. The Myofibroblast. Am. J. Pathol. 2007, 170 (6), 1807–1816. https://doi.org/10.2353/ajpath.2007.070112.

(46) Derynck, R.; Zhang, Y. E. Smad-Dependent and Smad-Independent Pathways in TGF-Beta Family Signalling. Nature 2003, 425 (6958), 577–584. https://doi.org/10.1038/nature02006.

(47) Ryu, J.-K.; Suh, J.-K.; Burnett, A. L. Research in Pharmacotherapy for Erectile Dysfunction. Transl. Androl. Urol. 2017, 6 (2), 207–215.

(48) Tomasek, J. J.; Gabbiani, G.; Hinz, B.; Chaponnier, C.; Brown, R. A. Myofibroblasts and Mechano-Regulation of Connective Tissue Remodelling. Nat. Rev. Mol. Cell Biol. 2002, 3 (5), 349–363. https://doi.org/10.1038/nrm809.

(49) Tang, P. M.-K.; Zhang, Y.-Y.; Mak, T. S.-K.; Tang, P. C.-T.; Huang, X.-R.; Lan, H.-Y. Transforming Growth Factor-β Signalling in Renal Fibrosis: From Smads to Non-Coding RNAs. J. Physiol. 2018, 596 (16), 3493–3503. https://doi.org/10.1113/JP274492.

(50) Shen, M. M.; Abate-Shen, C. Molecular Genetics of Prostate Cancer: New Prospects for Old Challenges. Genes Dev. 2010, 24 (18), 1967–2000. https://doi.org/10.1101/gad.1965810.

(51) Haas, G. P.; Delongchamps, N.; Brawley, O. W.; Wang, C. Y.; de la Roza, G. The Worldwide Epidemiology of Prostate Cancer: Perspectives from Autopsy Studies. Can. J. Urol. 2008, 15 (1), 3866–3871.

(52) Potosky, A. L.; Miller, B. A.; Albertsen, P. C.; Kramer, B. S. The Role of Increasing Detection in the Rising Incidence of Prostate Cancer. JAMA 1995, 273 (7), 548–552. https://doi.org/10.1001/jama.1995.03520310046028.

(53) Ferlay, J.; Steliarova-Foucher, E.; Lortet-Tieulent, J.; Rosso, S.; Coebergh, J. W. W.; Comber, H.; Forman, D.; Bray, F. Cancer Incidence and Mortality Patterns in Europe: Estimates for 40 Countries in 2012. Eur. J. Cancer Oxf. Engl. 1990 2013, 49 (6), 1374–1403. https://doi.org/10.1016/j.ejca.2012.12.027.

(54) Torre, L. A.; Bray, F.; Siegel, R. L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A. Global Cancer Statistics, 2012. CA. Cancer J. Clin. 2015, 65 (2), 87–108. https://doi.org/10.3322/caac.21262.

(55) McCullough, A. R. Sexual Dysfunction after Radical Prostatectomy. Rev. Urol. 2005, 7 (Suppl 2), S3–S10.

(56) Takenaka, A.; Murakami, G.; Matsubara, A.; Han, S. H.; Fujisawa, M. Variation in Course of Cavernous Nerve with Special Reference to Details of Topographic Relationships near Prostatic Apex: Histologic Study Using Male Cadavers. Urology 2005, 65 (1), 136–142. https://doi.org/10.1016/j.urology.2004.08.028.

(57) Alsaid, B.; Bessede, T.; Karam, I.; Abd-Alsamad, I.; Uhl, J.-F.; Benoît, G.; Droupy, S.; Delmas, V. Coexistence of Adrenergic and Cholinergic Nerves in the Inferior Hypogastric Plexus: Anatomical and Immunohistochemical Study with 3D Reconstruction in Human Male Fetus. J. Anat. 2009, 214 (5), 645–654. https://doi.org/10.1111/j.1469-7580.2009.01071.x.

(58) Yucel, S.; Erdogru, T.; Baykara, M. Recent Neuroanatomical Studies on the Neurovascular Bundle of the Prostate and Cavernosal Nerves: Clinical Reflections on Radical Prostatectomy. Asian J. Androl. 2005, 7 (4), 339–349. https://doi.org/10.1111/j.1745-7262.2005.00097.x.

(59) Burnett, A. L. Erectile Function Outcomes in the Current Era of Anatomic Nerve-Sparing Radical Prostatectomy. Rev. Urol. 2006, 8 (2), 47–53.

(60) Shaiji, T. A.; Domes, T.; Brock, G. Penile Rehabilitation Following Treatment for Prostate Cancer: An Analysis of the Current State of the Art. Can. Urol. Assoc. J. 2009, 3 (1), 37–48.

(61) Capogrosso, P.; Salonia, A.; Briganti, A.; Montorsi, F. Postprostatectomy Erectile Dysfunction: A Review. World J. Mens Health 2016, 34 (2), 73–88. https://doi.org/10.5534/wjmh.2016.34.2.73.

(62) Wang, R. Penile Rehabilitation after Radical Prostatectomy: Where Do We Stand and Where Are We Going? J. Sex. Med. 2007, 4 (4, Part 2), 1085–1097. https://doi.org/10.1111/j.1743-6109.2007.00482.x.

(63) Zippe, C. D.; Raina, R. Management of Erectile Dysfunction Following Radical Prostatectomy. In Management of Prostate Cancer; Springer, 2004; pp 431–458.

(64) Mulhall, J. P.; Morgentaler, A. Controversies in Sexual Medicine: Penile Rehabilitation Should Become the Norm for Radical Prostatectomy Patients. J. Sex. Med. 2007, 4 (3), 538–543. https://doi.org/10.1111/j.1743-6109.2007.00486.x.

(65) Moran, P. S.; O'Neill, M.; Teljeur, C.; Flattery, M.; Murphy, L. A.; Smyth, G.; Ryan, M. Robot-Assisted Radical Prostatectomy Compared with Open and Laparoscopic Approaches: A Systematic Review and Meta-Analysis. Int. J. Urol. 2013, 20 (3), 312–321. https://doi.org/10.1111/iju.12070.

(66) Burnett, A. L. Erectile Dysfunction Following Radical Prostatectomy. JAMA 2005, 293 (21), 2648–2653. https://doi.org/10.1001/jama.293.21.2648.

(67) Montorsi, F.; Guazzoni, G.; Strambi, L. F.; Da Pozzo, L. F.; Nava, L.; Barbieri, L.; Rigatti, P.; Pizzini, G.; Miani, A. Recovery of Spontaneous Erectile Function after Nerve-Sparing Radical Retropubic Prostatectomy with and without Early Intracavernous Injections of Alprostadil: Results of a Prospective, Randomized Trial. J. Urol. 1997, 158 (4), 1408–1410.

(68) Hatzimouratidis, K.; Burnett, A. L.; Hatzichristou, D.; McCullough, A. R.; Montorsi, F.; Mulhall, J. P. Phosphodiesterase Type 5 Inhibitors in Postprostatectomy Erectile Dysfunction: A Critical Analysis of the Basic Science Rationale and Clinical Application. Eur. Urol. 2009, 55 (2), 334–347. https://doi.org/10.1016/j.eururo.2008.10.028.

(69) Burnett, A. L. Rationale for Cavernous Nerve Restorative Therapy to Preserve Erectile Function after Radical Prostatectomy. Urology 2003, 61 (3), 491–497. https://doi.org/10.1016/S0090-4295(02)02271-9.

(70) Weyne, E.; Mulhall, J.; Albersen, M. Molecular Pathophysiology of Cavernous Nerve Injury and Identification of Strategies for Nerve Function Recovery after Radical Prostatectomy. Curr. Drug Targets 2015, 16 (5), 459–473.

(71) Nangle, M. R.; Keast, J. R. Reduced Efficacy of Nitrergic Neurotransmission Exacerbates Erectile Dysfunction after Penile Nerve Injury despite Axonal Regeneration. Exp. Neurol. 2007, 207 (1), 30–41. https://doi.org/10.1016/j.expneurol.2007.05.011.

(72) Padma-Nathan, H.; McCullough, A. R.; Levine, L. A.; Lipshultz, L. I.; Siegel, R.; Montorsi, F.; Giuliano, F.; Brock, G.; Study Group. Randomized, Double-Blind, Placebo-Controlled Study of Postoperative Nightly Sildenafil Citrate for the Prevention of Erectile Dysfunction after Bilateral Nerve-Sparing Radical Prostatectomy. Int. J. Impot. Res. 2008, 20 (5), 479–486. https://doi.org/10.1038/ijir.2008.33.

(73) Novara, G.; Ficarra, V. Re: Francesco Montorsi, Gerald Brock, Jay Lee, et al. Effect of Nightly versus on-Demand Vardenafil on Recovery of Erectile Function in Men Following Bilateral Nerve-Sparing Radical Prostatectomy. Eur Urol 2008;54:924-31. Eur. Urol. 2009, 55 (5), e95-96; author reply e97-98. https://doi.org/10.1016/j.eururo.2008.11.055.

(74) Pavlovich, C. P.; Levinson, A. W.; Su, L.-M.; Mettee, L. Z.; Feng, Z.; Bivalacqua, T. J.; Trock, B. J. Nightly vs On-Demand Sildenafil for Penile Rehabilitation after Minimally Invasive Nerve-Sparing Radical Prostatectomy: Results of a Randomized Double-Blind Trial with Placebo. BJU Int. 2013, 112 (6), 844–851. https://doi.org/10.1111/bju.12253.

(75) Montorsi, F.; Brock, G.; Stolzenburg, J.-U.; Mulhall, J.; Moncada, I.; Patel, H. R. H.; Chevallier, D.; Krajka, K.; Henneges, C.; Dickson, R.; Büttner, H. Effects of Tadalafil Treatment on Erectile Function Recovery Following Bilateral Nerve-Sparing Radical Prostatectomy: A Randomised Placebo-Controlled Study (REACTT). Eur. Urol. 2014, 65 (3), 587–596. https://doi.org/10.1016/j.eururo.2013.09.051.

(76) Mulhall, J. P.; Burnett, A. L.; Wang, R.; McVary, K. T.; Moul, J. W.; Bowden, C. H.; DiDonato, K.; Shih, W.; Day, W. W. A Phase 3, Placebo Controlled Study of the Safety and Efficacy of Avanafil for the Treatment of Erectile Dysfunction after Nerve Sparing Radical Prostatectomy. J. Urol. 2013, 189 (6), 2229–2236. https://doi.org/10.1016/j.juro.2012.11.177.

(77) Lue, T. F.; Mueller, S. C.; Jow, Y. R.; Hwang, T. I. Functional Evaluation of Penile Arteries with Duplex Ultrasound in Vasodilator-Induced Erection. Urol. Clin. North Am. 1989, 16 (4), 799–807.

(78) Nehra, A.; Goldstein, I.; Pabby, A.; Nugent, M.; Huang, Y. H.; de las Morenas, A.; Krane, R. J.; Udelson, D.; Saenz de Tejada, I.; Moreland, R. B. Mechanisms of Venous Leakage: A Prospective Clinicopathological Correlation of Corporeal Function and Structure. J. Urol. 1996, 156 (4), 1320–1329.

(79) Gresser, U.; Gleiter, C. H. Erectile Dysfunction: Comparison of Efficacy and Side Effects of the PDE-5 Inhibitors Sildenafil, Vardenafil and Tadalafil-Review of the Literature. Eur. J. Med. Res. 2002, 7 (10), 435–446.

(80) Martins, F. E.; Padma-Nathan, H. Diffuse Veno-Occlusive Dysfunction: The Underlying Hemodynamic Abnormality Resulting

in Failure to Respond to Intracavernous Pharmacotherapy. J. Urol. 1996, 156 (6), 1942–1946. https://doi.org/10.1016/S0022-5347(01)65400-4.

(81) Mulhall, J.; Barnas, J.; Aviv, N.; Anderson, M.; Parker, M. ORIGINAL RESEARCH—ED PHARMACOTHERAPY: Sildenafil Citrate Response Correlates with the Nature and the Severity of Penile Vascular Insufficiency. J. Sex. Med. 2005, 2 (1), 104–108. https://doi.org/10.1111/j.1743-6109.2005.20110.x.

(82) Padma-Nathan, H.; Yeager, J. L. An Integrated Analysis of Alprostadil Topical Cream for the Treatment of Erectile Dysfunction in 1732 Patients. Urology 2006, 68 (2), 386–391. https://doi.org/10.1016/j.urology.2006.02.027.

(83) Usta, M. F.; Sanabriav, J.; Bivalacqua, T. J.; Hellstrom, W. J. G. Feline Penile Erection Induced by Topical Glans Penis Application of Combination Alprostadil and SEPA (Topiglan). Int. J. Impot. Res. 2004, 16 (1), 73–77. https://doi.org/10.1038/sj.ijir.3901145.

(84) Leungwattanakij, S.; Flynn Jr, V.; Hellstrom, W. J. G. INTRACAVERNOSAL INJECTION AND INTRAURETHRAL THERAPY FOR ERECTILE DYSFUNCTION. Urol. Clin. North Am. 2001, 28 (2), 343–354. https://doi.org/10.1016/S0094-0143(05)70143-9.

(85) Harding, L. M.; Adeniyi, A.; Everson, R.; Barker, S.; Ralph, D. J.; Baranowski, A. P. Comparison of a Needle-Free High-Pressure Injection System with Needle-Tipped Injection of Intracavernosal Alprostadil for Erectile Dysfunction. Int. J. Impot. Res. 2002, 14 (6), 498–501.

(86) Eardley, I.; Donatucci, C.; Corbin, J.; El-Meliegy, A.; Hatzimouratidis, K.; McVary, K.; Munarriz, R.; Lee, S. W. Pharmacotherapy for Erectile Dysfunction. J. Sex. Med. 2010, 7 (1pt2), 524–540. https://doi.org/10.1111/j.1743-6109.2009.01627.x.

(87) Intracavernous Injections for Erectile Dysfunction in Patients with Cardiovascular Diseases and Failure or Contraindications for Sildenafil Citrate. Publ. Online 19 Febr. 2002 Doi101038sjijir3900812 2002, 14 (1). https://doi.org/10.1038/sj/ijir/3900812.

(88) Gupta, R.; Kirschen, J.; Barrow, R. C.; Eid, J. F. Predictors of Success and Risk Factors for Attrition in the Use of Intracavernous Injection. J. Urol. 1997, 157 (5), 1681–1686. https://doi.org/10.1016/S0022-5347(01)64834-1.

(89) Lehrfeld, T.; Lee, D. I. The Role of Vacuum Erection Devices in Penile Rehabilitation after Radical Prostatectomy. Int. J. Impot. Res. 2009, 21 (3), 158–164. https://doi.org/10.1038/ijir.2009.3.

(90) Sopko, N. A.; Burnett, A. L. Erection Rehabilitation Following Prostatectomy--Current Strategies and Future Directions. Nat. Rev. Urol. 2016, 13 (4), 216–225. https://doi.org/10.1038/nrurol.2016.47.

(91) Montague, D. K.; Angermeier, K. W. Penile Prosthesis Implantation. In Operative Urology at the Cleveland Clinic; MD, A. C. N., MD, J. S. J., MD, I. S. G., MD, I. S. G., MD, E. A. K., MD, R. R., MD, J. H. R., Eds.; Humana Press, 2006; pp 477–488. https://doi.org/10.1007/978-1-59745-016-4_46.

(92) Sadeghi-Nejad, H. Penile Prosthesis Surgery: A Review of Prosthetic Devices and Associated Complications. J. Sex. Med. 2007, 4 (2), 296–309.

(93) Katz, B. F.; Gaunay, G. S.; Barazani, Y.; Nelson, C. J.; Moreira, D. M.; Dinlenc, C. Z.; Nagler, H. M.; Stember, D. S. Use of a Preoperative Checklist Reduces Risk of Penile Prosthesis Infection. J. Urol. 2014, 192 (1), 130–135. https://doi.org/10.1016/j.juro.2013.12.044.

(94) Garber, B. B.; Bickell, M. Delayed Postoperative Hematoma Formation after Inflatable Penile Prosthesis Implantation. J. Sex. Med. 2015, 12 (1), 265–269. https://doi.org/10.1111/jsm.12728.

(95) Grewal, S.; Vetter, J.; Brandes, S. B.; Strope, S. A. A Population-Based Analysis of Contemporary Rates of Reoperation for Penile Prosthesis Procedures. Urology 2014, 84 (1), 112–116. https://doi.org/10.1016/j.urology.2014.02.028.

(96) Le, B.; Burnett, A. L. Evolution of Penile Prosthetic Devices. Korean J. Urol. 2015, 56 (3), 179–186. https://doi.org/10.4111/kju.2015.56.3.179.

(97) Darouiche, R. O.; Bella, A. J.; Boone, T. B.; Brock, G.; Broderick, G. A.; Burnett, A. L.; Carrion, R.; Carson III, C.; Christine, B.; Dhabuwala, C. B.; Hakim, L. S.; Henry, G.; Jones, L. A.; Khera, M.; Montague, D. K.; Nehra, A. North American Consensus

Document on Infection of Penile Prostheses. Urology 2013, 82 (4), 937–942. https://doi.org/10.1016/j.urology.2013.05.048.

(98) Wessells, H.; Levine, N.; Hadley, M. E.; Dorr, R.; Hruby, V. Melanocortin Receptor Agonists, Penile Erection, and Sexual Motivation: Human Studies with Melanotan II. Int. J. Impot. Res. 2000, 12 Suppl 4, S74-79. https://doi.org/10.1038/sj.ijir.3900582.

(99) Patel, M. V.; Kolasa, T.; Mortell, K.; Matulenko, M. A.; Hakeem, A. A.; Rohde, J. J.; Nelson, S. L.; Cowart, M. D.; Nakane, M.; Miller, L. N.; Uchic, M. E.; Terranova, M. A.; El-Kouhen, O. F.; Donnelly-Roberts, D. L.; Namovic, M. T.; Hollingsworth, P. R.; Chang, R.; Martino, B. R.; Wetter, J. M.; Marsh, K. C.; Martin, R.; Darbyshire, J. F.; Gintant, G.; Hsieh, G. C.; Moreland, R. B.; Sullivan, J. P.; Brioni, J. D.; Stewart, A. O. Discovery of 3-Methyl-N-(1-Oxy-3',4',5',6'-Tetrahydro-2'H-[2,4'-Bipyridine]-1'-Ylmethyl)Benzamide (ABT-670), an Orally Bioavailable Dopamine D4 Agonist for the Treatment of Erectile Dysfunction. J. Med. Chem. 2006, 49 (25), 7450–7465. https://doi.org/10.1021/jm060662k.

(100) Chan, J. S. W.; Kim, D. J.; Ahn, C. H.; Oosting, R. S.; Olivier, B. Clavulanic Acid Stimulates Sexual Behaviour in Male Rats. Eur. J. Pharmacol. 2009, 609 (1–3), 69–73. https://doi.org/10.1016/j.ejphar.2009.03.009.

(101) Heaton, J. P.; Morales, A.; Adams, M. A.; Johnston, B.; el-Rashidy, R. Recovery of Erectile Function by the Oral Administration of Apomorphine. Urology 1995, 45 (2), 200–206. https://doi.org/10.1016/0090-4295(95)80005-0.

(102) Ra, C.; M, S. Oral Trazodone Is Not Effective Therapy for Erectile Dysfunction: A Double-Blind, Placebo Controlled Trial. J. Urol. 1999, 161 (6), 1819–1822. https://doi.org/10.1016/s0022-5347(05)68813-1.

(103) Goldstein, I.; Carson, C.; Rosen, R.; Islam, A. Vasomax for the Treatment of Male Erectile Dysfunction. World J. Urol. 2001, 19 (1), 51–56. https://doi.org/10.1007/s003450000178.

(104) Burnett, A. L.; Lue, T. F. Neuromodulatory Therapy to Improve Erectile Function Recovery Outcomes After Pelvic Surgery. J. Urol. 2006, 176 (3), 882–887. https://doi.org/10.1016/j.juro.2006.04.020.

(105) Lue, T. F.; Schmidt, R. A.; Tanagho, E. A. Electrostimulation and Penile Erection. Urol. Int. 1985, 40 (1), 60–64. https://doi.org/10.1159/000281034.

(106) Lue, T. F.; Gleason, C. A.; Brock, G. B.; Carroll, P. R.; Tanagho, E. A. Intraoperative Electrostimulation of the Cavernous Nerve: Technique, Results and Limitations. J. Urol. 1995, 154 (4), 1426–1428.

(107) Burnett, A. L.; Teloken, P. E.; Briganti, A.; Whitehurst, T.; Montorsi, F. Intraoperative Assessment of an Implantable Electrode Array for Cavernous Nerve Stimulation. J. Sex. Med. 2008, 5 (8), 1949–1954. https://doi.org/10.1111/j.1743-6109.2008.00865.x.

(108) Shafik, A. Extrapelvic Cavernous Nerve Stimulation in Erectile Dysfunction. Human Study. Andrologia 1996, 28 (3), 151–156. https://doi.org/10.1111/j.1439-0272.1996.tb02774.x.

(109) Klotz, L.; Herschorn, S. Early Experience with Intraoperative Cavernous Nerve Stimulation with Penile Tumescence Monitoring to Improve Nerve Sparing during Radical Prostatectomy. Urology 1998, 52 (4), 537–542. https://doi.org/10.1016/S0090-4295(98)00319-7.

(110) KLOTZ, L.; HEATON, J.; JEWETT, M.; CHIN, J.; FLESHNER, N.; GOLDENBERG, L.; GLEAVE, M. A RANDOMIZED PHASE 3 STUDY OF INTRAOPERATIVE CAVERNOUS NERVE STIMULATION WITH PENILE TUMESCENCE MONITORING TO IMPROVE NERVE SPARING DURING RADICAL PROSTATECTOMY. J. Urol. 2000, 164 (5), 1573–1578. https://doi.org/10.1016/S0022-5347(05)67031-0.

(111) Kim, H. L.; Stoffel, D. S.; Mhoon, D. A.; Brendler, C. B. A Positive Caver Map Response Poorly Predicts Recovery of Potency after Radical Prostatectomy. Urology 2000, 56 (4), 561–564. https://doi.org/10.1016/S0090-4295(00)00748-2.

(112) Kim, H. L.; Mhoon, D. A.; Brendler, C. B. Does the CaverMap Device Help Preserve Potency? Curr. Urol. Rep. 2001, 2 (3), 214–217.

(113) Schwartz, B. G.; Kloner, R. A. Cardiovascular Implications of Erectile Dysfunction. Circulation 2011, 123 (21), e609–e611.

https://doi.org/10.1161/CIRCULATIONAHA.110.017681.

(114) Solomon, H.; Man, J. W.; Jackson, G. Erectile Dysfunction and the Cardiovascular Patient: Endothelial Dysfunction Is the Common Denominator. Heart 2003, 89 (3), 251–253.

(115) Bivalacqua, T. J.; Usta, M. F.; Champion, H. C.; Kadowitz, P. J.; Hellstrom, W. J. G. Endothelial Dysfunction in Erectile Dysfunction: Role of the Endothelium in Erectile Physiology and Disease. J. Androl. 2003, 24 (S6), S17–S37. https://doi.org/10.1002/j.1939-4640.2003.tb02743.x.

(116) Dall'Era, J. E.; Meacham, R. B.; Mills, J. N.; Koul, S.; Carlsen, S. N.; Myers, J. B.; Koul, H. K. Vascular Endothelial Growth Factor (VEGF) Gene Therapy Using a Nonviral Gene Delivery System Improves Erectile Function in a Diabetic Rat Model. Int. J. Impot. Res. 2008, 20 (3), 307–314. https://doi.org/10.1038/ijir.2008.1.

(117) Gholami, S. S.; Rogers, R.; Chang, J.; Ho, H.-C.; Grazziottin, T.; Lin, C.-S.; Lue, T. F. The Effect of Vascular Endothelial Growth Factor and Adeno-Associated Virus Mediated Brain Derived Neurotrophic Factor on Neurogenic and Vasculogenic Erectile Dysfunction Induced by Hyperlipidemia. J. Urol. 2003, 169 (4), 1577–1581. https://doi.org/10.1097/01.ju.0000055120.73261.76.

(118) Lee, R. J.; Springer, M. L.; Blanco-Bose, W. E.; Shaw, R.; Ursell, P. C.; Blau, H. M. VEGF Gene Delivery to Myocardium: Deleterious Effects of Unregulated Expression. Circulation 2000, 102 (8), 898–901. https://doi.org/10.1161/01.cir.102.8.898.

(119) Ryu, J.-K.; Cho, C.-H.; Shin, H.-Y.; Song, S. U.; Oh, S.-M.; Lee, M.; Piao, S.; Han, J.-Y.; Kim, I.-H.; Koh, G. Y.; Suh, J.-K. Combined Angiopoietin-1 and Vascular Endothelial Growth Factor Gene Transfer Restores Cavernous Angiogenesis and Erectile Function in a Rat Model of Hypercholesterolemia. Mol. Ther. J. Am. Soc. Gene Ther. 2006, 13 (4), 705–715. https://doi.org/10.1016/j.ymthe.2005.10.016.

(120) Nishimatsu, H.; Suzuki, E.; Nomiya, A.; Niimi, A.; Suzuki, M.; Fujimura, T.; Fukuhara, H.; Homma, Y. Adrenomedullin and Angiopoietin-1 Additively Restore Erectile Function in Diabetic Rats: Comparison with the Combination Therapy of Vascular Endothelial Growth Factor and Angiopoietin-1. J. Sex. Med. 2013, 10 (7), 1707–1719. https://doi.org/10.1111/jsm.12177.

(121) Ryu, J.-K.; Han, J.-Y.; Chu, Y.-C.; Song, S. U.; Lee, K.-H.; Yoon, S.-M.; Suh, J.-K.; Kim, S.-J. Expression of Cavernous Transforming Growth Factor-B1 and Its Type II Receptor in Patients with Erectile Dysfunction. Int. J. Androl. 2004, 27 (1), 42–49. https://doi.org/10.1046/j.0105-6263.2003.00447.x.

(122) Kamiya, Y.; Miyazono, K.; Miyazawa, K. Smad7 Inhibits Transforming Growth Factor-β Family Type I Receptors through Two Distinct Modes of Interaction. J. Biol. Chem. 2010, 285 (40), 30804–30813. https://doi.org/10.1074/jbc.M110.166140.

(123) Song, K. M.; Chung, J.-S.; Choi, M. J.; Jin, H.-R.; Yin, G. N.; Kwon, M.-H.; Park, J.-M.; Kim, W. J.; Lee, S.-J.; Kim, S.-J.; Ryu, J.-K.; Suh, J.-K. Effectiveness of Intracavernous Delivery of Adenovirus Encoding Smad7 Gene on Erectile Function in a Mouse Model of Cavernous Nerve Injury. J. Sex. Med. 2014, 11 (1), 51–63. https://doi.org/10.1111/jsm.12329.

(124) Li, W. J.; Wang, H.; Zhou, J.; Li, B.; Zhang, J.; Lu, M.; Wang, Z. P144, A TGF-B1 Antagonist Peptide, Synergizes with Sildenafil and Enhances Erectile Response via Amelioration of Cavernosal Fibrosis in Diabetic Rats. J. Sex. Med. 2013, 10 (12), 2942–2951. https://doi.org/10.1111/jsm.12325.

(125) Quazi, R.; Palaniswamy, C.; Frishman, W. H. The Emerging Role of Apelin in Cardiovascular Disease and Health: Cardiol. Rev. 2009, 17 (6), 283–286. https://doi.org/10.1097/CRD.0b013e3181b3fe0d.

(126) Falcão-Pires, I.; Ladeiras-Lopes, R.; Leite-Moreira, A. F. The Apelinergic System: A Promising Therapeutic Target. Expert Opin. Ther. Targets 2010, 14 (6), 633–645. https://doi.org/10.1517/14728221003752743.

(127) Japp, A. G.; Newby, D. E. The Apelin–APJ System in Heart Failure: Pathophysiologic Relevance and Therapeutic Potential. Biochem. Pharmacol. 2008, 75 (10), 1882–1892. https://doi.org/10.1016/j.bcp.2007.12.015.

(128) Japp, A. G.; Newby, D. E. The Apelin-APJ System in Heart Failure: Pathophysiologic Relevance and Therapeutic Potential. Biochem. Pharmacol. 2008, 75 (10), 1882–1892. https://doi.org/10.1016/j.bcp.2007.12.015.

(129) Tatemoto, K.; Hosoya, M.; Habata, Y.; Fujii, R.; Kakegawa, T.; Zou, M. X.; Kawamata, Y.; Fukusumi, S.; Hinuma, S.; Kitada, C.;

Kurokawa, T.; Onda, H.; Fujino, M. Isolation and Characterization of a Novel Endogenous Peptide Ligand for the Human APJ Receptor. Biochem. Biophys. Res. Commun. 1998, 251 (2), 471–476. https://doi.org/10.1006/bbrc.1998.9489.

(130) Hosoya, M.; Kawamata, Y.; Fukusumi, S.; Fujii, R.; Habata, Y.; Hinuma, S.; Kitada, C.; Honda, S.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. Molecular and Functional Characteristics of APJ. Tissue Distribution of MRNA and Interaction with the Endogenous Ligand Apelin. J. Biol. Chem. 2000, 275 (28), 21061–21067. https://doi.org/10.1074/jbc.M908417199.

(131) Kawamata, Y.; Habata, Y.; Fukusumi, S.; Hosoya, M.; Fujii, R.; Hinuma, S.; Nishizawa, N.; Kitada, C.; Onda, H.; Nishimura, O.; Fujino, M. Molecular Properties of Apelin: Tissue Distribution and Receptor Binding. Biochim. Biophys. Acta 2001, 1538 (2–3), 162–171.

(132) Lee, D. K.; Cheng, R.; Nguyen, T.; Fan, T.; Kariyawasam, A. P.; Liu, Y.; Osmond, D. H.; George, S. R.; O'Dowd, B. F. Characterization of Apelin, the Ligand for the APJ Receptor. J. Neurochem. 2000, 74 (1), 34–41.

(133) Tatemoto, K.; Takayama, K.; Zou, M. X.; Kumaki, I.; Zhang, W.; Kumano, K.; Fujimiya, M. The Novel Peptide Apelin Lowers Blood Pressure via a Nitric Oxide-Dependent Mechanism. Regul. Pept. 2001, 99 (2–3), 87–92.

(134) Pchejetski, D.; Foussal, C.; Alfarano, C.; Lairez, O.; Calise, D.; Guilbeau-Frugier, C.; Schaak, S.; Seguelas, M.-H.; Wanecq, E.; Valet, P.; Parini, A.; Kunduzova, O. Apelin Prevents Cardiac Fibroblast Activation and Collagen Production through Inhibition of Sphingosine Kinase 1. Eur. Heart J. 2012, 33 (18), 2360–2369. https://doi.org/10.1093/eurheartj/ehr389.

(135) O'Carroll, A.-M.; Lolait, S. J.; Harris, L. E.; Pope, G. R. The Apelin Receptor APJ: Journey from an Orphan to a Multifaceted Regulator of Homeostasis. J. Endocrinol. 2013, 219 (1), R13–R35. https://doi.org/10.1530/JOE-13-0227.

(136) Kwon, M.-H.; Tuvshintur, B.; Kim, W. J.; Jin, H.-R.; Yin, G. N.; Song, K.-M.; Choi, M. J.; Kwon, K.-D.; Batbold, D.; Ryu, J.-K.; Suh, J.-K. Expression of the Apelin–APJ Pathway and Effects on Erectile Function in a Mouse Model of Vasculogenic Erectile Dysfunction. J. Sex. Med. 2013, 10 (12), 2928–2941. https://doi.org/10.1111/jsm.12158.

(137) Syme, D. B. y.; Corcoran, N. M.; Bouchier-Hayes, D. M.; Costello, A. J. Hope Springs Eternal: Cavernosal Nerve Regeneration. BJU Int. 2006, 97 (1), 17–21. https://doi.org/10.1111/j.1464-410X.2006.05767.x.

(138) Menorca, R. M. G.; Fussell, T. S.; Elfar, J. C. Peripheral Nerve Trauma: Mechanisms of Injury and Recovery. Hand Clin. 2013, 29 (3), 317–330. https://doi.org/10.1016/j.hcl.2013.04.002.

(139) Ide, C. Peripheral Nerve Regeneration. Neurosci. Res. 1996, 25 (2), 101–121.

(140) Terenghi, G. Peripheral Nerve Regeneration and Neurotrophic Factors. J. Anat. 1999, 194 (Pt 1), 1–14.

(141) Schmidt, C. E.; Leach, J. B. Neural Tissue Engineering: Strategies for Repair and Regeneration. Annu. Rev. Biomed. Eng. 2003, 5, 293–347. https://doi.org/10.1146/annurev.bioeng.5.011303.120731.

(142) Arslantunali, D.; Dursun, T.; Yucel, D.; Hasirci, N.; Hasirci, V. Peripheral nerve conduits: technology update https://www.dovepress.com/peripheral-nerve-conduits-technology-update-peer-reviewed-article-MDER (accessed Aug 8, 2020). https://doi.org/10.2147/MDER.S59124.

(143) Campbell, J. D.; Burnett, A. L. Neuroprotective and Nerve Regenerative Approaches for Treatment of Erectile Dysfunction after Cavernous Nerve Injury. Int. J. Mol. Sci. 2017, 18 (8). https://doi.org/10.3390/ijms18081794.

(144) Hsieh, P.-S.; Bochinski, D. J.; Lin, G. T.; Nunes, L.; Lin, C. S.; Lue, T. F. The Effect of Vascular Endothelial Growth Factor and Brain-Derived Neurotrophic Factor on Cavernosal Nerve Regeneration in a Nerve-Crush Rat Model. BJU Int. 2003, 92 (4), 470–475. https://doi.org/10.1046/j.1464-410x.2003.04373.x.

(145) Bella, A. J.; Lin, G.; Cagiannos, I.; Lue, T. F. Emerging Neuromodulatory Molecules for the Treatment of Neurogenic Erectile Dysfunction Caused by Cavernous Nerve Injury. Asian J. Androl. 2008, 10 (1), 54–59. https://doi.org/10.1111/j.1745-7262.2008.00368.x.

(146) Falls, D. L. Neuregulins: Functions, Forms, and Signaling Strategies. Exp. Cell Res. 2003, 284 (1), 14–30. https://doi.org/10.1016/s0014-4827(02)00102-7.

(147) Burnett, A. L.; Sezen, S. F.; Hoke, A.; Caggiano, A. O.; Iaci, J.; Lagoda, G.; Musicki, B.; Bella, A. J. GGF2 Is Neuroprotective in a Rat Model of Cavernous Nerve Injury-Induced Erectile Dysfunction. J. Sex. Med. 2015, 12 (4), 897–905. https://doi.org/10.1111/jsm.12834.

(148) Ifergan, I.; Kebir, H.; Terouz, S.; Alvarez, J. I.; Lécuyer, M.-A.; Gendron, S.; Bourbonnière, L.; Dunay, I. R.; Bouthillier, A.; Moumdjian, R.; Fontana, A.; Haqqani, A.; Klopstein, A.; Prinz, M.; López-Vales, R.; Birchler, T.; Prat, A. Role of Ninjurin-1 in the Migration of Myeloid Cells to Central Nervous System Inflammatory Lesions. Ann. Neurol. 2011, 70 (5), 751–763. https://doi.org/10.1002/ana.22519.

(149) Allaf, M. E.; Hoke, A.; Burnett, A. L. Erythropoietin Promotes the Recovery of Erectile Function Following Cavernous Nerve Injury. J. Urol. 2005, 174 (5), 2060–2064. https://doi.org/10.1097/01.ju.0000176808.94610.dd.

(150) Harraz, A.; Shindel, A. W.; Lue, T. F. Emerging Gene and Stem Cell Therapies for the Treatment of Erectile Dysfunction. Nat. Rev. Urol. 2010, 7 (3), 143–152. https://doi.org/10.1038/nrurol.2010.8.

(151) Hakim, L.; Van der Aa, F.; Bivalacqua, T. J.; Hedlund, P.; Albersen, M. Emerging Tools for Erectile Dysfunction: A Role for Regenerative Medicine. Nat. Rev. Urol. 2012, 9 (9), 520–536. https://doi.org/10.1038/nrurol.2012.143.

(152) Shan, H.; Chen, F.; Zhang, T.; He, S.; Xu, L.; Wei, A. Stem Cell Therapy for Erectile Dysfunction of Cavernous Nerve Injury Rats: A Systematic Review and Meta-Analysis. PLOS ONE 2015, 10 (4), e0121428. https://doi.org/10.1371/journal.pone.0121428.

(153) Milenkovic, U.; Albersen, M.; Castiglione, F. The Mechanisms and Potential of Stem Cell Therapy for Penile Fibrosis. Nat. Rev. Urol. 2019, 16 (2), 79–97. https://doi.org/10.1038/s41585-018-0109-7.

(154) Haahr, M. K.; Jensen, C. H.; Toyserkani, N. M.; Andersen, D. C.; Damkier, P.; Sørensen, J. A.; Lund, L.; Sheikh, S. P. Safety and Potential Effect of a Single Intracavernous Injection of Autologous Adipose-Derived Regenerative Cells in Patients with Erectile Dysfunction Following Radical Prostatectomy: An Open-Label Phase I Clinical Trial. EBioMedicine 2016, 5, 204–210. https://doi.org/10.1016/j.ebiom.2016.01.024.

(155) Yiou, R.; Hamidou, L.; Birebent, B.; Bitari, D.; Lecorvoisier, P.; Contremoulins, I.; Khodari, M.; Rodriguez, A.-M.; Augustin, D.; Roudot-Thoraval, F.; de la Taille, A.; Rouard, H. Safety of Intracavernous Bone Marrow-Mononuclear Cells for Postradical Prostatectomy Erectile Dysfunction: An Open Dose-Escalation Pilot Study. Eur. Urol. 2016, 69 (6), 988–991. https://doi.org/10.1016/j.eururo.2015.09.026.

(156) Huang, J.; Zhang, Y.; Lu, L.; Hu, X.; Luo, Z. Electrical Stimulation Accelerates Nerve Regeneration and Functional Recovery in Delayed Peripheral Nerve Injury in Rats. Eur. J. Neurosci. 2013, 38 (12), 3691–3701. https://doi.org/10.1111/ejn.12370.

(157) Willand, M. P.; Nguyen, M.-A.; Borschel, G. H.; Gordon, T. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 2016, 30 (5), 490–496. https://doi.org/10.1177/1545968315604399.

(158) Chan, K. M.; Curran, M. W. T.; Gordon, T. The Use of Brief Post-surgical Low Frequency Electrical Stimulation to Enhance Nerve Regeneration in Clinical Practice. J. Physiol. 2016, 594 (13), 3553–3559. https://doi.org/10.1113/JP270892.

(159) Gordon, T. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 2016, 13 (2), 295–310. https://doi.org/10.1007/s13311-015-0415-1.

(160) Gu, X.; Fu, J.; Bai, J.; Wang, J.; Pan, W.; Zhang, C. Low-Frequency Electrical Stimulation Induces the Proliferation and Differentiation of Peripheral Blood Stem Cells Into Schwann Cells. Am. J. Med. Sci. 2015, 349 (2), 157–161. https://doi.org/10.1097/MAJ.0000000000000385.

(161) Wang, L.; Sanford, M. T.; Xin, Z.; Lin, G.; Lue, T. F. Role of Schwann Cells in the Regeneration of Penile and Peripheral Nerves. Asian J. Androl. 2015, 17 (5), 776–782. https://doi.org/10.4103/1008-682X.154306.

(162) Haan, N.; Song, B. Therapeutic Application of Electric Fields in the Injured Nervous System. Adv. Wound Care 2014, 3 (2), 156–165. https://doi.org/10.1089/wound.2013.0450.

(163) Huang, J.; Lu, L.; Zhang, J.; Hu, X.; Zhang, Y.; Liang, W.; Wu, S.; Luo, Z. Electrical Stimulation to Conductive Scaffold Promotes

Axonal Regeneration and Remyelination in a Rat Model of Large Nerve Defect. PLOS ONE 2012, 7 (6), e39526. https://doi.org/10.1371/journal.pone.0039526.

(164) Elzinga, K.; Tyreman, N.; Ladak, A.; Savaryn, B.; Olson, J.; Gordon, T. Brief Electrical Stimulation Improves Nerve Regeneration after Delayed Repair in Sprague Dawley Rats. Exp. Neurol. 2015, 269, 142–153. https://doi.org/10.1016/j.expneurol.2015.03.022.

(165) Du, L.; Yang, M.; Wan, L.; Wang, X.; Li, S. Electrical Stimulation Promotes Regeneration of Injured Oculomotor Nerves in Dogs. Neural Regen. Res. 2016, 11 (10), 1666–1669. https://doi.org/10.4103/1673-5374.193248.

(166) Ray, W. Z.; Mahan, M. A.; Guo, D.; Guo, D.; Kliot, M. An Update on Addressing Important Peripheral Nerve Problems: Challenges and Potential Solutions. Acta Neurochir. (Wien) 2017, 1–9. https://doi.org/10.1007/s00701-017-3203-3.

(167) Kapoor, M. S.; Khan, S. A.; Gupta, S. K.; Choudhary, R.; Bodakhe, S. H. Animal Models of Erectile Dysfunction. J. Pharmacol. Toxicol. Methods 2015, 76, 43–54. https://doi.org/10.1016/j.vascn.2015.07.013.

(168) Musicki, B.; Liu, T.; Lagoda, G. A.; Strong, T. D.; Sezen, S. F.; Johnson, J. M.; Burnett, A. L. Hypercholesterolemia-Induced Erectile Dysfunction: Endothelial Nitric Oxide Synthase (ENOS) Uncoupling in the Mouse Penis by NAD(P)H Oxidase. J. Sex. Med. 2010, 7 (9), 3023–3032. https://doi.org/10.1111/j.1743-6109.2010.01880.x.

(169) Montorsi, F.; Briganti, A.; Salonia, A.; Deho', F.; Zanni, G.; Cestari, A.; Guazzoni, G.; Rigatti, P.; Stief, C. The Ageing Male and Erectile Dysfunction. BJU Int. 2003, 92 (5), 516–520. https://doi.org/10.1046/j.1464-410X.2003.04378.x.

(170) Xie, D.; Odronic, S. I.; Wu, F.; Pippen, A. M.; Donatucci, C. F.; Annex, B. H. A Mouse Model of Hypercholesterolemia-Induced Erectile Dysfunction. J. Sex. Med. 2007, 4 (4, Part 1), 898–907. https://doi.org/10.1111/j.1743-6109.2007.00518.x.

(171) Getz, G. S.; Reardon, C. A. Use of Mouse Models in Atherosclerosis Research. Methods Mol. Biol. Clifton NJ 2015, 1339, 1–16. https://doi.org/10.1007/978-1-4939-2929-0_1.

(172) Plump, A. S.; Smith, J. D.; Hayek, T.; Aalto-Setälä, K.; Walsh, A.; Verstuyft, J. G.; Rubin, E. M.; Breslow, J. L. Severe Hypercholesterolemia and Atherosclerosis in Apolipoprotein E-Deficient Mice Created by Homologous Recombination in ES Cells. Cell 1992, 71 (2), 343–353. https://doi.org/10.1016/0092-8674(92)90362-G.

(173) Zhang, S. H.; Reddick, R. L.; Piedrahita, J. A.; Maeda, N. Spontaneous Hypercholesterolemia and Arterial Lesions in Mice Lacking Apolipoprotein E. Science 1992, 258 (5081), 468–471. https://doi.org/10.1126/science.1411543.

(174) Chung, E.; De Young, L.; Brock, G. B. Investigative Models in Erectile Dysfunction: A State-of-the-Art Review of Current Animal Models. J. Sex. Med. 2011, 8 (12), 3291–3305. https://doi.org/10.1111/j.1743-6109.2011.02505.x.

(175) Behr-Roussel, D.; Darblade, B.; Oudot, A.; Compagnie, S.; Bernabé, J.; Alexandre, L.; Giuliano, F. ORIGINAL RESEARCH—BASIC SCIENCE: Erectile Dysfunction in Hypercholesterolemic Atherosclerotic Apolipoprotein E Knockout Mice. J. Sex. Med. 2011, 3 (4), 596–603. https://doi.org/10.1111/j.1743-6109.2006.00282.x.

(176) Quinlan, D. M.; Nelson, R. J.; Partin, A. W.; Mostwin, J. L.; Walsh, P. C. The Rat as a Model for the Study of Penile Erection. J. Urol. 1989, 141 (3), 656–661. https://doi.org/10.1016/s0022-5347(17)40926-8.

(177) Martínez-Piñeiro, L.; Brock, G.; Trigo-Rocha, F.; Hsu, G. L.; Lue, T. F.; Tanagho, E. A. Rat Model for the Study of Penile Erection: Pharmacologic and Electrical-Stimulation Parameters. Eur. Urol. 1994, 25 (1), 62–70.

(178) Mills, T. M.; Stopper, V. S.; Wiedmeier, V. T. Effects of Castration and Androgen Replacement on the Hemodynamics of Penile Erection in the Rat. Biol. Reprod. 1994, 51 (2), 234–238. https://doi.org/10.1095/biolreprod51.2.234.

(179) Mullerad, M.; Donohue, J. F.; Li, P. S.; Scardino, P. T.; Mulhall, J. P. Functional Sequelae of Cavernous Nerve Injury in the Rat: Is There Model Dependency. J. Sex. Med. 2006, 3 (1), 77–83. https://doi.org/10.1111/j.1743-6109.2005.00158.x.

(180) Canguven, O.; Burnett, A. Laboratory Forum: Cavernous Nerve Injury Using Rodent Animal Models. J. Sex. Med. 2008, 5 (8), 1776–1785. https://doi.org/10.1111/j.1743-6109.2008.00955.x.

(181) Mulhall, J. P.; Müller, A.; Donohue, J. F.; Mullerad, M.; Kobylarz, K.; Paduch, D. A.; Tal, R.; Li, P. S.; Cohen-Gould, L.; Scardino,

P. T. The Functional and Structural Consequences of Cavernous Nerve Injury Are Ameliorated by Sildenafil Citrate. J. Sex. Med. 2008, 5 (5), 1126–1136. https://doi.org/10.1111/j.1743-6109.2008.00794.x.

(182) Iacono, F.; Giannella, R.; Somma, P.; Manno, G.; Fusco, F.; Mirone, V. Histological Alterations in Cavernous Tissue after Radical Prostatectomy. J. Urol. 2005, 173 (5), 1673–1676. https://doi.org/10.1097/01.ju.0000154356.76027.4f.

(183) Vignozzi, L.; Filippi, S.; Morelli, A.; Ambrosini, S.; Luconi, M.; Vannelli, G. B.; Donati, S.; Crescioli, C.; Zhang, X.-H.; Mirone, V.; Forti, G.; Maggi, M. Effect of Chronic Tadalafil Administration on Penile Hypoxia Induced by Cavernous Neurotomy in the Rat. J. Sex. Med. 2006, 3 (3), 419–431. https://doi.org/10.1111/j.1743-6109.2006.00208.x.

(184) Ferrini, M. G.; Kovanecz, I.; Sanchez, S.; Umeh, C.; Rajfer, J.; Gonzalez-Cadavid, N. F. FIBROSIS AND LOSS OF SMOOTH MUSCLE IN THE CORPORA CAVERNOSA PRECEDE CORPORAL VENO-OCCLUSIVE DYSFUNCTION (CVOD) INDUCED BY EXPERIMENTAL CAVERNOSAL NERVE DAMAGE IN THE RAT. J. Sex. Med. 2009, 6 (2), 415–428. https://doi.org/10.1111/j.1743-6109.2008.01105.x.

(185) Hannan, J. L.; Matsui, H.; Sopko, N. A.; Liu, X.; Weyne, E.; Albersen, M.; Watson, J. W.; Hoke, A.; Burnett, A. L.; Bivalacqua, T. J. Caspase-3 Dependent Nitrergic Neuronal Apoptosis Following Cavernous Nerve Injury Is Mediated via RhoA and ROCK Activation in Major Pelvic Ganglion. Sci. Rep. 2016, 6. https://doi.org/10.1038/srep29416.

Chapter 2 Apelin-13 Protects Corpus Cavernosum against Fibrosis induced by High-Fat Diet in an MMP-dependent mechanism

Mikael Sturny[1], Léa Anguenot[1], Fabiana P. Costa-Fraga[1], Maiia E. Bragina[1], Augusto Martins Lima[1], Rafaela F. da Silva[2], Rodrigo A. Fraga-Silva[1*], Nikolaos Stergiopulos[1*].

[1] *Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland.*

[2] *Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte, Brazil.*

* Equal contribution

Running title: Apelin protects corpus cavernosum against fibrosis

Manuscript submitted to the Journal of Sexual Medicine (2020 May 8th)

Corresponding Authors: Rodrigo Araujo Fraga-Silva, PhD
Ecole Polytechnique Fédérale de Lausanne
Institute of Bioengineering,
Station 09, MED 3.2526, CH-1015, Lausanne, Switzerland
Phone: (+41) 21 693 9654
Fax: (+41) 21 693 1120
Email: rodrigo.fragasilva@epfl.ch

2.1 Abstract

Background: An increased fibrosis of the corpora cavernosa is a prevalent process that underlies most cases of erectile dysfunction (ED). Apelin, an endogenous circulating peptide, has been documented as an important effector on cardiovascular homeostasis, controlling vascular function and reducing fibrosis in multiple pathological conditions. Recently, initial studies have shown that Apelin, acting through the APJ receptor, also modulates penile erection, however, the role of this system on penile structure and intracorporal collagen remodeling has not been investigated yet.

Aims: Here we sought to investigate the effect of chronic Apelin treatment on the corpus cavernosum structure of hypercholesterolemic mice.

Methods: Apolipoprotein gene-deleted ($ApoE^{-/-}$) mice were fed with a Western diet for 11 weeks and received Apelin-13 (2mg/kg/day) or vehicle during the last 3 weeks. Penile samples were obtained for histological and biochemical analyses to assess the intracorporal collagen content and key proteins expression. Furthermore, the effect of Apelin-13 was evaluated in cultured NIH3T3 mouse fibroblasts stimulated with TGF-β.

Outcome: Local expression of Apelin-13 in mouse corpus cavernosum and its protective effect against fibrosis.

Results: Apelin and APJ receptor were expressed (gene and protein) within the corpus cavernosum of $ApoE^{-/-}$ mice, indicating a local modulation of the Apelin system. Interestingly, three weeks of Apelin-13 treatment strongly reduced intracavernosal collagen content. In addition, Apelin-13 enhanced total matrix metalloproteinase (MMP) activity in the mice penis, which was associated with an increased protein expression of MMP-1, MMP-3, MMP-8 and MMP-9, while tissue inhibitor of metalloproteinase (TIMP) were unaltered. These beneficial actions were not associated with changes in nNOS or eNOS protein expression, intracavernosal ROS content, or atherosclerotic plaque deposition. Additionally, in cultured fibroblast, Apelin-13 inhibited TGF-β-induced fibroblast to myofibroblast differentiation and collagen production, possibly through the activation of ERK1/2 kinase.

Clinical Translation: These results point out Apelin/APJ system as a potential target to treat intracavernosal fibrosis-related disorders.

Strength & Limitations: These results provide the first evidence of the Apelin system's positive role on erectile tissue structure/remodeling. Nevertheless, additional functional study addressing erectile response would bring extended validation regarding the relevance of such effect.

Conclusion: These results suggest a local modulation of the Apelin system within the corpus cavernosum. Remarkably, Apelin-13 reduced intracavernosal fibrosis in hypercholesterolemic mice by: 1) enhancing MMPs expression and activity; and 2) inhibiting fibroblast differentiation into myofibroblast. Altogether, these results suggest an essential protective role of Apelin, indicating Apelin/APJ system as a promising candidate for the development of fibrosis-associated ED treatments.

Key Words: Erectile dysfunction; hypercholesterolemia; Apelin; APJ receptor; fibrosis; matrix metalloproteinase.

2.2 Introduction

An increased fibrosis of the corpora cavernosa and the media of penile arteries is a highly prevalent process that underlies most cases of erectile dysfunction (ED)[1,2] and hypercholesterolemia is one of the most frequent cause of vasculogenic ED. One of the major mechanisms involved has been attributed to oxidative stress within the corpus cavernosum, which triggers a higher vasoconstrictor tonus and a reduction of nitric oxide (NO) bioavailability[3,4]. At long-term, prolonged oxidative stress within the corpus cavernosum leads to hypoxia and inflammatory response[5], triggering the release of mitogenic and pro-fibrotic cytokines[6] inducing fibrosis. The excess of collagen deposition of stiff fibers and decrease of elastic fibers content impairs the function of the penile erectile structures[7,8]. Indeed, elastic properties and expandability of the corpus cavernosum are required to insure tissue compliance and accommodation of blood within the cavernosal sinuses during erection and is essential for proper compression of subtunical venules, guaranteeing the pro-erectile veno-occlusive mechanism[9]. Therefore, at long-term, hypercholesterolemia may lead to severe ED by promoting fibrosis and exaggerated collagen deposition[2,8].

The fibrosis process is characterized by fibroblast activation and differentiation into myofibroblast. The activation and differentiation of fibroblasts to myofibroblasts, major cells responsible for the collagen production, represent a key event in the fibrosis establishment and progression[6,10]. Myofibroblast differentiation is known to be initiated by multiple inflammatory factors and especially by

transforming growth factor beta (TGF-β), leading to a phenotype modification characterized by the acquirement of a stellate shape, expression of alpha smooth muscle actin (α-SMA), and augmented secretion of collagen type-I fibers[11]. Activated myofibroblasts are then able to secrete TGF-β themselves, amplifying the pro-fibrotic process[6]. At the contrary, matrix metalloproteinase family (MMP) are enzymes known to degrade structural components of the extracellular matrix (ECM), regulating the repartition, type and content of collagen fibers within tissues[12]. 23 and 24 different MMPs have been identified in humans and in mice respectively. These essential collagenases are required during development in mammals, wound healing or tissue remodeling process and deficiency or over-expression of MMP's have been revealed in many pathological conditions such as rheumatoid arthritis or cancer[12]. The activity and expression of MMPs is controlled at many levels and the regulation of MMP activity remains a topic of intense research, nevertheless, it is known to be at least partially regulated by tissue inhibitor of metalloproteinase (TIMP) family. This endogenous proteins family, composed of four conserved enzymes in mammals (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) act as significant regulators of the activities of MMPs and, in some instances, of other metalloendopeptidases[13].

The Apelin-APJ system has emerged in the last years as an important regulator of the cardiovascular function. Apelin is an endogenous circulating peptide family, which acts through the activation of the G protein-coupled receptor APJ[14], evoking a wide range of actions in the cardiovascular system including vasodilation, nitric oxide release and angiogenesis[15,16]. Apelin and APJ are expressed in the vascular system, as well as in the cerebellum, heart, lung and kidney, where high concentrations have been reported[15–18]. Apelin peptide family derives from a 77 amino-acids precursor and is present in different active circulating isoforms denoted by their amino-acid sequence length, such as apelin-12, apelin-13, apelin-17 and apelin-36, respectively corresponding to 12, 13, 17 and 36 amino-acids[17,18]. Apparently, all these isoforms may activate APJ; however, apelin-13 exhibits higher affinity to APJ and promotes stronger physiologic response[14,19].

The role of Apelin–APJ system in cardiovascular physiology has not been fully elucidated yet. However, several studies, predominantly performed in animals, indicate that the Apelin system plays an important role in the cardio-vascular remodeling and homeostasis[20–22]. For instance, Apelin inhibits TGF-β-stimulated activation of cardiac fibroblasts and prevents structural remodeling and fibrosis of the myocardium, avoiding ventricular dysfunction in an aortic banding model[23]. In accordance, Apelin was shown to reduce fibrosis in other pathological conditions, such as renal injury or pulmonary hypertension[24–28]. Recently, the Apelin system was shown to be down-regulated in hypercholesterolemic mice and hyperglycemic mice, two well-known vasculogenic ED models, while intracavernosal acute injection of Apelin restored erectile functions of such model[29]. However, its action modulating penile structural composition, particularly collagen deposition, is unknown.

Therefore, here we aimed to investigate the role and involved mechanism of Apelin in the modulation of cavernosal structural composition and fibrosis, using a well-known mouse model of vasculogenic ED[30].

2.3 Materials and Methods

In vivo experimental design

A well-established animal model of hypercholesterolemia-induced ED was used. The experimental protocol was performed as previously described[31]. Briefly, apolipoprotein-E knockout (ApoE$^{-/-}$) male mice on a C57BL/6J background (n = 40) were obtained from Jackson Laboratories. All animals at 15-weeks-old were fed for 11 weeks with a Western-type diet consisting in 15% (wt/wt) cocoa butter and 0.25% (wt/wt) cholesterol (Diet W; abDiets). During the final 3 weeks of experimental protocol, the animals were randomly divided into two groups to receive intraperitoneal injections of Apelin-13 (2 mg/kg, in a volume of 100 µl, 5 days/week – Cat# 4029109, Bachem, Switzerland) or vehicle (saline 0.9%). At the end of the experimental protocol, animals were anaesthetized (ketamine 100 mg/Kg, Xylazine 10 mg/Kg – Ketasol/Xylasol, Graeub, Switzerland) and blood samples were collected by cardiac puncture followed by perfusion with phosphate buffered solution (PBS). Blood samples were centrifuged at 2500 rpm for 10 min to obtain the serum and stored at -80 °C. Immediately after the cardiac puncture, the corpora cavernosa were carefully dissected from skin, dorsal vessels and urethra, snap-frozen in liquid nitrogen and stored at -80 °C for protein measurements, or frozen in cryoembedding medium for histological analysis. Aorta was dissected from the ascending thoracic arch until the abdominal portion and fixed in 4% paraformaldehyde (PFA, Sigma) for 24 hours for atherosclerotic lesion analyses. The animal experimentation was approved by the local ethics committee and Swiss regulatory authorities (license number 2026.5) in accordance with the guidelines from the directive 2010/63/EU of the European parliament on the protection of animals used for scientific purposes.

Intracavernosal collagen content

Total intracavernosal collagen deposition was assessed by Sirius red staining (Sigma Chemical Co, St Louis, MO) as previously reported[32]. Briefly, 6 µm mouse penis cryosections were rinsed with water and the nuclei stained with Weigert's hematoxylin for 10 min. After washing in tap water, the slides were incubated with 0.1% Sirius red in saturated picric acid for 60 min. Sequentially, the sections were rinsed twice with 5% acetic acid for 10 seconds, then immersed in absolute ethanol three times before clearing in xylene twice and cover-slipping. The sections were photographed using identical exposure settings under normal light microscopy. Intracavernosal total collagen content was quantified by Image J software (NIH). Data were calculated as percentages of stained area to total area.

Intracavernosal matrix metalloproteinase activity

Total matrix metalloproteinase (MMP) activity of mice penile protein was assessed using a commercial kit (cat# ab112146, Abcam, Inc) based on a fluorescence resonance energy transfer (FRET) peptide as generic MMP activity indicator. The assay was performed according to the manufacturer's instructions. Penile protein extraction from Apelin-13 treated or untreated mice was first incubated with the assay buffer containing p-aminophenylmercuric acetate. Consecutively, the fluorescent substrate was added and incubated for 2h at 37 °C. The total fluorescence intensity (490/525 nm of excitation/emission) was read in a fluorescence plate reader. The relative total MMP activity was determined as total detected fluorescence. Each sample was run as a technical triplicate.

Intracavernosal MMP and TIMP protein expression

The relative protein expression level of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, and tissue inhibitor of metalloproteinase-(TIMP)-1, TIMP-2, TIMP-4 in mouse penile tissue were measured using a commercially available MMP array kit (cat# ab134004, Abcam, Inc). The assay was performed as described in the instructions manual with minor modifications to allow infrared wavelengths system detection (Odyssey Imaging System, LiCor Biosciences). Briefly, frozen penile tissue of mice treated or not with Apelin-13 were mechanically disrupted in lysis buffer containing protease inhibitor cocktail (cat# 11 836 170 001, Roche Diagnostics GmbH, Mannheim, Germany) for protein extraction. After centrifugation (10 min, 14000 rpm at 4 °C), the protein concentration of supernatant was determined by Bradford assay. Each kit membrane was placed into a well tray and blocked at RT for 1h (cat#927-40000, Odyssey, Inc. with 0.05% Tween-20 (Sigma)). After pouring the blocking buffer, 250 µg of penile protein extract diluted in 1 mL of blocking buffer was placed and incubated at 4°C overnight. Next, the membranes were washed with assay buffer and incubated with biotin-conjugated antibody diluted in blocking buffer for 1h. After washing with assay buffer, IRDye® infrared dye-labeled streptavidin (dilution 1:2000, cat# 925-32230, LiCor Biosciences, Inc.) diluted in blocking solution was added to the membranes and incubated for 45 min at RT. Finally, the membranes were washed with assay buffer and scanned using a fluorescence imaging system. The fluorescence intensity of acquired digital images was quantified by Image J software. The relative protein expression level was determined by normalizing the signal of each group with the signal of positive control (biotin-conjugated IgG protein printed on each membrane).

mRNA expression analysis by RT-qPCR

Total RNA was extracted from corpus cavernosum of ApoE$^{-/-}$ mice by TRIzol® Reagent (ThermoFischer Scientific). Quantity and purity of the extracted RNA was measured by spectrophotometry (NanoDrop; Thermo Scientific). To minimize DNA contamination, total RNA extracts were treated with DNAse (Roche) in the presence of RNAse inhibitor (Roche). Following DNAse treatment, 1 μg of total RNA was reverse transcribed with ImProm-II™ System (Promega) according to the manufacturer's protocol. Obtained cDNA was used to run qPCR using PowerUP™ SYBR™ Green Master Mix (ThermoFisher Scientific) on the QuantStudio 6 Flex Real-Time PCR System (Thermofisher Scientific). 1.5 μl of cDNA at 1:10 dilution was used. qPCR reaction started with initial steps of 2 min @ 50 °C and 2 min @ 95 °C, followed by 40 cycles of denaturation @ 95 °C for 15 sec and annealing/extention @ 60 °C for 1 min. Non-reverse-transcribed RNA samples were used to confirm absence of genomic DNA contamination. Following primers were used in the current study: Apelin receptor 5'-CTTCTAGCTGTGCCTGTCATG-3' (forward), 5'-TGAAGGGCACCACAAAGC-3' (reverse); Apelin precursor 5'-CACTGATGTTGCCTCCAGATG-3' (forward), 5'-CCCTTCAATCCTGCTTTAGAAAG-3' (reverse). Threshold cycles (C_t) were recorded. Size of the DNA fragments generated by PCR reaction and absence of additional bands were confirmed by agarose gel electrophoresis (10 μl/lane).

Western Blotting

Protein expression levels of APJ receptor, neuronal and endothelial nitric oxide synthase (nNOS and eNOS, respectively) within the corpus cavernosum of mice and, protein expression of APJ, alpha smooth muscle actin (α-SMA), collagen type I (Col-I), total and phosphorylated extracellular signal-regulated kinases (ERK1/2 and P-ERK1/2 respectively, phosphorylation sites: ERK1-T-202 and ERK2-T185) from fibroblasts culture were assessed by Western blotting. After running 40 µg of mice penis protein extraction or whole cell lysate on a 10% SDS-PAGE gel, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilion™,

cat# IPFL00010, Merck Millipore, 0.45 µm pore size). After blocking for 1h, the membranes were probed with one of the following primary antibodies: anti-APJ (dilution 1:1000, cat# ab-84296, Abcam, Inc.); anti-eNOS (dilution 1:200, cat# sc-654, Santa Cruz Biotechnology, Inc.); anti-nNOS (dilution 1:200, cat# sc-8309, Santa Cruz Biotechnology, Inc.); anti-α-SMA (dilution 1:1500, cat# ab-7817, Abcam, Inc.); anti-Col-I (dilution 1:500, cat#AB765P, Merck Millipore, Inc.); anti-ERK1/2 (dilution 1:500, cat#ab-54230, Abcam, Inc.); anti-P-ERK1/2 (dilution 1:500, cat# ab-201015, Abcam, Inc.); anti-α-actin (dilution 1:600, cat#32251, Santa Cruz Biotechnology, Inc.); or anti-GAPDH (dilution 1:1000, cat# sc-48167, Santa Cruz Biotechnology, Inc.). Membranes were washed 3 times for 10 min with Tris-buffered saline-Tween (TBS-T) and incubated with the following secondary antibodies: anti-mouse IgG conjugated with IRDye 680RD (dilution 1:5000, cat# 926-68072, LiCor Biosciences, Inc.), anti-Rabbit IgG conjugated with IRDye 680RD (dilution 1:5000, cat# 926-68073, LiCor Biosciences, Inc.), anti-goat IgG conjugated with IRDye 800CW (dilution 1:5000, cat# 926-32212, LiCor Biosciences, Inc.) or anti-rabbit IgG conjugated with IRDye 800CW (dilution 1:2000, cat# 926-32213, LiCor Biosciences, Inc.) for 2h at RT. After three washes of 10 min with TBS-T, the fluorescence signal of the membranes was detected by a fluorescence detector. The total amount of loaded protein and the membrane transfer quality were assessed by loading control or 5% Ponceau S staining (Sigma). The 300 dpi acquired images from an optical scanner were converted in 16-bit grey images using Image J software and analyzed similarly to the blot images using Image Studio Lite software (LiCor Biosciences, Inc.). The relative protein expression level was determined by normalizing referring to control group.

Reactive oxygen species (ROS) levels in the corpus cavernosum

To assess ROS production within mice corpus cavernosum, cryosections were stained with dihydroethidium (DHE; Cat# 37291, Sigma-Aldrich, USA) as previously described[33]. Slides containing mice penis transversal cryosections of 6 µm were thawed at RT and washed with PBS. Next, the sections were incubated with DHE at 2 µmol/L in PBS for 20 min in a dark humid chamber at 37 °C. After three washes, the slides were coverslip-sealed with DAPI containing mounting medium (Fluoroshield™, cat#F6057, Sigma) and examined on a Confocal microscope (Carl Zeiss LSM 700, 20x magnification). DHE fluorescence intensity of acquired digital images was quantified by Image J software.

Measurement of the atherosclerotic lesion area

Atherosclerotic lesion area on the aortic arch and thoracic aorta were quantified by *en face* Oil-Red-O staining as previously reported[31]. After tissue dissection and fixation in PFA4%, the aorta was opened longitudinally to expose the lumen surface. After staining with Oil-Red-O, the aorta was positioned between a slide and a coverslip to expose the internal lumen for visualization on the binocular (6x magnification). The images of the aorta *en face* were captured and the stained area quantified by Image J software.

Dosage of serum lipid profile

The lipids were measured by photometric enzymatic reaction using commercial kits (glucose cat.# 11447513; triglycerides cat.# 12016648; total cholesterol cat.# 12016630; low-density lipoprotein cat.# 03038661; high-density lipoprotein cat.# 03030024 122; free fatty acids cat.# 11383175001; from Roche Diagnostics GmbH, Mannheim, Germany) and the chemistry analyzer Roche Hitachi 902 (Roche Diagnostics GmbH, Mannheim, Germany). Concentration was expressed in mmol/L.

Cell culture & collagen synthesis measurement

NIH3T3 mouse fibroblasts collagen synthesis was assessed by Sirius red staining followed by dye elution for spectrophotometric analysis as previously reported[34]. In brief, cells cultured on 12-well plates were washed with cold PBS and fixed with 100% methanol for 15 min. After that, cells were incubated with 0.1% Sirius red F3B (Cat# 365548, Sigma) in saturated aqueous picric acid solution (Cat# 197378, Sigma) for 1h at room temperature (RT). Sequentially, the dye solution was removed and the preparation washed twice with 0.1% acetic acid. The stained dye was then eluted in 200 µl of 0.1 M sodium hydroxide for 1h at RT. Finally, the eluted solution was transferred into a 96-well plate and the optical density absorbance was recorded at 540 nm by a microplate reader (Wallac Victor2, PerkinElmer, Waltham, MA). Data in triplicate were normalized to the control group (non-treated cells).

Induction of fibroblast to myofibroblast differentiation

NIH3T3 mouse fibroblasts (Cat# 93061524, PHE, Salisbury, UK) were cultured in Dulbecco's Modified Eagle's medium (DMEM) with high glucose (cat# D6429, Sigma) and supplemented with 10% fetal bovine serum (FBS, cat# F6178, Sigma) and 1% antibiotics (penicillin 10'000 Unit/mL and streptomycin 10'000 µg/mL, cat# 15140-122, Gibco). Cells were seeded into 8-well chamber slides (cat# 154534, Thermo-Fisher) for immunostaining, 12-well plates for collagen synthesis measurement, or 150mm diameter petri-dishes for protein collection.

For experimental protocol, fibroblasts were cultured with 1% FBS supplemented DMEM for at least 24h and/or until 50% of confluence. Cells were pretreated with Apelin-13 (100 nM) or vehicle (fresh medium) for 20 min. Sequentially, TGF-β (10 ng/mL, Cat# 100-21C, Peprotech, USA) was added for 24h, as previously described elsewhere [23]. All cell culture and incubation were performed under humidified atmosphere at 37°C in 95% air and 5% CO_2.

Quantification of differentiated fibroblast

Identification of naïve and differentiated fibroblasts was assessed by immunofluorescence. In brief, NIH3T3 fibroblasts were cultured on 8-well chamber slides. After Apelin or vehicle incubation for 20 min, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% PFA for 10 min at RT. Cells were then permeabilized with 0.1% Triton X-100 (Sigma) for 10 min and sequentially washed with PBS. The slides were then blocked for 30 min with 3% bovine serum albumin (BSA, Sigma) and incubated for two hours at RT with the primary anti-α-SMA antibody (dilution 1:100, cat# ab-7817, Abcam, Inc.), diluted in PBS containing 1% BSA. Following three washing steps of 5 min with PBS, the cells were incubated with secondary antibody conjugated Alexa Fluor® 555 (dilution: 10 µg/mL, cat# A21422, Thermo- Fisher) for 1h at RT. Final washing steps were done with PBS before mounting samples with DAPI containing mounting medium. Slides were examined on a Confocal microscope (Carl Zeiss LSM 700, 20x magnification). Fluorescence intensity of acquired digital images was quantified by Image J software. Data were calculated as percentages of stained area normalized by cell number.

Data Analysis

Statistical analyses for all measurements were performed using unpaired Student t test or one-way analysis of variance (ANOVA) comprising intergroup comparisons by Tukey's post-hoc test. Statistical analysis was performed using GraphPad Prism V6.0 software (GraphPad, San Diego, CA, USA). A *P* value <0.05 was considered significant and the results are expressed as mean ± SEM.

2.4 Results

APJ system is present in mice corpus cavernosum

In order to understand the regulation of the APJ system within erectile tissue, we first evaluated the local expression of Apelin and APJ receptor. Interestingly, Apelin precursor and APJ mRNA were both expressed within the corpus cavernosum of ApoE$^{-/-}$ mice (Figure 2:1 A). Accordingly, APJ protein expression was also detected by Western Blot, which revealed a single band of approximately 58 kDa (Figure 2:1 B). Therefore, it appears that Apelin peptides and receptor are locally regulated within male erectile tissues.

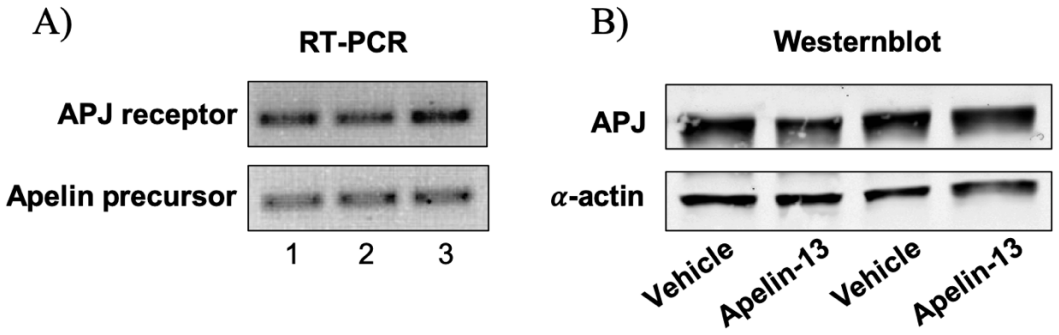

Figure 2:1 - Expression of APJ receptor and Apelin precursor in the corpus cavernosum of ApoE$^{-/-}$ mice and ApoE$^{-/-}$ treated with apelin-13. Western blotting and RT-PCR analyses were performed to detect APJ receptor and Apelin precursor in ApoE$^{-/-}$ mice penis. A) mRNA expression of APJ receptor and Apelin precursor in corpus cavernous tissue of ApoE$^{-/-}$ mice. The experiment was performed with RNA extract obtained from three different mice. Average C_t for Apelin receptor and Apelin precursor were 27.92±0.26 and 30.05±0.08, respectively. B) Representative Western Blotting picture showing APJ protein expression levels in ApoE$^{-/-}$ mice penis treated and non-treated (vehicle) with Apelin-13 (in duplicate), revealing a specific single band at the expected molecular weight of 58kDa. Expression of α-actin protein is used as control. Expression of APJ and Apelin precursor in the penile tissue suggests existence of the "local" Apelin system in the tissue and its independent regulation.

Apelin-13 attenuates fibrosis in the corpus cavernosum of hypercholesterolemic mice

The Apelin system has shown protective actions against pathological collagen deposition in diverse organs[23,24,26]. Therefore, as the next step, we evaluated the long-term effect of Apelin-13 on penile fibrosis in hypercholesterolemic ApoE$^{-/-}$ mice, a well-known ED animal model associated with exaggerated intracavernosal collagen deposition[32].

Remarkably, treatment with Apelin-13 for 3 weeks, produced a robust decrease in the collagen content within the corpus cavernosum of ApoE$^{-/-}$ mice when compared to vehicle group (Figure 2:2 A, B and C). Interestingly, this anti-fibrotic effect was associated with a significant increase in the total MMP activity (Figure 2:2 D, 35% of increase, p = 0.008). In accordance, the protein expression level of MMP-1, MMP-3, MMP-8 and MMP-9 was significantly increased in the Apelin-13-treated group compared to vehicle (Figure 2:2 E), while MMP-2, MMP-10 and MMP-13 expression was unchanged. Moreover, the increase of MMPs expression was not followed by changes in TIMP-1, TIMP-2 or TIMP-4 expression (Figure 2:2 F).

Together, these data show that chronic treatment with Apelin-13 significantly decreases intracavernosal collagen deposition by increasing MMP expression and activity.

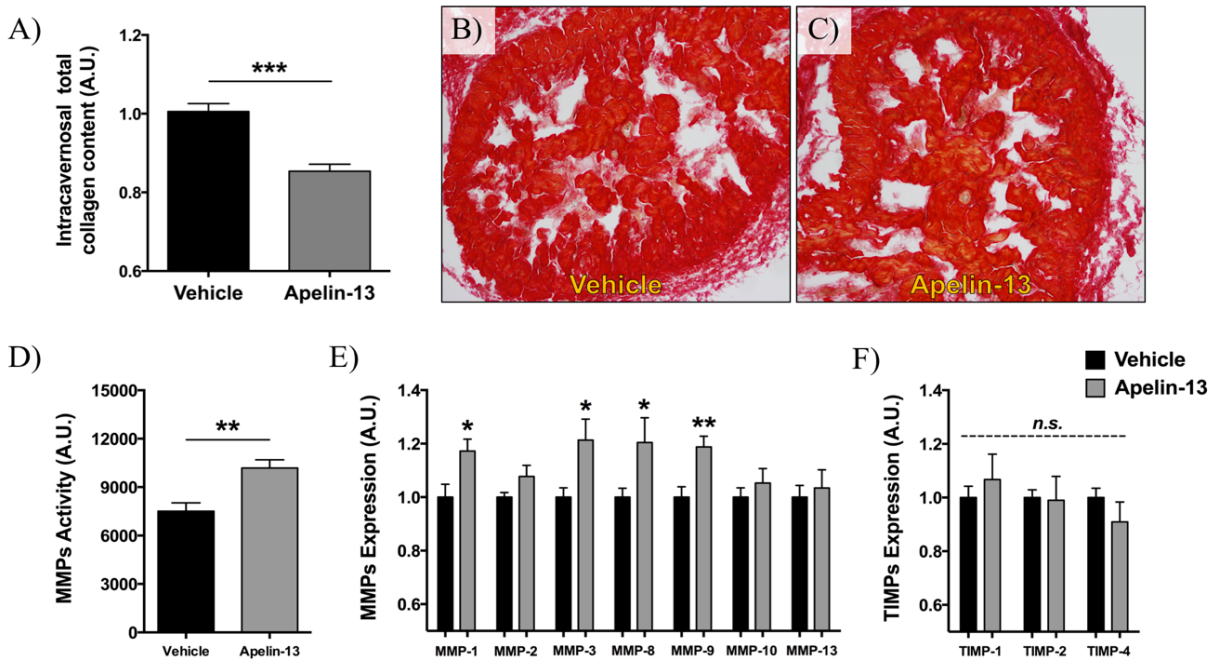

Figure 2:2 - Treatment with Apelin-13 strongly reduces hypercholesterolemia-induced cavernosal fibrosis by increasing total activity and protein expression of MMPs. Collagen content was assessed by Sirius red staining in penis from ApoE-/- mice treated or not with Apelin-13. Total MMPs activity was evaluated by MMP activity assay. MMP-1, -2, -3, -8, -9, -10, -13 and TIMP-1, -2, -4 expression levels were assessed by human MMP Antibody Array-Membrane. A) Quantification of intracavernosal total collagen content (n=12). B) and C) Representative images of corpus cavernosum from ApoE-/- mice sections under bright field microscope showing the collagen content (red staining) of untreated (vehicle) and Apelin-13-treated D) Quantification of MMPs activity (n=6). E) and F) Quantification of MMPs (E) and TIMPs (F) expression normalized by controls (n=6). * p<0.05, ** p<0.01, *** p<0.001 (Unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative collagen content or fluorescence in arbitrary unit (A.U.).

Apelin-13 inhibits TGF-β-induced fibroblast differentiation and collagen production

The differentiation of fibroblasts to myofibroblasts under inflammatory response has been pointed as an important event in fibrosis progression[10]. In order to understand the mechanism by which Apelin-13 reduces intracavernosal fibrosis, we next evaluated Apelin-13 action on TGF-β-induced fibroblast differentiation and collagen production. For that, NIH3T3 fibroblasts were cultured and stimulated with TGF-β to induce differentiation and collagen production. As expected, TGF-β stimulation induced a pronounced increase in collagen production and fibroblast to myofibroblast differentiation (assessed by α-SMA expression, a myofibroblast marker). Strikingly, pretreatment with Apelin-13 significantly reduced α-SMA expression assessed by immunostaining (Figure 2:3 A and B) and Western blot (Figure 2:3 D and E). Furthermore, augmented total collagen production induced by TGF-β was completely reversed by Apelin-13 pretreatment, when evaluated by Sirius red staining assay (Figure 2:3 C), while TGF-β-induced increase in collagen type-I

expression was significantly attenuated (Figure 2:3 H and I). Together, these results suggest that apelin-13 decreases collagen production due to, at least in part, inhibition of the pro-fibrotic inflammatory response.

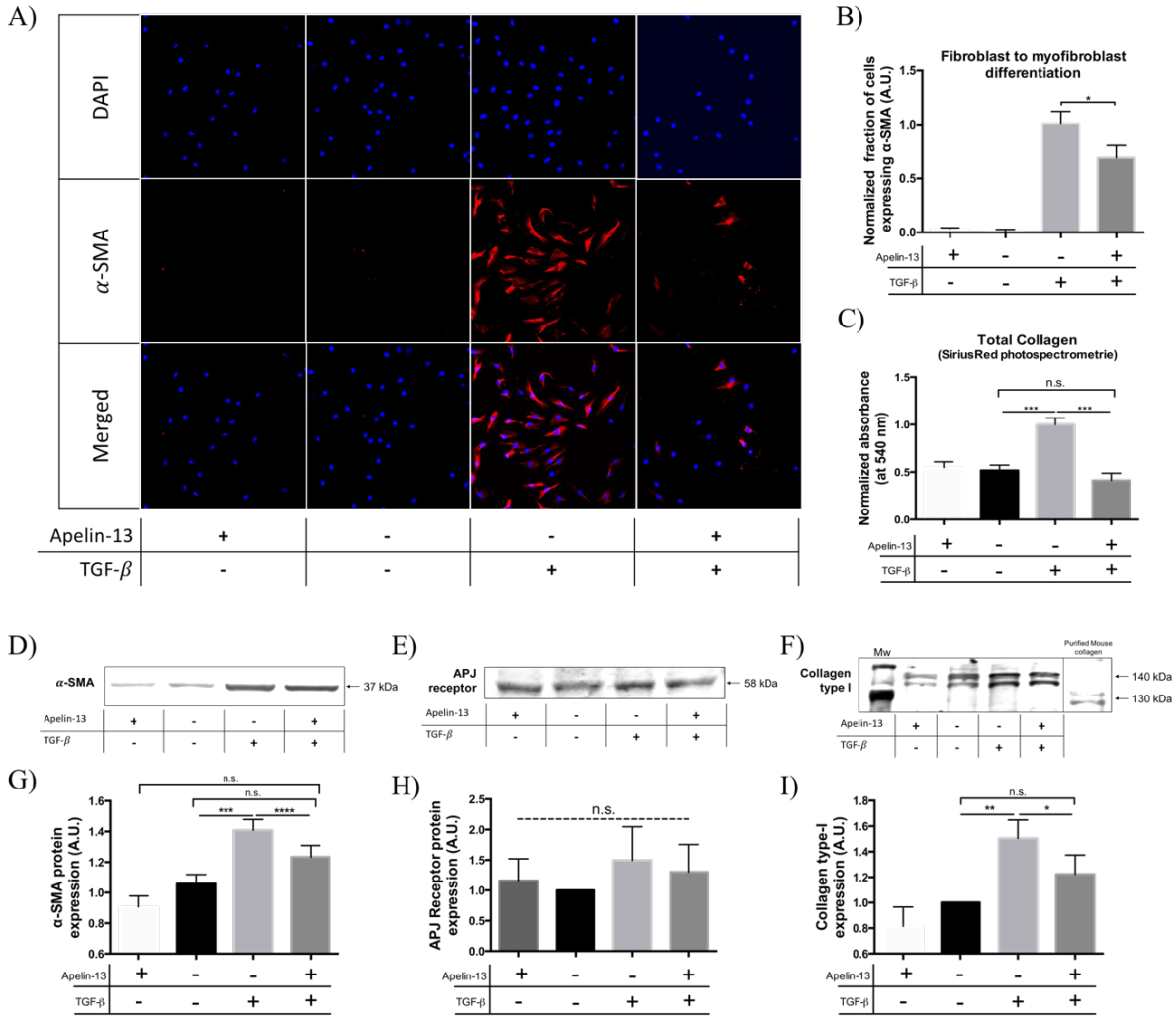

Figure 2:3 - **Apelin-13 pretreatment significantly reduces fibroblast to myofibroblast differentiation and collagen production in NIH3T3 fibroblasts stimulated by TGF-β.** The number of cells undergoing differentiation from fibroblast to myofibroblast after TGF-β stimulation and pretreatment or not with Apelin-13 was assessed by immunostaining and western blot. The total collagen produced by the different cultures treated and non-treated was determined by Picro-Sirius Red staining of the total collagen, elution of the dye and photospectrometry. In addition, the expression of Collagen type-I was assessed by western blot. A) Representative immunofluorescence images of cells showing the nucleus in blue, α-SMA in red and the merged images. Cells expressing α-SMA are differentiated myofibroblast. B) Quantification of the number of differentiated myofibroblast compared to total number of cells after TGF-β stimulation and pretreatment or not with Apelin-13 (n=15). C) Quantification of the optical absorbance of the eluted Sirius Red dye at 540nm for each culture after TGF-β stimulation and pretreatment or not with Apelin-13 (n=12). The absorbance is directly representative of the dye concentration in each sample reflecting the total collagen production. D) and E) Representative images of the Western blotting of APJ receptor and α-SMA expression showing a specific single band at the expected molecular weight. F) Representative image of the Western blotting of Collagen type I showing two bands at the expected molecular weight representing the α1 and α2 fibers of the type I collagen. Mouse endogenous collagen type-I was used as positive control. The molecular weight of the two subunits of the endogenous collagen is slightly lower ($\sim$130 kDa) due to post-translational modifications and cleavage of the pro-collagen fibers when secreted [35]. G), H) and I) Quantification of APJ receptor, α-SMA and Collagen Type-I expression respectively, in fibroblast stimulated with TGF-β and pretreated or not with Apelin-13 (n=9). * p<0.05, ** p<0.01, *** p<0.001 (Unpaired Student t-test). Each column represents the mean ± SEM. Mw = Molecular weight markers.

Of note, the presence of APJ receptor expression in NIH3T3 cell line was confirmed by western blot (Figure 2:3 F and G), but not affected by Apelin-13 treatment nor TGF-β stimulation. Moreover, pretreatment with only Apelin-13 did not affect fibroblast differentiation or collagen production.

Apelin-13 increases ERK phosphorylation, an important downstream kinase for TGF-β signaling

ERK MAP kinase is a well-known signaling cascade of the Smad-independent TGF-β pathways[36,37]. A key component of the signaling cascade activation is the phosphorylation of the ERK1 and ERK2 kinases. Analysis of the ratio between phospho-ERK1/2 proteins and total ERK1/2 expression showed a significant increase of the phosphorylated form of ERK1/2 when pretreated with Apelin-13 followed by TGF-β stimulation (Figure 2:4 A and B, 194% increase compared with the non-treated group, *P*=0.0001). Surprisingly, the ratio between P-ERK1/2 and ERK1/2 was not affected by TGF-β stimulation alone. These results suggest that the anti-fibrotic effect of Apelin-13 might be due to blocking or alteration of the TGF-β signaling through activation of ERK1/2 phosphorylation.

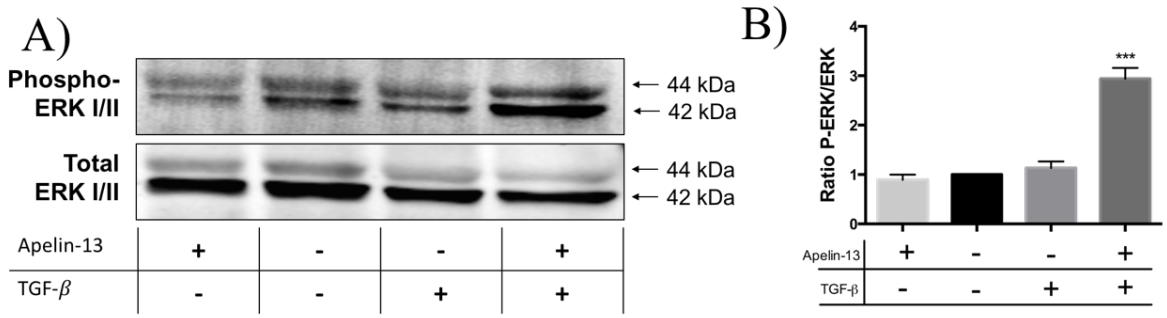

Figure 2:4 - Apelin-13 pretreatment in fibroblasts stimulated by TGF-β induces phosphorylation of ERK1/2. A) Representative images of the Western blotting of phosphorylated-ERK1/2 and total-ERK1/2 showing two expected bands at 42 and 44 kDa. B) Quantification of the ratio between phosphorylated and total ERK1/2 expression (phosphorylation sites: ERK1-T202 and ERK2-T185) (n=9). *** p<0.001 (Unpaired Student t-test). Each column represents the mean ± SEM.

Chronic Apelin-13 treatment does not affect the oxidative stress balance

It was previously reported that acute Apelin-13 injection restores erectile function of hypercholesterolemic mice by increasing the NO bioavailability and reducing ROS production, however, ROS and NO levels were reported to return to normal levels within a few days [29]. Therefore, we evaluated whether this beneficial action would take place at long-term treatment. In contrast to acute injections, 3 weeks of Apelin-13 treatment did not change the intracavernosal ROS content compared to vehicle (Figure 2:5 C, E and F), while no statistical difference was observed in the protein expression level of eNOS and nNOS in the penis of hypercholesterolemic mice treated with Apelin-13 compared to vehicle (Figure 2:5 A, B and D).

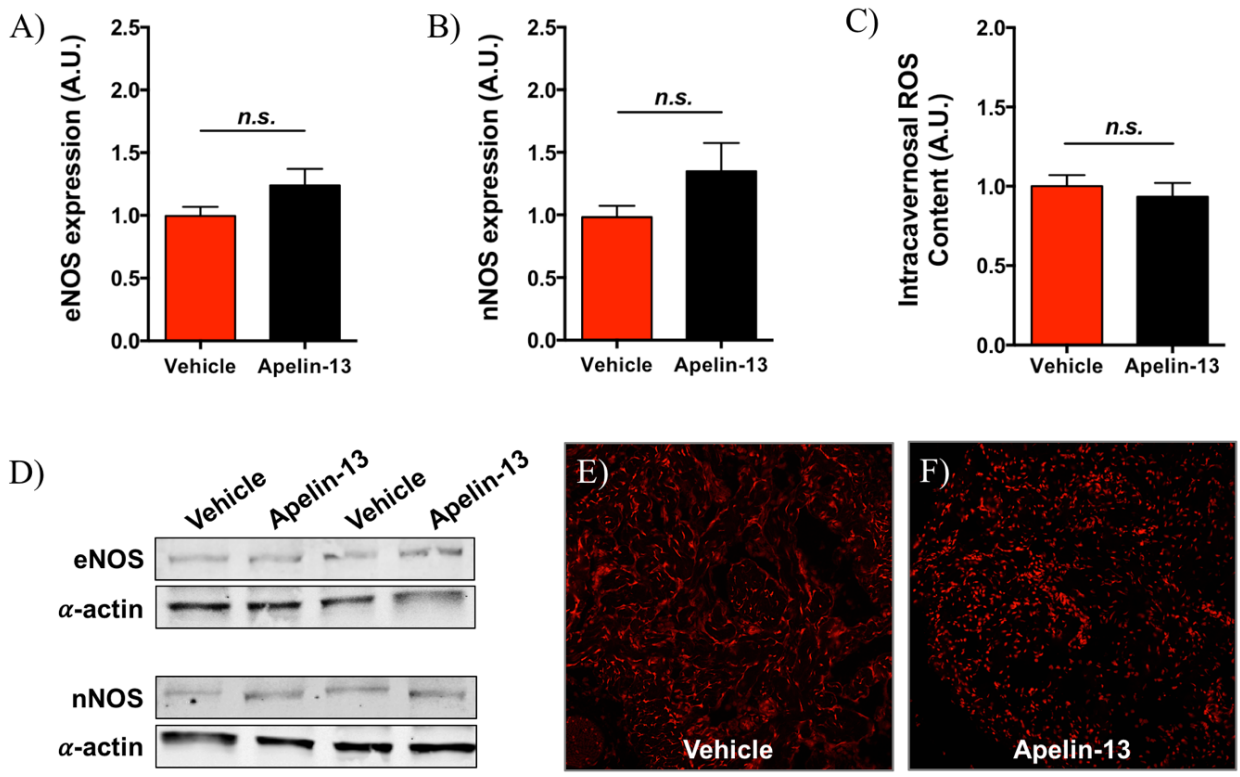

Figure 2:5 - Chronic treatment with Apelin-13 does not affect eNOS, nNOS expression and ROS production in penis from hypercholesterolemic ApoE-/- mice. Apelin-13 treatment does not affect the eNOS and nNOS expression levels in corpus cavernosum of ApoE-/- mice. A) and B) Quantification of eNOS (A) and nNOS (n=12) (B) protein expression was performed by Western blotting of total proteins extracted from penis of vehicle- or Apelin-13-treated ApoE-/- mouse. C) Quantification of intracaverosal ROS production (n=15). D) Representative Western blotting images of eNOS and nNOS with α-actin control. E) and F) Representative DHE staining images of treated and non-treated corpus cavernosum section of ApoE-/- mouse representing the intracavernosal ROS content. Each column represents the mean ± SEM of relative protein expression or relative fluorescence in arbitrary unit (A.U.).

Anti-fibrotic action of Apelin-13 was not associated with reduction in atherosclerotic plaque size

Despite the 3 weeks treatment with Apelin-13 which significantly decreased the total cholesterol, LDL and free fatty acids serum levels of hypercholesterolemic ApoE$^{-/-}$ mice (Figure 2:6 C, D and F), no significant changes were observed in the size of atherosclerotic plaques developed in the aortic arch and thoracic aorta of Apelin-13-treated group compared to untreated mice (Figure 2:6 A and B). This data suggests that the beneficial actions of Apelin-13 on the cavernosal fibrosis might not be associated with atherosclerotic plaque reduction.

No significant difference was observed in HDL, glucose or triglyceride levels (Figure 2:6 E, G and H).

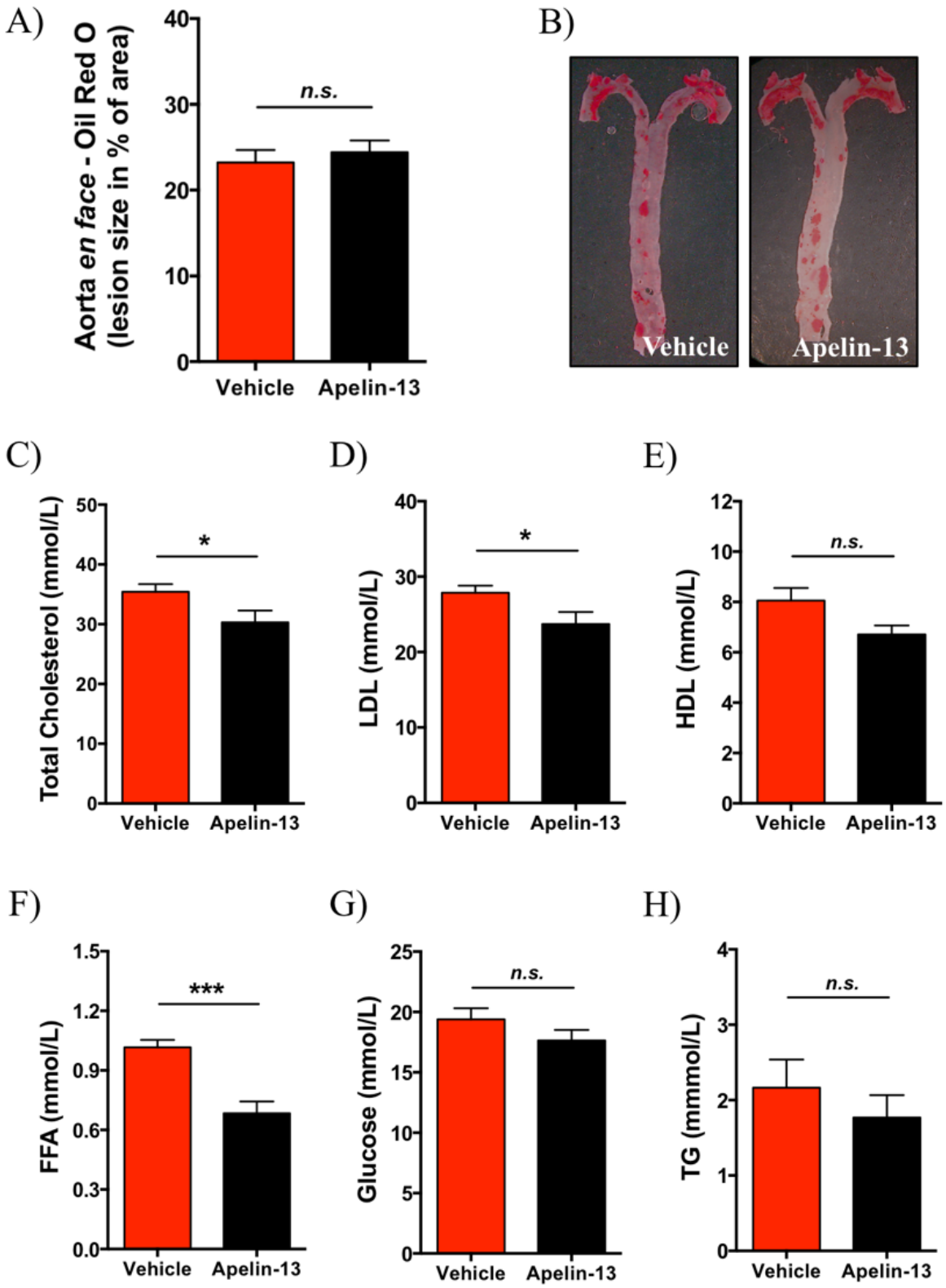

Figure 2:6 - **Apelin-13 treatment improves the total cholesterol, LDL and free fatty acids serum levels of hypercholesteremic mice, without affecting atherosclerotic aortic plaque lesion.** Blood serum profile and aortic plaque of ApoE$^{-/-}$ mice treated or not with Apelin-13 were measured. A) Quantification of the atherosclerotic plaque size was obtained using Oil Red O staining and it is expressed in % of the total aortic area (n=16). B) Representative bright field images of the aorta *en face* with atherosclerotic plaques stained in red from Apelin-13-treated or non-treated hypercholesterolemic mice. C, D, E, F, G and H) Total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL), free fatty acids (FFA), glucose and triglycerides levels of blood serum collected from Apelin-13-treated or non-treated hypercholesterolemic mice (n=15). * $p<0.05$ (Unpaired Student t-test). Each column represents the mean ± SEM of the lesion size in % of area or the serum concentration in mmol/L.

2.5 Discussion

Our study brings the first evidence that chronic administration of Apelin-13 modulates and protects penile structures within the corpus cavernosum of hypercholesterolemic ApoE$^{-/-}$ mice, which is associated with an enhanced MMP expression and activity. Furthermore, we observed that Apelin-13 reduced the fibroblast to myofibroblast differentiation and collagen expression, possibly through an ERK1/2 dependent signaling mechanism.

Hypercholesterolemia is largely known to induce ED[38]. This condition impairs penile erection mainly due to increased oxidative stress within the corpus cavernosum[39]. Moreover, in a long-term, it may induce remodeling of the corpus cavernosum, impairing the mechanical properties of the penis[8,40]. In the present study, a well-established model of hypercholesterolemia inducing ED was used[30]. This mouse model presents erection deficiency, which has been linked to an increased oxidative stress, reduction of NO release and exaggerated collagen deposition within the corpus cavernosum and penile arteries[30,41]. Therefore, this model exhibits a phenotype closely related to the human ED associated with hypercholesterolemia[38,42]. Interestingly, here we reported that Apelin-13 treatment for 3 weeks significantly reduced the collagen content within the cavernosal tissues of this animal model compared to non-treated animals, which is in accordance with previous studies evaluating the anti-fibrotic action of Apelin-13 in other organs, such as heart[23], liver[24], kidney[26] and lungs[28]. The reduction in collagen content observed in this study was not associated with oxidative stress or atherosclerotic plaque reduction, although evidences reveal that augmented oxidative stress within the corpus cavernosum and atherosclerotic plaque formation play a central role in ED development[38,43]. Indeed, beyond the decrease of NO bioavailability and/or increasing smooth muscle contractile tonus, chronic oxidative stress and inflammation contribute to the exaggerated collagen deposition and penile fibrosis, worsen erectile function and lead to severe ED[1,38,43]. Several recent studies have shown that Apelin is able to mitigate ROS production and enhance NO production in different tissues[44–46]. In another study, *Kwon et al.* observed such beneficial effect of Apelin on ED when administered acutely to an hypercholesteremic model. The mechanism behind the beneficial effects of Apelin was mainly due to an acute activation of eNOS and increase in NO bioavailability, however, this beneficial effect was reported to be only transient[29]. In our study, the Apelin treatment neither reduced the size of atherosclerotic plaques nor changed the ROS production, or eNOS and nNOS expression in ApoE$^{-/-}$ mouse penis, but efficiently reduced collagen content. Therefore, it is plausible to assume that changes in NO bioavailability and ROS production are observed only immediately after the administration of Apelin, while this study indicates an additional beneficial effect of Apelin-13 in the corpus cavernosum when chronically administered.

The reduction of collagen content following Apelin treatment observed here could be attributed to a decrease of LDL levels and increase MMP's activity. Indeed, it has been shown that an improvement of serum lipid profile is associated with modulation of collagen synthesis, MMP-1 expression and MMP-1 activity in cultured fibroblasts[47]. In the current study, a significant increase of MMP-1, -3, -8 and -9 protein expression and activity was observed after Apelin treatment. In accordance with our results, overexpression of MMP-1, also named collagenase-1, was shown to reduce fibrosis in models of induced liver-fibrosis as well as in heart and muscle fibrosis[48,49]. Similarly, MMP-8 (Collagenase-2) cleaves interstitial collagen type-I and type-III, and its expression was able to reduce fibrotic tissues in the liver of rats even after the establishment of fibrosis[50]. It is suggested that MMP-8, in addition, decreases inflammation and modulate cytokine and fibroblast activation[12,51]. Contrarily, MMP-3 and MMP-9 are known to process latent TGF-β1 to its active form, inducing epithelial cells EMT and generating myofibroblast, contributing to a pro-fibrotic effect[12,51]. Historically, MMPs were thought to function mainly as enzymes that degrade structural components of the ECM, however, MMP's functions during fibrosis are not limited to effects on ECM turnover[51]. For example, MMP proteolysis creates space for cells to migrate, produces specific substrate-cleavage fragments with independent biological activity, regulates tissue architecture through effects on the ECM and activates, deactivates or modifies the activity of signaling molecules, both directly and indirectly[12]. Although the literature shows contradictory pro- or anti-fibrotic effects of these enzymes regarding different organs or animal models[5]19, based on our findings and previous publications, it is conceivable to assume that the anti-fibrotic effect of Apelin-13 observed in this study is correlated to a serum lipid profile improvement and an increase of MMP's activity and expression.

In addition to its effect on collagen remodeling through MMP's activation and increased expression, Apelin was shown to inhibit TGF-β-induced phenotypic differentiation of fibroblast to myofibroblasts, preventing extracellular collagen accumulation[23]. TGF-β is considered a major factor in the induction of fibrosis in numerous tissues, including corpus cavernosum[2]. TGF-β level was shown to increase in the corpus cavernosum of men with vasculogenic ED, especially in men with dyslipidemia or atherosclerosis[52]. In addition, TGF-β was shown to be involved in MMP's regulation through ERK1/2 activation[36,53], while TGF-β-induced Smad3 dependent signaling is necessary for collagen type-I expression[37]. Therefore, TGF-β appears to be a central modulator of fibrotic process and it is reasonable to speculate that the anti-fibrotic effect of Apelin-13 in the corpus cavernosum of hypercholesterolemic mice could also be associated with inhibition of TGF-β signaling. In this study, we observed that Apelin-13 pretreatment was able to block the TGF-β-induced NIH3T3 fibroblast activation, preventing their differentiation into myofibroblasts and inhibiting collagen production, which appeared to be mediated by ERK1/2 phosphorylation.

In this study, an increase of ERK1/2 phosphorylation was observed only under the combination of Apelin-13 and TGF-β stimulus. In contrast with previous studies[36,54], no variation of the phosphorylation of ERK1/2 was observed when treated with TGF-β alone. ERK is known to be an important non-canonical signaling pathway of TGF-β signaling[37,55] and it has been documented that ERK signaling is involved in collagen production and myofibroblast differentiation. Phosphorylation of ERK1/2 is then commonly reported as associated with a pro-fibrotic response[36,54], however, it was also shown that ERK1/2 activation is required for the cardio-protective effect of Apelin-13 after ischemia-reperfusion injury, as inhibition of ERK signaling pathway abrogated such beneficial effect[19], corroborating our findings. In addition, ERK1/2 was shown to directly phosphorylate Smads proteins to regulate their activities[36,55]. TGF-β signaling is a complex molecular pathway with differential role regarding the cell type and state, numerous parallel signaling branches and multiple crosstalk that have been reported between the different components of this signaling cascade[37,53,55,56]. An increased activation of ERK1/2 by Apelin-13 could then eventually modulate the Smads signaling cascade of TGF-β, inhibiting the fibroblast to myofibroblast differentiation and/or collagen expression (Figure 2:7), however, further study is required to confirm the complete molecular mechanism of Apelin on TGF-β signaling.

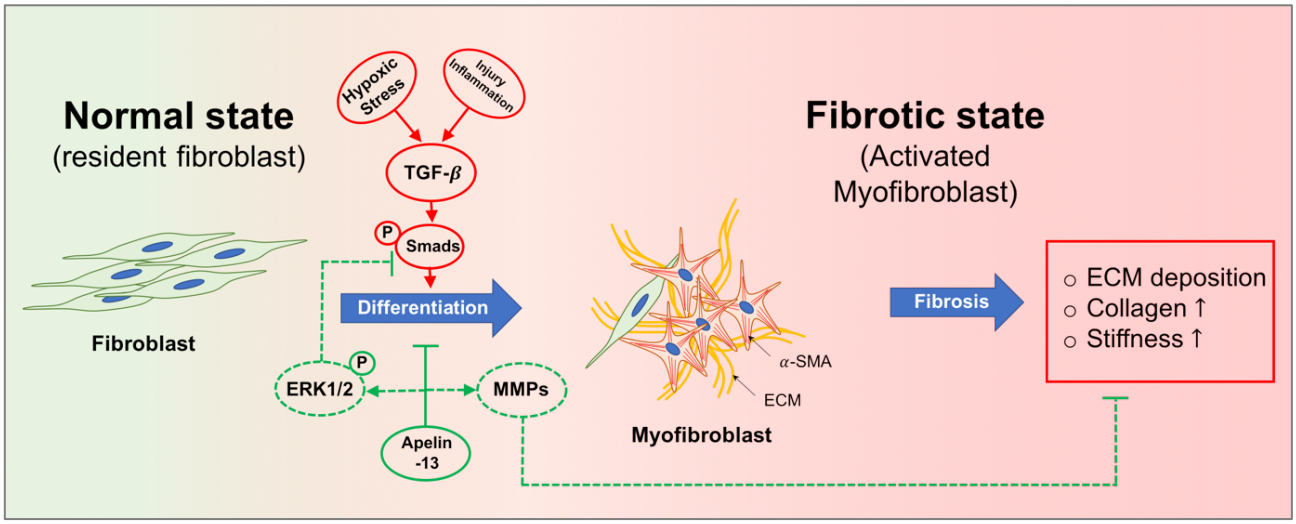

Figure 2:7 - Illustration of the possible molecular mechanism of Apelin-13 on the differentiation of fibroblast into activated hyper-secretory myofibroblast, leading to collagen expression and ECM fibers deposition involved in Fibrosis. Apelin-13 pretreatment inhibited the pro-fibrotic effect of TGF-β stimulation, preventing the differentiation of fibroblast to myofibroblast and the subsequent collagen production, possibly by the phosphorylation of ERK1/2 and the modulation of TGF-β downstream signal transducers kinases SMADs, and by increasing MMPs expression and activity that in turn modulate the ECM and collagen. ECM = Extracellular matrix, MMP = matrix metalloproteinase.

Although showing promising results of the protective effect of Apelin-13 on cavernosal structures, the major limitation of this study is that only molecular and immunohistochemical analysis of ApoE$^{-/-}$ mice corpus cavernosum samples were performed. Additional functional study addressing erectile response, *in vivo* and/or *ex vivo,* would bring extended validation regarding the relevance of such beneficial effect of Apelin-13 at long-term treatment and its potential in the management of ED.

2.6 Conclusion

In summary, our findings indicate that long-term treatment with Apelin-13 produces significant beneficial effects against the deleterious hypercholesterolemia-induced corpus cavernosum fibrosis. Apelin-13 behaves as a potent suppressor of increased collagen deposition within the cavernosal tissues, and such effect involves regulation of MMPs expression and activity and potentially the reduction of fibroblast to myofibroblast differentiation. Altogether, these results suggest an essential role of the Apelin system modulating cavernosal structure, indicating the Apelin/APJ system as a promising target for the development of new treatment against fibrosis-associated ED.

2.7 Conflict of Interest

None to be declared.

2.8 Acknowledgements

This research was funded by the Swiss National Science Foundation [SNSF-Grant 310030_156859 to Prof. N. Stergiopulos]. We thank the histology core facility of the EPFL Faculty of Life Science for helping with the histological sectioning and Sirius red staining.

2.9 Supplementary Material

Amongst its pro-fibrotic effect, TGF-β has been reported to affect multiple proteins expression, including some common "housekeeping" proteins like GAPDH or tubulin-β in fibroblasts, thenceforth negatively influencing western blot analysis of protein expression when normalized by a loading control[57–59]. In our case, GAPDH expression was significantly affected by TGF-β and Apelin-13 stimulation in NIH3T3 fibroblast cultures (Figure 1:8 C and D - supplementary material – *P*=0.0080). The western blot data were then normalized regarding the non-treated group, while the accuracy of the protein loading and transfer quality for each blot were assessed by Ponceau S staining (Figure 1:8 E and F), a more stable control as suggested by previous studies[60,61].

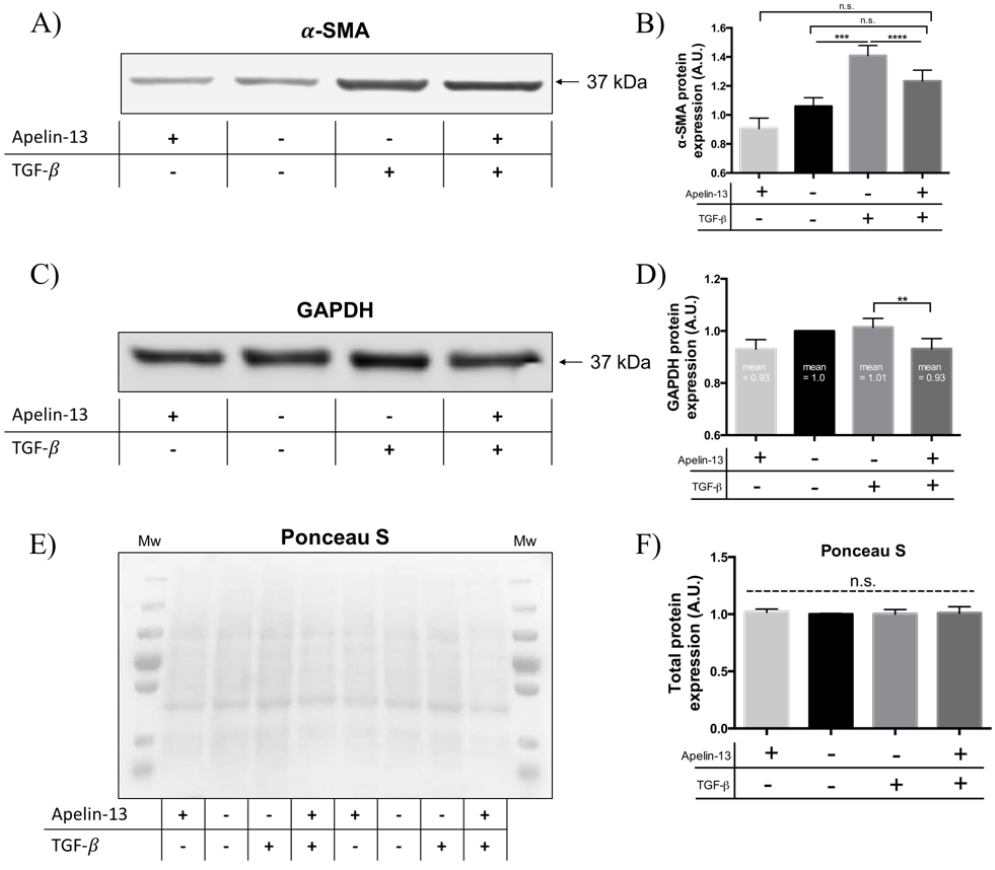

Figure 2:8 - Expression of GAPDH was significantly affected by TGF-β and Apelin-13 treatment in NIH 3T3 fibroblast cultures, defending the use of Ponceau S staining for total protein expression as a loading control in Western blotting analysis. A) Representative image of the Western blotting of α-SMA showing clear visual difference of expression between control and treated groups. B) Quantification of α-SMA expression. The possible errors induced by different levels of proteins loading or transfer issues are minimized by division of each result by the total amount of protein determined by Ponceau S staining. C) Representative image of the Wester blotting of GAPDH showing an increased expression of the housekeeping protein when stimulated with TGF-β compared with Apelin-13 pretreatment. D) Quantification of the GAPDH expression showing significant alteration of GAPDH expression between groups. E) Representative scan image of the Ponceau S total protein staining. F) Quantification of the total protein expression for each fibroblast culture group stimulated with TGF-β and pretreated or not with Apelin-13. * p<0.05, ** p<0.01, *** p<0.001 (Unpaired Student t-test). Each column represents the mean ± SEM. Mw = Molecular weight markers.

2.10 References

(1) El-Sakka, A. I. Reversion of Penile Fibrosis: Current Information and a New Horizon. Arab J. Urol. 2011, 9 (1), 49–55. https://doi.org/10.1016/j.aju.2011.03.013.

(2) Gonzalez-Cadavid, N. F. Mechanisms of Penile Fibrosis. J. Sex. Med. 2009, 6 (Supplement 3), 353–362. https://doi.org/10.1111/j.1743-6109.2008.01195.x.

(3) Behr-Roussel, D.; Bernabe, J.; Compagnie, S.; Rupin, A.; Verbeuren, T. J.; Alexandre, L.; Giuliano, F. Distinct Mechanisms Implicated in Atherosclerosis-Induced Erectile Dysfunction in Rabbits. Atherosclerosis 2002, 162 (2), 355–362.

(4) Yesilli, C.; Yaman, Ö.; Anafarta, K. Effect of Experimental Hypercholesterolemia on Cavernosal Structures. Urology 2001, 57, 1184–1188. https://doi.org/10.1016/S0090-4295(01)00974-8.

(5) Grosche, J.; Meißner, J.; Eble, J. A. More than a Syllable in Fib-ROS-Is: The Role of ROS on the Fibrotic Extracellular Matrix and on Cellular Contacts. Mol. Aspects Med. https://doi.org/10.1016/j.mam.2018.03.005.

(6) Kisseleva, T.; Brenner, D. A. Mechanisms of Fibrogenesis. Exp. Biol. Med. Maywood NJ 2008, 233 (2), 109–122. https://doi.org/10.3181/0707-MR-190.

(7) Musicki, B.; Liu, T.; Lagoda, G. A.; Strong, T. D.; Sezen, S. F.; Johnson, J. M.; Burnett, A. L. Hypercholesterolemia-Induced Erectile Dysfunction: Endothelial Nitric Oxide Synthase (ENOS) Uncoupling in the Mouse Penis by NAD(P)H Oxidase. J. Sex. Med. 2010, 7 (9), 3023–3032. https://doi.org/10.1111/j.1743-6109.2010.01880.x.

(8) El-Sakka, A. I.; Yassin, A. A. Amelioration of Penile Fibrosis: Myth or Reality. J. Androl. 2010, 31 (4), 324–335. https://doi.org/10.2164/jandrol.109.008730.

(9) Dean, R. C.; Lue, T. F. Physiology of Penile Erection and Pathophysiology of Erectile Dysfunction. Urol. Clin. North Am. 2005, 32 (4), 379–395.

(10) Hinz, B.; Gabbiani, G. Fibrosis: Recent Advances in Myofibroblast Biology and New Therapeutic Perspectives. F1000 Biol. Rep. 2010, 2. https://doi.org/10.3410/B2-78.

(11) Hinz, B.; Phan, S. H.; Thannickal, V. J.; Galli, A.; Bochaton-Piallat, M.-L.; Gabbiani, G. The Myofibroblast. Am. J. Pathol. 2007, 170 (6), 1807–1816. https://doi.org/10.2353/ajpath.2007.070112.

(12) Page-McCaw, A.; Ewald, A. J.; Werb, Z. Matrix Metalloproteinases and the Regulation of Tissue Remodelling. Nat. Rev. Mol. Cell Biol. 2007, 8 (3), 221–233. https://doi.org/10.1038/nrm2125.

(13) Murphy, G. Tissue Inhibitors of Metalloproteinases. Genome Biol. 2011, 12 (11), 233. https://doi.org/10.1186/gb-2011-12-11-233.

(14) Tatemoto, K.; Hosoya, M.; Habata, Y.; Fujii, R.; Kakegawa, T.; Zou, M. X.; Kawamata, Y.; Fukusumi, S.; Hinuma, S.; Kitada, C.; Kurokawa, T.; Onda, H.; Fujino, M. Isolation and Characterization of a Novel Endogenous Peptide Ligand for the Human APJ Receptor. Biochem. Biophys. Res. Commun. 1998, 251 (2), 471–476. https://doi.org/10.1006/bbrc.1998.9489.

(15) Lee, D. K.; Cheng, R.; Nguyen, T.; Fan, T.; Kariyawasam, A. P.; Liu, Y.; Osmond, D. H.; George, S. R.; O'Dowd, B. F. Characterization of Apelin, the Ligand for the APJ Receptor. J. Neurochem. 2000, 74 (1), 34–41.

(16) Tatemoto, K.; Takayama, K.; Zou, M. X.; Kumaki, I.; Zhang, W.; Kumano, K.; Fujimiya, M. The Novel Peptide Apelin Lowers Blood Pressure via a Nitric Oxide-Dependent Mechanism. Regul. Pept. 2001, 99 (2–3), 87–92.

(17) Hosoya, M.; Kawamata, Y.; Fukusumi, S.; Fujii, R.; Habata, Y.; Hinuma, S.; Kitada, C.; Honda, S.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. Molecular and Functional Characteristics of APJ. Tissue Distribution of MRNA and Interaction with the Endogenous Ligand Apelin. J. Biol. Chem. 2000, 275 (28), 21061–21067. https://doi.org/10.1074/jbc.M908417199.

(18) Kawamata, Y.; Habata, Y.; Fukusumi, S.; Hosoya, M.; Fujii, R.; Hinuma, S.; Nishizawa, N.; Kitada, C.; Onda, H.; Nishimura, O.; Fujino, M. Molecular Properties of Apelin: Tissue Distribution and Receptor Binding. Biochim. Biophys. Acta 2001, 1538 (2–3), 162–171.

(19) Simpkin, J. C.; Yellon, D. M.; Davidson, S. M.; Lim, S. Y.; Wynne, A. M.; Smith, C. C. T. Apelin-13 and Apelin-36 Exhibit Direct Cardioprotective Activity against Ischemiareperfusion Injury. Basic Res. Cardiol. 2007, 102 (6), 518. https://doi.org/10.1007/s00395-007-0671-2.

(20) Quazi, R.; Palaniswamy, C.; Frishman, W. H. The Emerging Role of Apelin in Cardiovascular Disease and Health: Cardiol. Rev. 2009, 17 (6), 283–286. https://doi.org/10.1097/CRD.0b013e3181b3fe0d.

(21) Falcão-Pires, I.; Ladeiras-Lopes, R.; Leite-Moreira, A. F. The Apelinergic System: A Promising Therapeutic Target. Expert Opin. Ther. Targets 2010, 14 (6), 633–645. https://doi.org/10.1517/14728221003752743.

(22) Japp, A. G.; Newby, D. E. The Apelin–APJ System in Heart Failure: Pathophysiologic Relevance and Therapeutic Potential. Biochem. Pharmacol. 2008, 75 (10), 1882–1892. https://doi.org/10.1016/j.bcp.2007.12.015.

(23) Pchejetski, D.; Foussal, C.; Alfarano, C.; Lairez, O.; Calise, D.; Guilbeau-Frugier, C.; Schaak, S.; Seguelas, M.-H.; Wanecq, E.; Valet, P.; Parini, A.; Kunduzova, O. Apelin Prevents Cardiac Fibroblast Activation and Collagen Production through Inhibition of Sphingosine Kinase 1. Eur. Heart J. 2012, 33 (18), 2360–2369. https://doi.org/10.1093/eurheartj/ehr389.

(24) Melgar-Lesmes, P.; Casals, G.; Pauta, M.; Ros, J.; Reichenbach, V.; Bataller, R.; Morales-Ruiz, M.; Jimenez, W. Apelin Mediates the Induction of Profibrogenic Genes in Human Hepatic Stellate Cells. Endocrinology 2010, 151 (11), 5306–5314. https://doi.org/10.1210/en.2010-0754.

(25) Kisseleva, T.; Brenner, D. A. Anti-Fibrogenic Strategies and the Regression of Fibrosis. Best Pract. Res. Clin. Gastroenterol. 2011, 25 (2), 305–317. https://doi.org/10.1016/j.bpg.2011.02.011.

(26) Chen, H.; Wan, D.; Wang, L.; Peng, A.; Xiao, H.; Petersen, R. B.; Liu, C.; Zheng, L.; Huang, K. Apelin Protects against Acute Renal Injury by Inhibiting TGF-B1. Biochim. Biophys. Acta BBA - Mol. Basis Dis. 2015, 1852 (7), 1278–1287. https://doi.org/10.1016/j.bbadis.2015.02.013.

(27) Nishida, M.; Okumura, Y.; Oka, T.; Toiyama, K.; Ozawa, S.; Itoi, T.; Hamaoka, K. The Role of Apelin on the Alleviative Effect of Angiotensin Receptor Blocker in Unilateral Ureteral Obstruction-Induced Renal Fibrosis. Nephron Extra 2012, 2 (1), 39–47. https://doi.org/10.1159/000337091.

(28) Andersen, C. U.; Hilberg, O.; Mellemkjær, S.; Nielsen-Kudsk, J. E.; Simonsen, U. Apelin and Pulmonary Hypertension. Pulm. Circ. 2011, 1 (3), 334–346. https://doi.org/10.4103/2045-8932.87299.

(29) Kwon, M.-H.; Tuvshintur, B.; Kim, W. J.; Jin, H.-R.; Yin, G. N.; Song, K.-M.; Choi, M. J.; Kwon, K.-D.; Batbold, D.; Ryu, J.-K.; Suh, J.-K. Expression of the Apelin–APJ Pathway and Effects on Erectile Function in a Mouse Model of Vasculogenic Erectile Dysfunction. J. Sex. Med. 2013, 10 (12), 2928–2941. https://doi.org/10.1111/jsm.12158.

(30) Xie, D.; Odronic, S. I.; Wu, F.; Pippen, A. M.; Donatucci, C. F.; Annex, B. H. A Mouse Model of Hypercholesterolemia-Induced Erectile Dysfunction. J. Sex. Med. 2007, 4 (4, Part 1), 898–907. https://doi.org/10.1111/j.1743-6109.2007.00518.x.

(31) Fraga-Silva, R. A.; Costa-Fraga, F. P.; Montecucco, F.; Sturny, M.; Faye, Y.; Mach, F.; Pelli, G.; Shenoy, V.; da Silva, R. F.; Raizada, M. K.; Santos, R. A. S.; Stergiopulos, N. Diminazene Protects Corpus Cavernosum Against Hypercholesterolemia-Induced Injury. J. Sex. Med. 2015, 12 (2), 289–302. https://doi.org/10.1111/jsm.12757.

(32) Fraga-Silva, R. A.; Costa-Fraga, F. P.; Savergnini, S. Q.; De Sousa, F. B.; Montecucco, F.; da Silva, D.; Sinisterra, R. D.; Mach, F.; Stergiopulos, N.; da Silva, R. F.; Santos, R. A. S. An Oral Formulation of Angiotensin-(1-7) Reverses Corpus Cavernosum Damages Induced by Hypercholesterolemia. J. Sex. Med. 2013, 10 (10), 2430–2442. https://doi.org/10.1111/jsm.12262.

(33) Fraga-Silva, R. A.; Costa-Fraga, F. P.; Faye, Y.; Sturny, M.; Santos, R. A. S.; da Silva, R. F.; Stergiopulos, N. An Increased Arginase Activity Is Associated with Corpus Cavernosum Impairment Induced by Hypercholesterolemia. J. Sex. Med. 2014, 11 (5), 1173–1181. https://doi.org/10.1111/jsm.12482.

(34) Xu, Q.; Norman, J. T.; Shrivastav, S.; Lucio-Cazana, J.; Kopp, J. B. In Vitro Models of TGF-Beta-Induced Fibrosis Suitable for High-Throughput Screening of Antifibrotic Agents. Am. J. Physiol. Renal Physiol. 2007, 293 (2), F631-640. https://doi.org/10.1152/ajprenal.00379.2006.

(35) Risteli, J.; Niemi, S.; Kauppila, S.; Melkko, J.; Risteli, L. Collagen Propeptides as Indicators of Collagen Assembly. Acta Orthop. Scand. Suppl. 1995, 266, 183–188.

(36) Zhang, Y. E. Non-Smad Pathways in TGF-β Signaling. Cell Res. 2009, 19 (1), 128–139. https://doi.org/10.1038/cr.2008.328.

(37) Leask, A.; Abraham, D. J. TGF-β Signaling and the Fibrotic Response. FASEB J. 2004, 18 (7), 816–827. https://doi.org/10.1096/fj.03-1273rev.

(38) Musicki, B.; Liu, T.; Lagoda, G. A.; Strong, T. D.; Sezen, S. F.; Johnson, J. M.; Burnett, A. L. Hypercholesterolemia-Induced Erectile Dysfunction: Endothelial Nitric Oxide Synthase (ENOS) Uncoupling in the Mouse Penis by NAD(P)H Oxidase. J. Sex. Med. 2010, 7 (9), 3023–3032. https://doi.org/10.1111/j.1743-6109.2010.01880.x.

(39) Bivalacqua, T. J.; Usta, M. F.; Champion, H. C.; Kadowitz, P. J.; Hellstrom, W. J. G. Endothelial Dysfunction in Erectile Dysfunction: Role of the Endothelium in Erectile Physiology and Disease. J. Androl. 2003, 24 (S6), S17–S37. https://doi.org/10.1002/j.1939-4640.2003.tb02743.x.

(40) Montorsi, F.; Briganti, A.; Salonia, A.; Deho', F.; Zanni, G.; Cestari, A.; Guazzoni, G.; Rigatti, P.; Stief, C. The Ageing Male and Erectile Dysfunction. BJU Int. 2003, 92 (5), 516–520. https://doi.org/10.1046/j.1464-410X.2003.04378.x.

(41) Chung, E.; De Young, L.; Brock, G. B. Investigative Models in Erectile Dysfunction: A State-of-the-Art Review of Current Animal Models. J. Sex. Med. 2011, 8 (12), 3291–3305. https://doi.org/10.1111/j.1743-6109.2011.02505.x.

(42) Behr-Roussel, D.; Darblade, B.; Oudot, A.; Compagnie, S.; Bernabé, J.; Alexandre, L.; Giuliano, F. ORIGINAL RESEARCH—BASIC SCIENCE: Erectile Dysfunction in Hypercholesterolemic Atherosclerotic Apolipoprotein E Knockout Mice. J. Sex. Med. 2011, 3 (4), 596–603. https://doi.org/10.1111/j.1743-6109.2006.00282.x.

(43) Fraga-Silva, R. A.; Costa-Fraga, F. P.; Montecucco, F.; Faye, Y.; Savergnini, S. Q.; Lenglet, S.; Mach, F.; Steffens, S.; Stergiopulos, N.; Souza dos Santos, R. A.; da Silva, R. F. Treatment with CB2 Agonist JWH-133 Reduces Histological Features Associated with Erectile Dysfunction in Hypercholesterolemic Mice. Clin. Dev. Immunol. 2013, 2013. https://doi.org/10.1155/2013/263846.

(44) Chun, H. J.; Ali, Z. A.; Kojima, Y.; Kundu, R. K.; Sheikh, A. Y.; Agrawal, R.; Zheng, L.; Leeper, N. J.; Pearl, N. E.; Patterson, A. J.; Anderson, J. P.; Tsao, P. S.; Lenardo, M. J.; Ashley, E. A.; Quertermous, T. Apelin Signaling Antagonizes Ang II Effects in Mouse Models of Atherosclerosis. J. Clin. Invest. 2008, 118 (10), 3343–3354. https://doi.org/10.1172/JCI34871.

(45) Foussal, C.; Lairez, O.; Calise, D.; Pathak, A.; Guilbeau-Frugier, C.; Valet, P.; Parini, A.; Kunduzova, O. Activation of Catalase by Apelin Prevents Oxidative Stress-Linked Cardiac Hypertrophy. FEBS Lett. 2010, 584 (11), 2363–2370. https://doi.org/10.1016/j.febslet.2010.04.025.

(46) Than, A.; Zhang, X.; Leow, M. K.-S.; Poh, C. L.; Chong, S. K.; Chen, P. Apelin Attenuates Oxidative Stress in Human Adipocytes. J. Biol. Chem. 2014, 289 (6), 3763–3774. https://doi.org/10.1074/jbc.M113.526210.

(47) Joseph, J.; Ranganathan, S.; Mehta, J. L. Low Density Lipoproteins Modulate Collagen Metabolism in Fibroblasts. J. Cardiovasc. Pharmacol. Ther. 2003, 8 (2), 161–166. https://doi.org/10.1177/107424840300800209.

(48) Iimuro, Y.; Nishio, T.; Morimoto, T.; Nitta, T.; Stefanovic, B.; Choi, S. K.; Brenner, D. A.; Yamaoka, Y. Delivery of Matrix Metalloproteinase-1 Attenuates Established Liver Fibrosis in the Rat. Gastroenterology 2003, 124 (2), 445–458. https://doi.org/10.1053/gast.2003.50063.

(49) Iimuro, Y.; Brenner, D. A. Matrix Metalloproteinase Gene Delivery for Liver Fibrosis. Pharm. Res. 2008, 25 (2), 249–258. https://doi.org/10.1007/s11095-007-9311-7.

(50) Siller-López, F.; Sandoval, A.; Salgado, S.; Salazar, A.; Bueno, M.; Garcia, J.; Vera, J.; Gálvez, J.; Hernández, I.; Ramos, M.; Aguilar-Cordova, E.; Armendariz-Borunda, J. Treatment with Human Metalloproteinase-8 Gene Delivery Ameliorates Experimental Rat Liver Cirrhosis. Gastroenterology 2004, 126 (4), 1122–1133. https://doi.org/10.1053/j.gastro.2003.12.045.

(51) Giannandrea, M.; Parks, W. C. Diverse Functions of Matrix Metalloproteinases during Fibrosis. Dis. Model. Mech. 2014, 7 (2), 193–203. https://doi.org/10.1242/dmm.012062.

(52) Ryu, J.-K.; Han, J.-Y.; Chu, Y.-C.; Song, S. U.; Lee, K.-H.; Yoon, S.-M.; Suh, J.-K.; Kim, S.-J. Expression of Cavernous Transforming Growth Factor-B1 and Its Type II Receptor in Patients with Erectile Dysfunction. Int. J. Androl. 2004, 27 (1), 42–49. https://doi.org/10.1046/j.0105-6263.2003.00447.x.

(53) Gomes, L. R.; Terra, L. F.; Wailemann, R. A.; Labriola, L.; Sogayar, M. C. TGF-B1 Modulates the Homeostasis between

MMPs and MMP Inhibitors through P38 MAPK and ERK1/2 in Highly Invasive Breast Cancer Cells. BMC Cancer 2012, 12, 26. https://doi.org/10.1186/1471-2407-12-26.

(54) Xiao, L.; Du, Y.; Shen, Y.; He, Y.; Zhao, H.; Li, Z. TGF-Beta 1 Induced Fibroblast Proliferation Is Mediated by the FGF-2/ERK Pathway. Front. Biosci. Landmark Ed. 2012, 17, 2667–2674.

(55) Derynck, R.; Zhang, Y. E. Smad-Dependent and Smad-Independent Pathways in TGF-β Family Signalling. Nature 2003, 425 (6958), 577–584. https://doi.org/10.1038/nature02006.

(56) Funaba, M.; Zimmerman, C. M.; Mathews, L. S. Modulation of Smad2-Mediated Signaling by Extracellular Signal-Regulated Kinase. J. Biol. Chem. 2002, 277 (44), 41361–41368. https://doi.org/10.1074/jbc.M204597200.

(57) Aldridge, G. M.; Podrebarac, D. M.; Greenough, W. T.; Weiler, I. J. The Use of Total Protein Stains as Loading Controls: An Alternative to High-Abundance Single-Protein Controls in Semi-Quantitative Immunoblotting. J. Neurosci. Methods 2008, 172 (2), 250–254. https://doi.org/10.1016/j.jneumeth.2008.05.003.

(58) Mathur, D.; Urena-Peralta, J. R.; Lopez-Rodas, G.; Casanova, B.; Coret-Ferrer, F.; Burgal-Marti, M. Bypassing Hazard of Housekeeping Genes: Their Evaluation in Rat Granule Neurons Treated with Cerebrospinal Fluid of Multiple Sclerosis Subjects. Front. Cell. Neurosci. 2015, 9. https://doi.org/10.3389/fncel.2015.00375.

(59) Glare, E.; Divjak, M.; Bailey, M.; Walters, E. SS-Actin and GAPDH Housekeeping Gene Expression in Asthmatic Airways Is Variable and Not Suitable for Normalising MRNA Levels. Thorax 2002, 57 (9), 765–770. https://doi.org/10.1136/thorax.57.9.765.

(60) Romero-Calvo, I.; Ocón, B.; Martínez-Moya, P.; Suárez, M. D.; Zarzuelo, A.; Martínez-Augustin, O.; de Medina, F. S. Reversible Ponceau Staining as a Loading Control Alternative to Actin in Western Blots. Anal. Biochem. 2010, 401 (2), 318–320. https://doi.org/10.1016/j.ab.2010.02.036.

(61) Eaton, S. L.; Roche, S. L.; Hurtado, M. L.; Oldknow, K. J.; Farquharson, C.; Gillingwater, T. H.; Wishart, T. M. Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting. PLOS ONE 2013, 8 (8), e72457. https://doi.org/10.1371/journal.pone.0072457.

Chapter 3 Novel Concept Enabling an Old Idea: A Flexible Electrode Array to Treat Neurogenic Erectile Dysfunction

Spyridon Skoufias[a*], Mikaël Sturny[b*], Rodrigo Fraga-Silva[b], Theodore G. Papaioannou[c], Nikolaos Stergiopoulos[b], Ioannis Adamakis[a], Constantinos A. Constantinides[a].

[a] *National and Kapodistrian University of Athens, Medical School, 1st Urology Dept., Laikon Hospital, Athens, Greece*

[b] *Ecole Polytechnique Fédérale De Lausanne, Institute of Bioengineering, Lausanne, Switzerland*

[c] *National and Kapodistrian University of Athens, Medical School, 1st Cardiology Dept., Biomedical Engineering Unit, Hippokration Hospital, Athens, Greece;*

* M. Sturny and S. Skoufias contributed equally to the work.

Running title: Neuromodulation for erectile dysfunction.

Manuscript published in the Journal of Sexual Medicine
(*J Sex Med*. 2018;15(11):1558-1569. doi:10.1016/j.jsxm.2018.09.003)

This version is the approved manuscript following peer review remarks.

Corresponding Authors: Nikolaos Stergiopulos, Prof.
Ecole Polytechnique Fédérale de Lausanne
Institute of Bioengineering,
Station 09, MED 3.2526, CH-1015, Lausanne, Switzerland
Phone: (+41) 21 693 83 46
Email: nikolaos.stergiopulos@epfl.ch

3.1 Abstract

Background: Many studies have shown that electrostimulation of the cavernosal nerve can induce and maintain penile erection. Based on these discoveries, neurostimulation to activate the erectile response has been considered a potential solution to treat erectile dysfunction (ED). However, despite recognized potential, this technology has not been further developed. The barrier is the complex anatomy of the human cavernous nerve, which challenges the intraoperative identification of the cavernosal nerves for electrodes placement.

Aim: To overcome this major barrier, we proposed a practical solution: a 2-dimensional flexible electrode array which can cover the entire plexus area, ensuring that at least one of the electrodes will be in optimal contact with the cavernosal nerve, without the need of intraoperative identification. The present study aims to evaluate this concept intraoperatively.

Methods: Twenty-four (24) patients enrolled for open radical prostatectomy were recruited. During the surgical procedures, the electrode array was positioned on the pelvic-plexus (on the prostatic apex or pelvic wall) and electrical stimulation was applied to induce penile erection. Penile erectile response was assessed by (i) visual change of penile tumescence and (ii) by a penile plethysmograph system.

Outcomes: Ability and success rate of evoking penile response by applying electrical stimulation using the developed electrode array.

Results: Electrical stimulation produced immediate penile response in all cases when tested before (on prostatic apex) or after prostate removal (on pelvic wall). Clear visual penile engorgement was observed in 75% of the cases, whereas 25% showed minimal to moderate penile tumescence. As expected, patients with lower IIEF-5 score presented a reduced response, while stimulation before prostate removal showed greater response than following removal. Interestingly, erectile response was potentiated by bilateral stimulation (circumference increase [mm]: 2.7 ± 1.02 vs. 8.2 ± 1.9, p = 0.01).

***Clinical Translation*:** These data bring sufficient proof of concept of a conceivable novel medical implant for the treatment of ED caused by mechanical nerve injury, such as prostatectomy and spinal cord injury.

***Strengths & Limitations*:** This is the first approach that can assure the optimal site stimulation of the erectogenic neuronal path within the lower pelvic area and overcome the major barrier of individual anatomical variability. However, since this study was performed intraoperatively in an acute scenario, further studies are needed to evaluate its chronic efficacy for clinical practice.

Conclusion: The flexible electrode array concept can ensure the electro-stimulation of erectogenic neuronal path when positioned on the prostate apex or pelvic floor.

Key words: Electrodes array; Erectile dysfunction; Neuromodulation; Neurostimulation; Post-prostatectomy; Spinal cord injury.

3.2 Introduction

Phosphodiesterase-5 (PDE5) inhibitors represents the first line in the treatment of ED, demonstrating substantial effectiveness and safety; however, these drugs are ineffective in at least 30% of patients[1]. As alternative, individuals that are non-responsive to PDE5 inhibitors mostly resort to intrapenial injection and penile implants. Intrapenial injection of prostaglandin E1 is effective in over 80% of patients, but is associated with considerable side effects, such as pain, hematoma, priapism, discomfort and penile fibrosis[2,3], while penile prosthesis requires an irreversible and destructive surgery, which often requires surgery revision[4]. Therefore, improved modalities in the treatment of ED are needed.

Scientific reports have shown that electrostimulation of the cavernosal nerve can induce and maintain penile erection in animals and humans[5–7]. Since then, neurostimulation to activate the erectile response has been considered as a potential solution for erectile dysfunction (ED), particularly for spinal cord injury and post-prostatectomy patients[6,7]. The principle is based on applying an electrostimulus using conductive electrodes placed in contact or close vicinity with the cavernosal nerve[6]. However, despite the recognized potential, this technology has not been further developed for use in clinical practice. The barrier is the complex anatomy of human cavernous nerve, which is embedded in the pelvic-plexus. Locating the optimal site for stimulation has proven to be rather difficult, since the human cavernous nerve travels from the pelvic-plexus to the penis through a complex anastomosis and it is not macroscopically visible, while, the cavernosal nerve pathway presents a strong variability between individuals[8–10]. In addition to intrinsic anatomical issues, difficulties faced during surgical procedure when assessing the pelvic cavity, such as presence of overlying tissue, blood obscuring the surgical field, and poor exposure due to bodily habitus, all contribute to the challenges faced with optimal location placement[11]. Taken together, the main difficulty in applying neurostimulation to treat ED is the identification of the cavernosal nerve within the surgical field, thus leading to difficulties in identifying optimal and specific location for electrode placement.

In pioneering efforts, *Lue et al.* used an oval electrode consisting of a stainless-steel mesh to apply neurostimulation on the neurovascular bundle posterolateral to the prostate[5], while *Burnett et al.* applied a curved tripolar electrode comprised of a central cathode and two adjacent anodes to be positioned on the pelvic neurovascular bundle[7]. These electrode designs aimed to trigger the proerectile nerve located within or near the neurovascular bundle. However, only half of the cases resulted in penile erectile response, which could be due to the fact that the cavernosal nerve may present different patterns along the prostate. Additionally, it is possible that the cavernosal nerve travels outside the vascular bundle[8,9,12]. Importantly, the large electrode surface area used in previous studies may lead to a diffusion of electrical stimulus and thus, reducing the activation potential, while smaller electrodes with minimal spacing would increase stimulation selectivity and produce greater activation potentials[13,14].

To overcome this major barrier in locating the cavernous nerve within the variable pelvic plexus anatomy, our research group proposed a practical solution: a 2D flexible flat patch with multiple electrodes of smaller dimension to be positioned on the plexus area. The overlying concept is to cover the entire plexus area using a 2D multi-electrode patch so that at least one of the electrode pairs will be in optimal contact with the cavernosal nerve. The multi-electrode patch aims to be implanted without intraoperative identification of the nerve path and, in a post-operative ambulatory setting, a scan can be performed to identify the electrode(s) yielding the best erectile response. The electrodes in contact with the cavernosal nerve (those evoking the penile erection) can then be selected and stored for stimulation/therapy.

In order to evaluate the principle and feasibility of this new concept, the present study aimed to assess if the proposed multi-electrode patch can ensure the nerve stimulation and evoke penile erection, without the need of finding the best stimulation site, intraoperatively. Therefore, the testable hypothesis herein is to determine if the positioning of electrodes array on the pelvic plexus (prostate apex or pelvic floor) for electro-stimulation, without cavernous path pre-identification, can ensure the activation and stimulation of the cavernous nerve to induce penile erection.

3.3 Materials and Methods

Subject Population

Patients that were in preparation to undergo open radical prostatectomy for local or locally advanced prostate cancer routinely assessed and confirmed at the National and Kapodistrian University of Athens, Medical School, Laïkon Hospital of Athens, Greece, were enrolled for this study. The experiments were performed between September 2014 to June 2017. In total, twenty-four patients, aged between 49 to 75 years old, were recruited (table 3:1). Four cases were excluded due to technical or surgical issues (system failure or erroneous placement of the electrode, for instance, laterally to the prostate instead of on the prostatic apex). The International Index of Erectile Function-5 (IIEF-5) score was used to assess the patient ED history, such as the possible use of erectile function medication (PDE5i). Patients were counselled and consented to participate (written consent). The study was conducted according to the Helsinki Declaration and protocol was approved by the Institutional Review Board of Laikon Hospital in Athens (protocol n.: ES798 – ID: NCT03425240).

Experimental Setup

The device developed for the present study was not proposed for chronic implantation, but exclusively for temporary assessment, and the entire evaluating procedure lasted maximum 20 minutes. The setup was designed to be a non-risk device for acute use. It was built to guarantee that only standard biocompatible materials in contact with patients (medical grade silicone and platinum electrodes), and standard safety tests were performed prior experimentation.

The experimental setup (figure 3:1 A), was composed of: i) a stimulation system, comprised of a stimulator (battery-powered pulse generator), a switch able to select unilateral or bilateral stimulation, and the multi-electrode patch; ii) a penile plethysmograph system (PPS, model MP36R, Biopac Systems Inc) able to record precise changes in penile circumference through a flexible transducer (PPS gauge, model TSD205, Neurospec AG) placed around the penis base, and continuously monitoring the circumference using an acquisition data system (software AcqKnowledge 4.4.1 from Biopac Systems Inc.).

The flexible multi-electrode patches were made of platinum coated electrodes embedded in flexible silicone matrix and were comprised of two identical mirrored parts, designed to deliver bilateral stimulation on right and left segments of the pelvic-plexus (figure 3:1 B, C and 3:2). Two multi-electrode patch designs, with distinct on electrode numbers, were used (figure 3:1 B): i) 64-electrodes array, which is comprised of two rod shaped projections, containing 32 electrodes each part (2x32); and ii) 24-electrodes array, comprised of two L-type shaped projections, containing 12 electrodes each part (2x12). The initial cases were performed using the 64-

electrodes array (cases #1 to #9), while the remaining with 24-electrodes array (cases #10 to #24). The stimulator was able to generate bipolar and monophasic electrical square pulses with control of amplitude (current), pulse width and frequency. Additionally, the unit is able to stimulate each individual electrode pair independently or execute pre-programed automatic stimulatory sequences (scan modes) in which every electrode pair stimulation (bipolar stimulation) is performed in between the interpulse interval of the other electrode pairs. The scan modes were applied from case #3 onwards and were able to optimize the experiment, as it was not possible to independently scan all electrode pairs of the array within the maximal experimental period of 20 minutes. The stimulator was connected to the electrode array by a 1.5-meter cable to secure the sterile field and a oscillate in between allowed to set unilateral or bilateral stimulation.

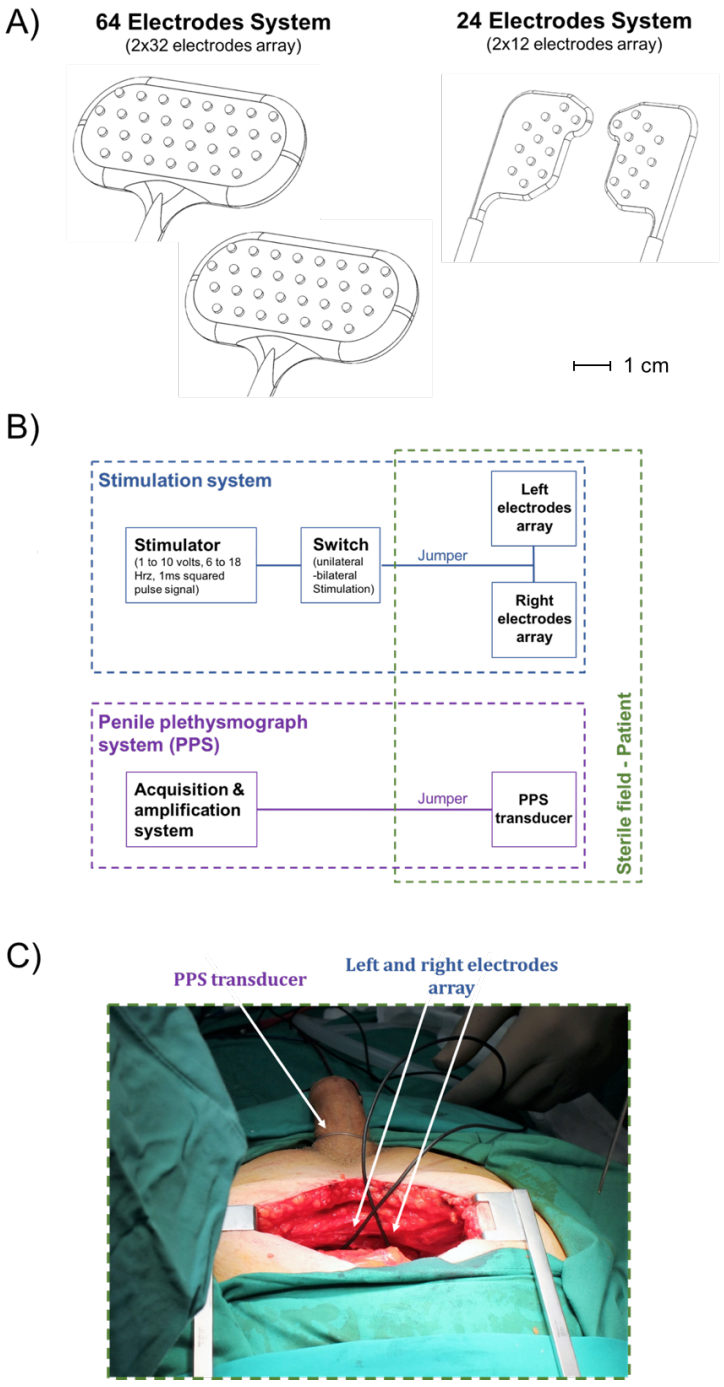

Figure 3:1 - Experimental setup and electrodes array placement. A) Schematic of the two-different models of electrodes arrays used: 2x32 electrodes array (left) and 2x12 electrodes array (right) B). Schematic representation of the stimulation system and recording setup. C) Photograph showing the experiment setup: white arrows indicate the PPS transducer around the penis base, and the cables of the left and right electrodes arrays placed on the pelvic wall. PPS: penile plethysmograph system.

Design Protocol

Under general anaesthesia and complete muscle relaxation, the incision was made vertically on the lower abdomen and the procedure progressed following a typical protocol for open radical prostatectomy. After locating and dissecting both pelvic fascias, creating space adjacent to the prostate, the electrode patches were positioned bilaterally on the prostatic apex, prior prostate removal (PR), or on the pelvic wall, near the urethra, after PR and before the urethrovesical anastomosis (figure 3:2). The electrode patches were maintained in place holding by dry surgical gauzes (no sutures), while electrical stimulation was applied to induce penile erection. Erectile response was assessed qualitatively by visual change of penile tumescence state, and quantitatively by variation of penile circumference recorded using the PPS (case #7 onwards). Continuous electro-stimulation was performed at 12Hz of frequency, 1 millisecond of pulse width, and 7 or 10 volts of potential, which provided 6mA of current amplitude in average. Usually the stimulation started unilaterally followed by bilateral stimulation. In few cases, due to limitation in time, only bilateral or unilateral stimulation was performed. Electrical stimulus was applied from 1 to 3 minutes for each parameter set, until a PPS measured plateau was reached. Upon stimulation completion, electrode patches and PPS gauge were removed and the surgery routinely concluded. One of the most important aspects during patch positioning was the dry surgical field, so that in all cases the haemostasis was meticulous in order to ensure optimal contact between the patch and the tissues.

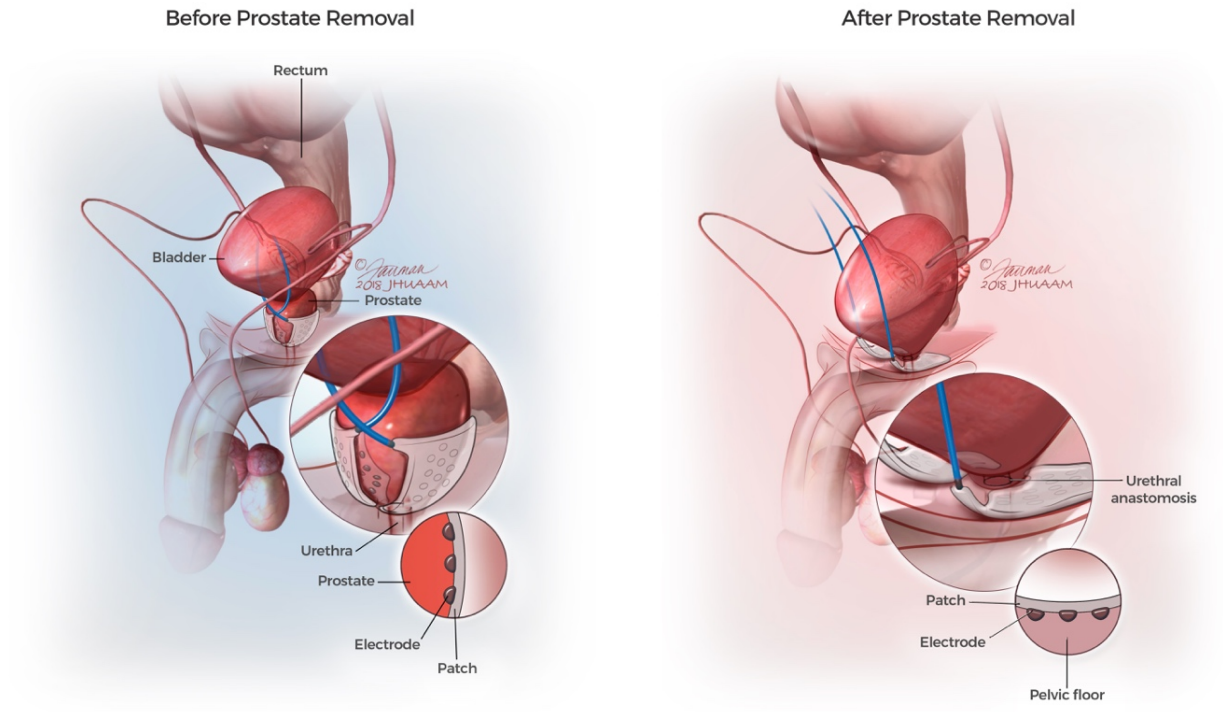

Figure 3:2 - **Flexible electrode array placement.** Illustration representing the electrode array positioned bilaterally on the prostatic apex, prior prostate removal, or on the pelvic wall, near the urethra, after prostate removal and before the urethrovesical anastomosis.

Data Analysis

Qualitative visual assessment data were defined as: i) large tumescence, referring to a clear visual change of penile engorgement (matched with circumference increase greater than 3mm by PPS); ii) small response, referring to a minor penile engorgement, not clearly defined visually, but detected by the PPS measures (matched with circumference increase less than 3 mm by PPS); and iii) no response, no variation detected visually or by PPS. Penile circumference variation data, obtained by PPS recording, present variables intrinsic to each single PPS gauge, therefore, just after experimentation, a calibration was performed using a metallic cylinder comprising precise standard circumference measures for linear regression (5 measurements of 5mm circumference increment).

Two-tailed paired t-test was used to compare the penile circumference change between unilateral and bilateral stimulation (performed same individuals), while two-tailed unpaired t-test was used to compare the maximal penile circumference changes before

and after PR (not always perform in the same individuals), changes between 64- and 24- electrode array, and changes observed for each individual compared with previous results, as reported by Burnett et al.[7]. Statistical analysis was performed using GraphPad Prism software (GraphPad 6.0, San Diego, CA, USA). A value of $p < 0.05$ was considered significant and the results are expressed as mean ± SEM.

3.4 Results

The study cohort and stimulation protocol for each case is shown on table 3:1. Remarkably, erectile response was observed in all cases (20 cases, 100% response). Considering the entire cohort, 75% (15 of 20) presented large tumescence (clear visual engorgement, higher than 3 mm increase in circumference), 25% (5 of 20) showed small response (detected by PPS, and smaller than 3 mm increase in circumference), while no case presented lack of response (figure 3:3 A). No statistically relevant difference was observed when comparing the protocols using 64-electrodes array vs. 24-electrodes array (64-electrodes: 86% large tumescence, n = 6, and 14% small response, n = 1, max. penile circumference increases 6.13 ± 2.05, n=3; 24-electrodes array: 69% large tumescence, n = 9, and 31% small response, n = 4, max. penile circumference increase 7.99 ± 2.02, n=9; P value 0.63) (figure 3:3 B). These results demonstrate that the 2D flexible electrode array concept can guarantee the nerve stimulation, without the need to locate it intraoperatively.

Table 3:1 - **Study cohort & demographic**

Case #	Demographics		Erectile Function	Stimulation Protocol					Maximal Penile response
	Age [year]	Comorbidities	IIEF-5 score	System (# of electrodes)	Unilateral	Bilateral	Before PR	After PR	
2	75	Coronary disease Smoker	12	64	Yes	No	Yes	No	Large tumescence
3	71	Hypertension	22	64	Yes	No	Yes	No	Large tumescence
4	70	Dyslipidemia Smoker	22	64	Yes	No	Yes	No	Large tumescence
6	63	-	15	64	Yes	No	Yes	No	Large tumescence
7	75	Hypertension	23	64	Yes	Yes	Yes	No	Small response
8	75	Diabetes	22	64	Yes	Yes	Yes	Yes	Large Tumescence
9	73	Hypertension, Arrhythmias Previous myocardial infraction	23	64	Yes	Yes	Yes	Yes	Large tumescence
10	63	Hypertension, Smoker Coronary disease	6	24	Yes	Yes	Yes	No	Small response
12	75	Hypertension, Coronary disease Previous cerebral stroke	5	24	No	Yes	Yes	No	Small response
13	63	Hypertension Dyslipidemia	22	24	Yes	Yes	Yes	No	Small response
14	74	Hypertension, Smoker Dyslipidemia	23	24	Yes	Yes	Yes	Yes	Large tumescence
16	67	Smoker Arrhythmias	12	24	Yes	Yes	Yes	Yes	Large tumescence
17	60	Hypertension, Smoker Dyslipidemia, Diabetes	25	24	Yes	Yes	No	Yes	Large tumescence
18	67	Hyperthyroidism Smoker	25	24	No	Yes	Yes	Yes	Large tumescence
19	61	Dyslipidemia Previous cerebral stroke	23	24	Yes	Yes	Yes	Yes	Small response
20	48	-	25	24	Yes	Yes	Yes	No	Large tumescence
21	73	Previous aortic aneurysm	25	24	No	Yes	Yes	No	Large tumescence
22	68	Dyslipidemia Arrhythmias	22	24	Yes	Yes	Yes	Yes	Large tumescence
23	64	-	22	24	Yes	Yes	Yes	Yes	Large tumescence
24	70	Hypertension Coronary disease	25	24	Yes	Yes	Yes	Yes	Large tumescence

Considering the location of electrodes array placement before and after PR, stimulation on the prostate apex (before PR) demonstrated a slightly higher response compared to pelvic wall placement (after PR), despite no observed statistical difference (before PR:

7.17 ± 1.70 mm, n=14; vs. after PR: 5.76 ± 2.13 mm, n=9; P = 0.61) (figure 3:3 C). Considering the visual increase classification, which is a non-quantitative measure, the response after PR was smaller (before PR: 68% large tumescence and 32% small response; vs. after PR: 40% large tumescence and 60% small response) (figure 3:3 C).

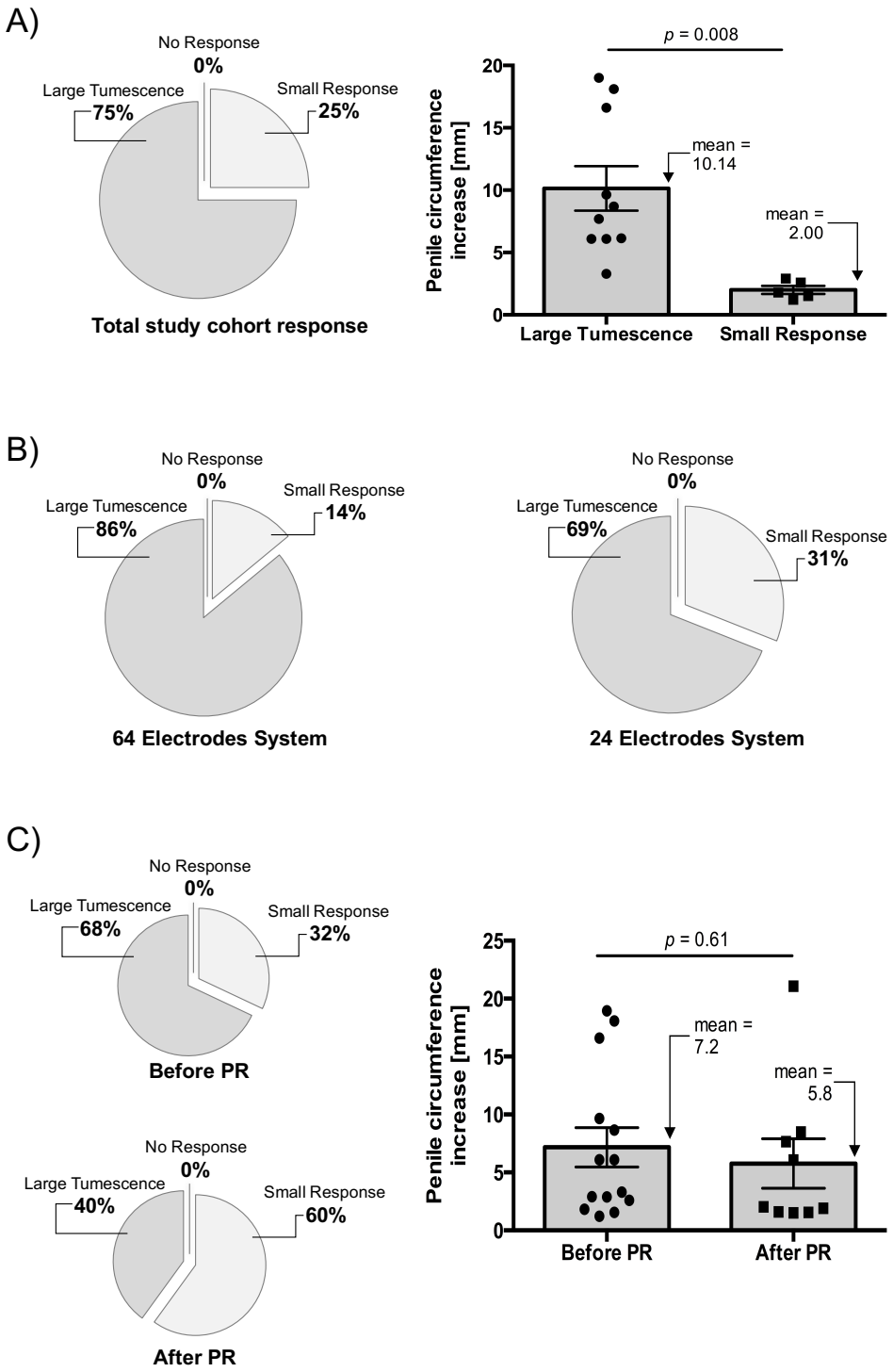

Figure 3:3 - **Penile response in different setups.** A) Left chart shows the penile response achieved in the entire cohort when evaluated by the qualitative measure of visual change in penile tumescence. Right graphic shows the maximal circumference increase assessed by PPS and compares the qualitative measures (large tumescence vs. small response). B) Charts of visual penile response when comparing 64 electrodes array (left, n=7) with 24 electrodes array (right, n=13). C) Left charts show visual penile response prior and after prostate removal, while right graphic shows respective maximal circumference increase.

Interestingly, bilateral stimulation potentiated erectile response compared to unilateral stimulation (figure 3:4). The figure 3:4 A displays a typical PPS recording, in which the protocol is initiated with unilateral stimulation, followed by bilateral stimulation after reaching a response plateau. Bilateral stimulation clearly evoked an amplification on penile circumference enlargement. Indeed, analysis of the data in which the protocol of unilateral stimulation followed by bilateral stimulation had been executed on the same patient, unilateral stimulation produced a mean increase of 2.7 ± 1.02 mm in penile circumference, while bilateral stimulation enhanced the response to 8.2 ± 1.9 mm (figure 3:4 B, n = 13, p = 0.01). Additionally, a 10-volt stimulus produced a higher response than 7 volts, except in one case in which 7 volts stimulation showed greater response. The current applied for 10 volts was measured as 6 mA, and no collateral stimulation was observed in the pelvic wall (e.g. no shaking of pelvic muscles, as previously described when higher intensity of stimulation was used[15]).

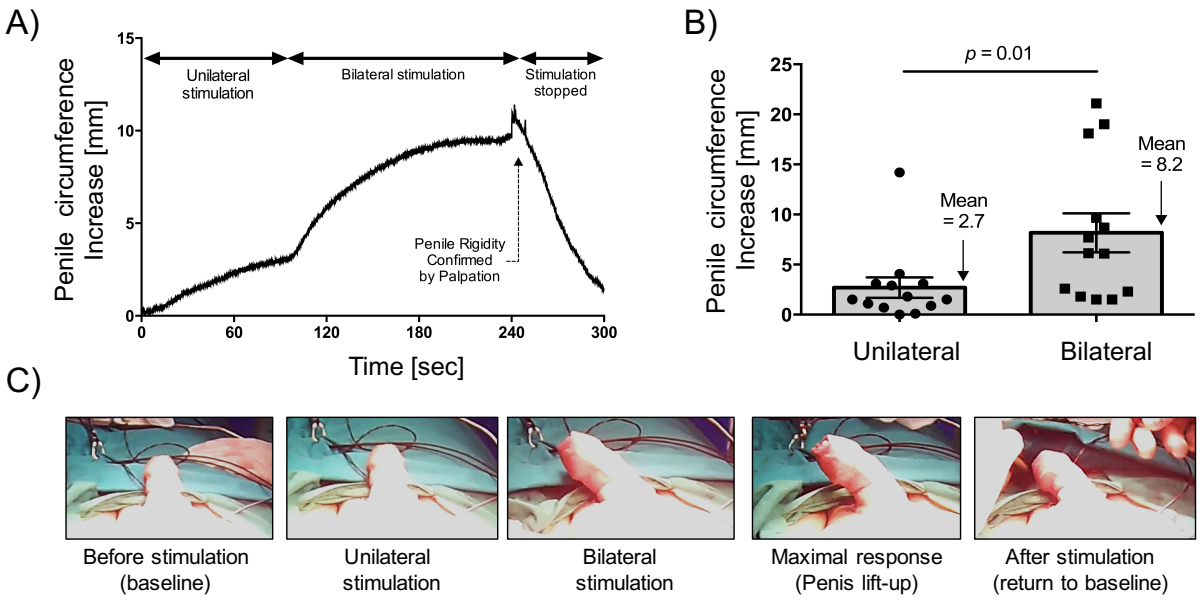

Figure 3:4 - **Bilateral stimulation potentiated electrostimulation-induced penile circumference increase.** A) Typical PPS recording data. Penile circumference increases during unilateral and bilateral electrostimulation, demonstrating a clear potentiation of the response when bilateral stimulation is applied, and rapid return to baseline when interrupted. B) Penile circumference increases in response to bilateral stimulation compared to unilateral stimulation (n=13). C) Time-frame photographs of a typical case, showing a clear visual penile engorgement following bilateral stimulation.

The majority of the patients recruited in the study reported intact erectile function status, when assessed by IIEF-5 score (IIEF-5 score of 22 or higher). Two subjects (2 out of 5) presenting a previous ED history showed minor penile response (table 3:1). When only considering patients with intact erectile function status (table 3:2), 80% (12 out of 15) evoked large penile tumescence, 20% (3 out of 15) small response and none without response. Interestingly, these results demonstrate more robust effectiveness than previous, similar attempts[7], which documented 42% of large tumescence, 8% of small response, and 50% no response in a cohort of non-ED patients (figure 3:5). Accordingly, the maximum average increase in penile circumference was also significantly higher in our study (7.5 ± 1.6 mm vs. 2.5 ± 0.85 mm, n=12, p value = 0.01).

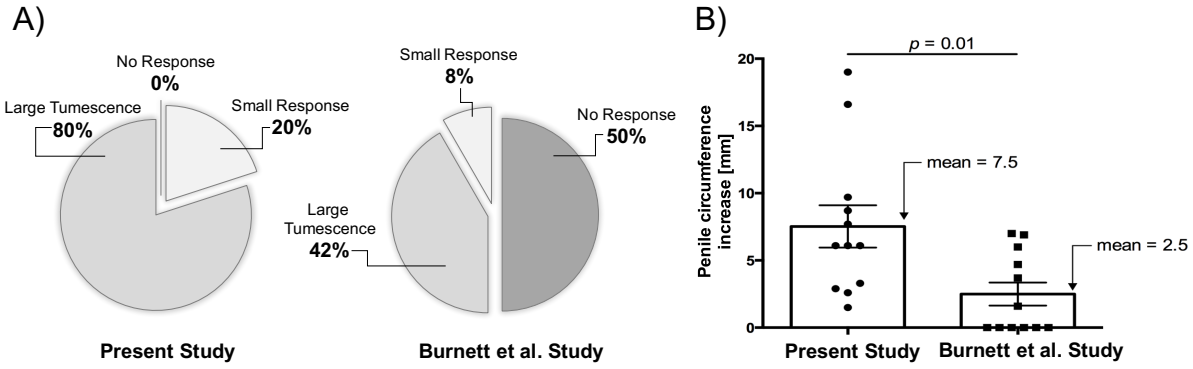

Figure 3:5 - Penile response (visual tumescene state) and penile circumference increase in patients without ED history compared with previous study. A) Chart of penile response comparing patients without ED history from the present study (left, n=15) with reported data from publication [7] (right, n=12). B) Penile circumference increase, present study compared to reported data [7].

Table 3:2 - Penile circumference increase in patients without previous ED

Case #	Maximal penile circumference increase [mm]	Maximal Penile response
2	*	Large tumescence
3	*	Large tumescence
7	2.6	Small response
8	6.1	Large tumescence
9	9.7	Large tumescence
13	2.9	Small response
14	19.0	Large tumescence
17	7.7	Large tumescence
18	6.1	Large tumescence
19	1.5	Small response
20	8.7	Large tumescence
21	**	Large tumescence
22	3.3	Large tumescence
23	16.6	Large tumescence
24	6.1	Large tumescence

* No PPS system used
** Numerical data not available due to PPS calibration error

3.5 Discussion

The present study describes a novel concept, a 2D flexible electrode array to guarantee the blind identification of the cavernosal nerve path within the pelvic plexus, without the need of extensive intraoperative maneuver. The use of electro-stimulation to induce penile erection has been extensively reported and suggested as potential therapy against ED[5–7,16]. However, the difficulty to identify the optimal location for electrode placement, considering the anatomical variability between individuals, distinguishability of overlying tissue and blood obscuring the surgical field, has blocked further development and reaching clinic use. The proposed flexible electrode array was able to induce penile response in all patients (20 cases), providing substantial evidence that our approach can guarantee the nerve activation, without requirement of precise identification of the nerve fiber.

The first study in human proposing electrostimulation to treat ED was done by Lue and colleagues[5]. In an intraoperative environment, they used an oval device consisting of a stainless-steel mesh with a broad stimulating surface to stimulate the neurovascular bundle posterolateraly to the prostate. Penile response was reported in 8 of 16 patients (50%)[5]. In another study, Burnett et al.[7] used a curved tripolar electrode comprised of a central cathode and two adjacent anodes to be positioned on the pelvic neurovascular bundle. They observed an increase in penile circumference in 50% of the patients, assessed by a penile plethysmograph, while visual penile engorgement was observed only in 25% of the patients[7]. These previous studies demonstrated that electrical stimulation applied on the prostatic apex[5] or pelvic neurovascular bundle[7] can induce penile response in human, supporting the therapeutic concept. However, these previous approaches presented low efficacy (25-50% visual engorgement – 50% no response). A plausible explanation could be the inadvertent induction of sympathetic nerve firing, which has erectolytic effects, as previous studies used electrodes with large surface areas. Our approach covers a holistic area for stimulation, however, each electrical pulse is applied only between two electrodes within the array, providing punctual stimulus and, therefore, minimizing the chances to trigger other nerves and favoring the desired activation potential. Large electrode area applications, as used in previous studies, may lead to a diffusion of electrical stimulus and, therefore, reduce activation potential, while smaller electrodes with minimal spacing increases stimulation selectivity and leads to greater activation potentials of the adjacent tissue, requiring a lower stimulation intensity to achieve better neuronal activation[13,14]. Indeed, comparing the stimulatory parameters used, our study was able to evoke penile erection with 7-10 volts and 6mA stimulus, while others required a higher intensity to attain penile response (40 mA to 60 mA[7], or 12 to 32 volts[5]). Additionally, the low efficacy of previous studies might be related to electrode positioning. Lue et al. applied stimulus to the prostatic apex[5], while Burnett et al. applied the stimulus on the neurovascular bundle following prostate removal although it has been documented that the cavernosal nerve may present different patterns along the prostate, in addition to the possibility that the cavernosal nerve travels outside the vascular bundle[8,9,12]. The cavernosal nerve travels along the posterolateral aspect of the prostate, in a complex anatomical pattern within the pelvic plexus, and starts converging in the prostatic apex when approaching the membranous urethra, at the 3 and 9 o'clock positions, prior to crossing the pelvic floor muscle near the urethra[8,10,17]. Sequentially, the fiber merge and form the cavernosal nerve, which traverses within the penile structures. Thus, when considering the site of injury (prostatectomy) and the general nerve path, we proposed the pelvic floor area, in close proximity to the urethra, as best location for the electrode array. In this area, the neuronal path starts to converge and is distal to the injured area. Indeed, our trial was executed by positioning the electrodes array in such region, following PR, and the results demonstrate the higher probability of targeting the nerve with such approach. Importantly, these results demonstrated that our approach can stimulate the distal portion of the nerves and evoke penile response, even after PR (positioned on the pelvic wall).

It is plausible to assume that more nerves will be injured once the prostate is removed and poorer response would be reached, implying efficacy issues to future clinical practice application in prostatectomy patients. However, in the present study, stimulation after RP was slightly lower and this comparable response suggests a favorable outcome for further development. Importantly, this data highlights the importance of performing nerve sparing techniques to improve efficacy of neuromodulation therapy in the treatment of ED in post-prostatectomy patients.

There are several concerns when considering nerve integrity during prostatectomy, and it is probable that some or many of the cavernosal nerves fibers may be injured by extirpation of the prostate and may eventually undergo Wallerian degeneration[18]. Therefore, one may argue that, despite the possibility to still being able to stimulate these nerves in an acute manner, they may eventually degenerate and lose the ability to be stimulated over the long-term, which would limit the therapeutic application. However, not all nerves will be damaged when considering the nerve sparing approach that might be used, and the remaining intact nerves could be sufficient to trigger penile erection which, for instance, could be potentiated by supplementation with PDE5 inhibitors. Importantly, it has recently been demonstrated that low frequency electrostimulation, similar to physiological neural mean firing frequency (10 to 20Hz), accelerates and enhances peripheral nerve regeneration[19,20]. The underlying mechanisms are still not fully elucidated, but they may involve i) the recruitment and guiding of Schwann cell migration[21]; ii) the favoring of neurotrophic factors expression and secretion[22]; iii) the facilitation of axons residue elimination from the Wallerian degeneration and acceleration of axonal growth[23]; and iv) the direct electrical guidance of the new growing axonal sprouts, facilitating and fastening the regeneration of the neural function[24]. Therefore, it is possible that electro-stimulation therapy, in addition to effect on evoking penile erection for sexual intercourse, could be used to prevent or minimize injured nerve degeneration or facilitate and fasten nerve regeneration. Nevertheless, all these assumptions still remain to be demonstrated in a chronic scenario, and the primary aim of the present study was to demonstrate the principle that an electrodes array can enable the cavernosal nerve activation without precise nerve localization and without extensive intraoperative exploration.

Although the results reported here add to previously published studies, the obtained maximal penile response observed in this study was preponderantly a large visual engorgement and not a fully rigid erection. The mean penile circumference increase during a fully rigid erection should average 20 mm in normal physiological conditions[25], while we observed a 7.5 mm increase in our study. One may argue such response is not sufficient for sexual intercourse; however, it is important to note that the intraoperative conditions

of the tests are not optimal to demonstrate efficacy, while these factors will not be present in the clinical setting. During surgery, loss of blood, hemodynamic depression, decrease in body temperature, peripheral vasoconstriction and, critically, the anesthesia effects, altogether may contribute to diminish final erectile response. For instance, it has been documented in animals that electrostimulation induced penile erection was inhibited to a certain degree by inhalation of anesthesia[5,26]. In our study, the patients were anesthetized with midazolam (3mg i.v.), fentanyl (100 mg i.v.) and propofol (1.5 to 3.0 mg/kg) for induction, and desflurane (6% of total gases) or sevoflurane (2% of total gases), which could have reduced the magnitude of response. One important factor to be considered is that anesthesia may significantly reduce vaso-occlusive mechanism of erection necessary for full erection. Following the neural response that initiates the vaso-occlusive mechanism, a muscular response occurs as well by the autonomous contraction of the perineal muscles (bulbo- and ischio-cavernous muscles) in order to reach complete rigidity[27,28]. Deep anesthesia and muscular relaxant agents may then contribute to the lack of full erectile rigidity by inhibiting such mechanism. In addition, the lack of a patient's natural or endogenous stimuli during general anesthesia and possible acute neuropraxia may also impair the erectile response. Finally, in the present study, the optimal stimulation parameters were not extensively investigated, mostly due to limitation of experimentation time. In the present study, frequency and pulse width were kept constant, while the potential varied between 7 and 10 volts. Considering the envisioned clinical application in which the parameters will be set after surgery recovery in an ambulatory environment, optimal parameters and electrodes pairs can be better established, since adequate time will be available for patient specific optimization. Taken together, we speculate that the magnitude of erectile response in the clinical scenario will be higher than the present intraoperative results, postulating promising gains in the advancement of this therapeutic option.

Beyond the intraoperative limitations that might have affected the magnitude of erectile response, it is reasonable to speculate that, during clinical application scenario using neuromodulation therapy, penile engorgement produced by electrostimulation could certainly be potentiated by PDE5 inhibitors, as the mechanisms of action are directly linked. PDE5 inhibitors act by amplifying the response evoked by the neurotransmitter nitric oxide, as it inhibits the degradation of the nitric oxide intracellular messenger cGMP, in the penile vasculature, potentiating its vascular reaction[1].

As discussed above, our data were taken during general anesthesia and muscle relaxation, and activation of somatic nerve is presumed to be inhibited under this condition. Consequently, it raises the question of the possible effects of this technology on somatic nerves stimulation that could represent limitations to future neuromodulation therapy for ED. Indeed, when anesthesia and muscle relaxant agents are not present, somatic nerves could be activated by electrostimulation of the pelvic floor. Certainly, somatic nerve stimulation is also possible when stimulating the apex area of the prostate. However, punctual stimulation, as permitted by the electrodes array principle (patient specific selected electrodes pair within the array), may reduce inadvertent somatic nerve activation and favor selection of autonomic nerves. In addition, by optimizing the stimulation parameters, which can be meticulously set in an ambulatory environment, it becomes feasible to maximize the activation potential of unmyelinated (parasympathetic cavernous nerves), while minimizing the activation of myelinated (somatic nerves). Indeed, similar techniques are applied in spinal cord stimulation paddles used for the treatment of chronic pain, in which, by optimizing the stimulus parameters, specific and selective targeting activates the dorsal column nerves and avoid dorsal root fibers in conditions of paresthesia[29]. Finally, the proportion of somatic nerves surrounding the prostate apex represent less than 5% of the autonomous nerves, comprising both sensory and motor nerves[30]. In the event of involuntary stimulation of motor nerves innervating the bladder or urinary sphincter, electrical stimulation would result in a contraction of those muscles and may represent a beneficiary effect against post-prostatectomy urinary incontinence, indicating another potential therapeutic application. However, no urethral sphincter contraction or pelvic floor electromyography were measured during this study and further investigations are required to understand such application. Inadvertent stimulation of the anal sphincter or rectum nerves is not expected as the main nerves fibers innervating those structures originate from the pudendal nerve that is traveling far enough away from the pelvic wall surrounding the urethra when considering the application of a punctual stimulation within the electrodes of the array.

As for limitations, the measurements of penile circumference started after case #6, reducing the number of quantitative measures. Moreover, four cases were excluded as the stimulatory protocol could not be performed: in two cases the stimulator showed error message and the experimentation was terminated, while two other cases, the electrodes array were misplaced and positioned laterally on the prostate instead of on the prostatic apex. It is important to mention that, due to limitation of time and to avoid safety issues with the ongoing surgery, unilateral and bilateral stimulation was not always performed in the same patient, as for stimulation before and after PR. In some cases, the unilateral stimulation or before PR was avoided to gain time during the testing, or the experiment was terminated before sufficient time needed to test bilateral or after PR stimulation. This time-based limitation was reduced to 13 cases for the comparison between unilateral vs. bilateral response (figure 3:4 B), and 14 and 9 cases for before and after PR respectively.

The study was performed with 2 different electrode arrays, 64- and 24-eletrodes, as we opted for a new array design following the initial cases in which we observed the more optimal areas to be covered. Most importantly, the results from the 24-electrode array

demonstrated a similar response to the 64-electrode array, thus this configuration is also suitable for further device development aimed for clinical practice.

In general, the present cohort grouped mainly patients without ED (IIEF score higher than 22). Therefore, it is still not clear if ED patients, particularly vasculogenic ED, would benefit from neuromodulation therapy. Most likely, as electro-stimulation can sole activate and/or restore the neuronal pathway, it may not overcome the penile vasculature dysfunction. In practice, this may restrict the group of patients that can potentially benefit from future clinical translation. Even considering such limitation, the envisioned medical implant may still benefit a large population of neurogenic ED patients, such as prostatectomy, spinal cord injury or multiple sclerosis patients. In addition, as discussed above, it is reasonable to speculate that a minor penile response could also be potentiated by PDE5-inhibitor, however, the benefit to vasculogenic ED remains to be answered in a clinical trial scenario.

The present study was performed during open radical prostatectomy; however, future application of this technology will not be limited to this procedure. The electrode array has features, such as flexibility (silicone matrix) and convenient dimensions, which are compatible for implantation in robotic-assisted surgery. The electrode array can be rolled and passed through standard trocars, while its fixation on the pelvic floor can be performed with standard surgical sutures.

On important aspect to consider in order to bring the present concept into the clinical practice is the available technology. The device envisioned here presents standard features and similar stimulation parameters of existing implantable neuromodulators used today for treating chronic pain and Parkinson[31]. Therefore, no major innovation is required in terms of implantable pulse generator for the clinical translation of this concept into a potential medical device. The envisioned device concept is to place the electrodes arrays on the pelvic floor and pulse generator subcutaneously, while the system is remotely controlled for activation.

3.6 Conclusion

In summary, our study demonstrates that the proposed 2D flexible electrode array can ensure the stimulation of the erectogenic neural path within the pelvic plexus when positioned on the prostatic apex or pelvic floor, since all patients responded to the desired extent. Importantly, these data open avenue for further development of a novel implantable medical device to alleviate ED, particularly in cases associated with mechanical neuronal damage, such as prostatectomy and spinal cord injury patients.

3.7 Conflict of Interest

Rodrigo Fraga-Silva and Nikos Stergiopulos are co-authors of the patent, which refers to the technology concept using a 2D flexible array of electrodes to restore erectile function (US-9821163-B2) and Mikael Sturny, Rodrigo Fraga-Silva, and Nikos Stergiopulos have stock ownership of Comphya Sarl, a start-up company that has the patent right of the technology.

All authors certify that no bias or lack of objectivity in research was induced.

3.8 Acknowledgements

The research being reported in this publication was supported by research grants from Swiss Commission for Technology and Innovation (CTI, grant no. 17162.1 PFLS-LS) and Wyss Center for Bio and Neuroengineering. We would like to thanks the Laikon Hospital staff for their friendly support and dedication that allowed the implementation of this study.

3.9 References

(1) Rotella, D. P. Phosphodiesterase 5 Inhibitors: Current Status and Potential Applications. Nat. Rev. Drug Discov. 2002, 1 (9), 674–682. https://doi.org/10.1038/nrd893.

(2) Linet, O. I.; Ogrinc, F. G. Efficacy and Safety of Intracavernosal Alprostadil in Men with Erectile Dysfunction. The Alprostadil Study Group. N. Engl. J. Med. 1996, 334 (14), 873–877. https://doi.org/10.1056/NEJM199604043341401.

(3) Purvis, K.; Egdetveit, I.; Christiansen, E. Intracavernosal Therapy for Erectile Failure--Impact of Treatment and Reasons for Drop-out and Dissatisfaction. Int. J. Impot. Res. 1999, 11 (5), 287–299.

(4) Sadeghi-Nejad, H. Penile Prosthesis Surgery: A Review of Prosthetic Devices and Associated Complications. J. Sex. Med. 2007, 4 (2), 296–309.

(5) Lue, T. F.; Gleason, C. A.; Brock, G. B.; Carroll, P. R.; Tanagho, E. A. Intraoperative Electrostimulation of the Cavernous Nerve: Technique, Results and Limitations. J. Urol. 1995, 154 (4), 1426–1428.

(6) Burnett, A. L.; Lue, T. F. Neuromodulatory Therapy to Improve Erectile Function Recovery Outcomes After Pelvic Surgery. J. Urol. 2006, 176 (3), 882–887. https://doi.org/10.1016/j.juro.2006.04.020.

(7) Burnett, A. L.; Teloken, P. E.; Briganti, A.; Whitehurst, T.; Montorsi, F. Intraoperative Assessment of an Implantable Electrode Array for Cavernous Nerve Stimulation. J. Sex. Med. 2008, 5 (8), 1949–1954. https://doi.org/10.1111/j.1743-6109.2008.00865.x.

(8) Takenaka, A.; Murakami, G.; Matsubara, A.; Han, S. H.; Fujisawa, M. Variation in Course of Cavernous Nerve with Special Reference to Details of Topographic Relationships near Prostatic Apex: Histologic Study Using Male Cadavers. Urology 2005, 65 (1), 136–142. https://doi.org/10.1016/j.urology.2004.08.028.

(9) Takenaka, A.; Tewari, A.; Hara, R.; Leung, R. A.; Kurokawa, K.; Murakami, G.; Fujisawa, M. Pelvic Autonomic Nerve Mapping Around the Prostate by Intraoperative Electrical Stimulation With Simultaneous Measurement of Intracavernous and Intraurethral Pressure. J. Urol. 2007, 177 (1), 225–229. https://doi.org/10.1016/j.juro.2006.08.104.

(10) Costello, A. J.; Dowdle, B. W.; Namdarian, B.; Pedersen, J.; Murphy, D. G. Immunohistochemical Study of the Cavernous Nerves in the Periprostatic Region. BJU Int. 2011, 107 (8), 1210–1215. https://doi.org/10.1111/j.1464-410X.2010.09711.x.

(11) Wang, R. Penile Rehabilitation after Radical Prostatectomy: Where Do We Stand and Where Are We Going? J. Sex. Med. 2007, 4 (4, Part 2), 1085–1097. https://doi.org/10.1111/j.1743-6109.2007.00482.x.

(12) Alsaid, B.; Bessede, T.; Karam, I.; Abd-Alsamad, I.; Uhl, J.-F.; Benoît, G.; Droupy, S.; Delmas, V. Coexistence of Adrenergic and Cholinergic Nerves in the Inferior Hypogastric Plexus: Anatomical and Immunohistochemical Study with 3D Reconstruction in Human Male Fetus. J. Anat. 2009, 214 (5), 645–654. https://doi.org/10.1111/j.1469-7580.2009.01071.x.

(13) Wei, X. F.; Grill, W. M. Current Density Distributions, Field Distributions and Impedance Analysis of Segmented Deep Brain Stimulation Electrodes. J. Neural Eng. 2005, 2 (4), 139. https://doi.org/10.1088/1741-2560/2/4/010.

(14) Holsheimer, J.; Wesselink, W. A. Optimum Electrode Geometry for Spinal Cord Stimulation: The Narrow Bipole and Tripole. Med. Biol. Eng. Comput. 1997, 35 (5), 493–497.

(15) Lue, T. F.; Schmidt, R. A.; Tanagho, E. A. Electrostimulation and Penile Erection. Urol. Int. 1985, 40 (1), 60–64. https://doi.org/10.1159/000281034.

(16) Facio, F.; Burnett, A. L. Penile Rehabilitation and Neuromodulation https://www.hindawi.com/journals/tswj/2009/474587/abs/ (accessed Sep 21, 2017). https://doi.org/10.1100/tsw.2009.86.

(17) Shafik, A. Extrapelvic Cavernous Nerve Stimulation in Erectile Dysfunction. Human Study. Andrologia 1996, 28 (3), 151–156. https://doi.org/10.1111/j.1439-0272.1996.tb02774.x.

(18) Sulaiman, W.; Gordon, T. Neurobiology of Peripheral Nerve Injury, Regeneration, and Functional Recovery: From Bench Top Research to Bedside Application. Ochsner J. 2013, 13 (1), 100–108.

(19) Willand, M. P.; Nguyen, M.-A.; Borschel, G. H.; Gordon, T. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 2016, 30 (5), 490–496. https://doi.org/10.1177/1545968315604399.

(20) Gordon, T. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 2016, 13 (2), 295–310. https://doi.org/10.1007/s13311-015-0415-1.

(21) Gu, X.; Fu, J.; Bai, J.; Wang, J.; Pan, W.; Zhang, C. Low-Frequency Electrical Stimulation Induces the Proliferation and

Differentiation of Peripheral Blood Stem Cells Into Schwann Cells. Am. J. Med. Sci. 2015, 349 (2), 157–161. https://doi.org/10.1097/MAJ.0000000000000385.

(22) Wang, L.; Sanford, M. T.; Xin, Z.; Lin, G.; Lue, T. F. Role of Schwann Cells in the Regeneration of Penile and Peripheral Nerves. Asian J. Androl. 2015, 17 (5), 776–782. https://doi.org/10.4103/1008-682X.154306.

(23) Chan, K. M.; Curran, M. W. T.; Gordon, T. The Use of Brief Post-surgical Low Frequency Electrical Stimulation to Enhance Nerve Regeneration in Clinical Practice. J. Physiol. 2016, 594 (13), 3553–3559. https://doi.org/10.1113/JP270892.

(24) Haan, N.; Song, B. Therapeutic Application of Electric Fields in the Injured Nervous System. Adv. Wound Care 2014, 3 (2), 156–165. https://doi.org/10.1089/wound.2013.0450.

(25) Suzuki, K.; Sato, Y.; Horita, H.; Adachi, H.; Kato, R.; Hisasue, S.; Itoh, N.; Tsukamoto, T. The Correlation between Penile Tumescence Measured by the Erectometer and Penile Rigidity by the RigiScan. Int. J. Urol. 8 (11), 594–598. https://doi.org/10.1046/j.1442-2042.2001.00390.x.

(26) Mueller, S. C.; Aboseif, S.; Fahey, M. R.; Lue, T. F.; Tanagho, E. A. Spinal Anesthesia and Electroerection in Dogs and Monkeys. J. Urol. 1989, 142 (1), 171–175.

(27) Lavoisier, P.; Proulx, J.; Courtois, F.; De Carufel, F.; Durand, L.-G. Relationship Between Perineal Muscle Contractions, Penile Tumescence, and Penile Rigidity During Nocturnal Erections. J. Urol. 1988, 139 (1), 176–179. https://doi.org/10.1016/S0022-5347(17)42348-2.

(28) Shafik, A.; Shafik, I.; El-Sibai, O.; Shafik, A. A. Effect of External Anal Sphincter Contraction on the Ischiocavernosus Muscle and Its Suggested Role in the Sexual Act. J. Androl. 2006, 27 (1), 40–44. https://doi.org/10.2164/jandrol.05049.

(29) Holsheimer, J.; Buitenweg, J. R. Review: Bioelectrical Mechanisms in Spinal Cord Stimulation. Neuromodulation Technol. Neural Interface 2015, 18 (3), 161–170. https://doi.org/10.1111/ner.12279.

(30) Reeves, F.; Battye, S.; Borin, J. F.; Corcoran, N. M.; Costello, A. J. High-Resolution Map of Somatic Periprostatic Nerves. Urology 2016, 97, 160–165. https://doi.org/10.1016/j.urology.2016.08.027.

(31) Hunter Peckham, P.; Michael Ackermann, D. Chapter 18 - Implantable Neural Stimulators. In Neuromodulation; Academic Press: San Diego, 2009; pp 215–228. https://doi.org/10.1016/B978-0-12-374248-3.00019-7.

Chapter 4 Low-intensity electrostimulation enhances neuroregeneration and improves erectile function in a rat model of cavernous nerve injury

Mikael Sturny[1,2*], Serkan Karakus[2*], Rodrigo Fraga-Silva[1], Nikolaos Stergiopulos[1], Arthur L. Burnett[2].

1. Ecole Polytechnique Fédérale de Lausanne (EPFL), Department of Bioengineering, Laboratory of Hemodynamics and Cardiovascular Technology (LHTC), Lausanne, Switzerland.

2. Johns Hopkins School of Medicine, The James Buchanan Brady Urological Institute and Department of Urology, Baltimore, MD, USA.

* Equal contribution

Running title: LIES improves EF recovery in rats.

Manuscript in preparation for publication

Corresponding Authors: Nikolaos Stergiopulos, Prof.
Ecole Polytechnique Fédérale de Lausanne
Institute of Bioengineering,
Station 09, MED 3.2526, CH-1015, Lausanne, Switzerland
Phone: (+41) 21 693 83 46
Email: nikolaos.stergiopulos@epfl.ch

4.1 Abstract

Background: Erectile dysfunction is a frequent complication of radical prostatectomy. In the long-term, the absence of pro-erectile nerve response may lead to corporal cavernosal remodelling and permanent ED. Low-intensity electrostimulation (LIES) has been

shown to enhance peripheral nerve regeneration, however, the application of LIES and its mechanisms of action on cavernous nerves (CN) have never been investigated.

Aims: This study aims to evaluate whether LIES can improve erectile function (EF) and prevent penile tissue remodelling induced by CN injury and to explore the potential underlying molecular mechanisms.

Methods: 36 male Sprague-Dawley rats were divided into Sham, BCNI, and BCNI + LIES (1V, 0.1ms, 12Hz, 1h/day). Bilateral cavernous nerve injury (BCNI) was induced by two consecutive pressure crushes using a haemostat clamp. Sham group had nerve bundles exposed but untouched. A bipolar electrode was then implanted on each CN bundle. After 7 days, EF (intracavernosal pressure) was measured, then penes and cavernous nerves were collected for molecular analyses of TGF-β1, Il-6, CRP, Collagen type-I and eNOS protein levels and histological analysis of corpus cavernosum and CN structure.

Outcome: Effects of LIES on EF, erectile tissue remodeling and CN structure.

Results: EF was decreased 7 days after BCNI and normalized by LIES. In accordance, LIES induced a reduction in intracavernosal oxidative stress, inflammation markers, pro-inflammatory cytokines, and fibrosis, preserving nitrergic CN fibers and myelination compared to control, indicating a protective effect of LIES on corpus cavernosum and CN following BCNI.

Clinical Translation: These results indicate LIES treatment as a promising new therapeutic tool to enhance EF recovery and minimize the side effects of radical prostatectomy.

Strengths & Limitations: This study provides evidence of the protective effect of LIES on EF and tissue remodeling following CN injury; nevertheless, this study has only been conducted on rat models, and the translation to humans remains to be demonstrated. Further research to fully identify the underlying mechanisms of action is required.

Conclusion: This study shows the first evidence of beneficial effects of LIES on EF recovery, corporal cavernosal remodeling, and CN structure protection following nerve injury, indicating a potential new tool for penile rehabilitation and ED management following radical prostatectomy.

Key Words: Neurogenic erectile dysfunction; postprostatectomy erectile dysfunction; cavernous nerves; LIES; nerve stimulation; nerve regeneration; penile rehabilitation.

4.2 Introduction

Penile erection is defined as a coordinated neurovascular event where both neuronal and vascular constituents are essential to induce and maintain an erection[1]. The release of neuronal nitric oxide synthase from the cavernous nerve (CN) terminals initiates the penile erection by activating the NO/cGMP pathway in endothelial and corporal smooth muscle cells. The CNs originate from the pelvic plexus and travel along the posterolateral side of the prostate to finally innervate the erectile tissues[2]. These fibers are accompanied by vascular structures traveling alongside them and together are defined as neurovascular bundles (NVBs)[3]. Pelvic surgery,particularly radical prostatectomy (RP) surgery, often leads to erectile dysfunction (ED)[4]. Despite the nerve-sparing technique and technological improvements, the surgical removal of the prostate is almost invariably associated with temporary neuropraxia of the nerves controlling erectile function (EF)[5]. In addition to direct injuries to the nerves, neuropraxia can be caused by traction, compression, coagulation, or inflammatory response[3]. This injury initiates Wallerian degeneration of the nerves, and the subsequent denervation of the corpora cavernosa, leading to ED, a loss in nocturnal erectile activity, penile hypoxia, fibrosis, and an increase of apoptosis, causing irreversible smooth-muscle cell loss within the erectile tissue[2]. The production of fibrogenic factors such as transforming growth factor-β1 (TGF-β1) and inflammation, responsible for structural changes in the erectile tissue, including impairment of the elasticity of the corpora cavernosa and the apoptosis of smooth muscle cells, may finally result in permanent veno-occlusive dysfunction[3]. Recovery of EF has been reported after 3 to 24 months following nerve injury[3], however, the chronic lack of erection period following neuropraxia, eventually leading to definitive damage of the corpora cavernosa, may preclude to regain the level of presurgical EF in patients[5]. To avoid permanent structural modifications of the corpora cavernosa due to the lack of erectile function following prostatectomy surgery, the concept of penile rehabilitation as emerged. It is based on the use of any therapy or combination of therapies able to induce erectile response and limit cavernosal hypoxia, to prevent fibrosis and smooth muscle loss and to preserve erectile function until some degree of neural function is restored[5]. As penile rehabilitation has not shown clear beneficial effects on EF recovery[6], it has brought attention to the need for new strategies to induce erectile response and enhance CN regeneration following RP. Many preclinical studies have provided promising results in EF recovery following CN injury by aspiring to improve and fasten nerve regeneration using neurotrophic factors, immunomodulatory, or stem cell therapy[5,6]. Unfortunately, these strategies have failed to demonstrate a clinically relevant effect or have not extended to the clinical practice yet.

Electrostimulation of the CN has been shown to induce penile erection in humans[7,8], which could be used as a way to produce penile response and promote penile rehabilitation following RP. In addition, it has been demonstrated recently that low-frequency electrostimulation (LIES), similar to physiological neural mean firing frequency with intensity lower than the nerve activation threshold, accelerate and enhance peripheral nerve regeneration[9–12]. The underlying mechanisms of LIES on nerve regeneration are still not fully elucidated, and may involve: the recruitment and guiding of Schwann cells migration[13]; the secretion or favoring of neurotrophic factors expression[14]; the facilitation of axons residue elimination from the Wallerian degeneration and subsequent speed-up of the axonal growth[11]; and/or the direct electrical guidance to the newly growing axonal sprouts, facilitating and fastening the regeneration of the neural function[15]. In order to understand the potential impact of this strategy in the treatment of post-prostatectomy ED, this study aims to evaluate the effects of LIES on CN regeneration, by means of penile structure protection in a cavernous nerve injury rat model which mimics nerve-sparing radical prostatectomy and neurogenic erectile dysfunction[16]. This study also intends to explore the cellular and molecular mechanisms underlying a potential beneficial effect of LIES.

4.3 Materials and Methods

In vivo experimental design

A well-established model of bilateral cavernous nerve injury (BCNI) was used[17]. Adult male Sprague-Dawley rats (325–350 g; Charles River Breeding Laboratories, Wilmington, MA, USA) were randomly separated into three groups (n=8–10/group): Sham, BCNI and BCNI+LIES. A bipolar microelectrode was implanted on the surface of each CN for all study groups. In the BCNI+LIES group, the electrodes were connected to an implantable pulse generator to deliver low-intensity electrostimulation for 1h per day for 7 days. Twenty four hours after the last treatment, the erectile function of each animal was assessed by penile erection studies as previously reported[18]. Following the penile erection studies, the animal was perfused with 0.9% saline solution. Sequentially, the penis shaft was collected and cut in 3 sections in 0.9% saline solution. 2 parts were snap-frozen for molecular analysis. In contrast, the third section was embedded in the cryoprotective matrix and frozen for cryosectioning and histological studies. The prostate gland was collected *en-bloc* with the implantable electrodes, incubated in 4% paraformaldehyde solution (PFA, Sigma) for 48h, washed with PBS solution, and stored in 70% ethanol solution before paraffin infiltration and paraffin embedding for histological analysis. The electrodes were used as markers for the tissue sectioning plan (Figure 4:1 D). A wash period of 5min was allowed after the last electrical stimulation and before perfusion and tissue collection to avoid confounding effects of repeated electrical stimuli of the cavernous nerve on the molecular measurements.

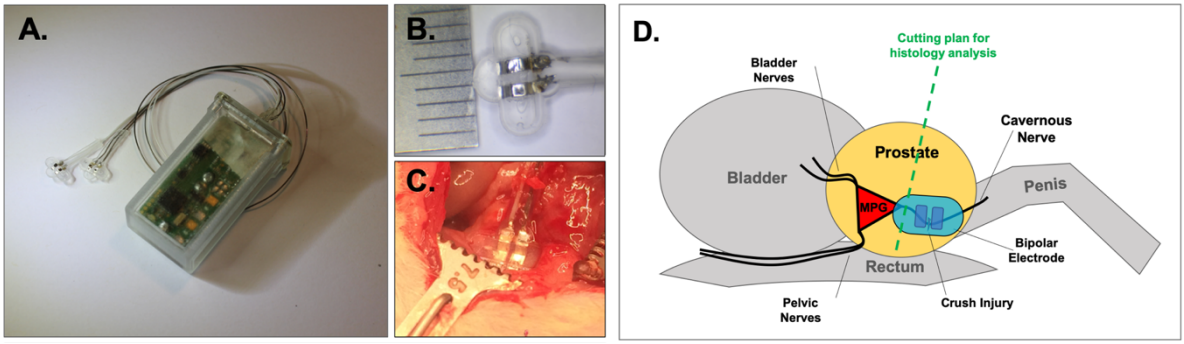

Figure 4:1 - Microelectrodes and implantable stimulator used to deliver LIES treatment. The implantable system used to deliver LIES treatment to bilateral CN after BCNI was manufactured in our laboratory using biocompatible 3D-printed plastic cases and injection-molding of medical grade silicone matrix to embed platinum contact electrodes. A) Picture of the implantable system used to deliver LIES showing two bipolar microelectrodes and an implantable stimulator. B) Bipolar microelectrode details. The exposed electrode area are two squares surfaces of platinum of 1mm x 1mm embedded in a medical grade silicone matrix. The ruler shows the length scale in mm. C) Microelectrode implanted over a rat CN bundle. Contact electrodes are placed at the site of crush injury. D) Schematic of the microelectrode position on top of the CN with the contact electrodes positioned over the crush injury site, 2-3mm from the MPG. For histology analysis of CN, the prostate was collected *en-block* with the microelectrodes in place allowing the use of the electrodes as marks to determine the slicing plan. Abbreviations: LIES: low-intensity electrostimulation, CN: cavernous nerve, BCNI: bilateral cavernous nerve injury, MPG: major pelvic ganglion.

The animal experimentation was approved by the local ethics committee and Swiss regulatory authorities (license number VD3431) under the guidelines from the directive 2010/63/EU of the European Parliament and by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee under the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Bilateral cavernous nerve crush injury (BCNI), bipolar electrodes implantation and low-intensity electrostimulation (LIES) treatment

To perform BCNI, rats were anesthetized with isoflurane inhalation (Oxygen flow rate of 0.8–1.5 l/min, 2.5% Isoflurane). Prostate and bilateral major pelvic ganglia were identified via a midline lower abdominal incision, and left and right cavernous nerves were isolated. Both nerves were crushed 1–2 mm distal to the major pelvic ganglia, as previously described[19]. To limit variability, all surgeries were completed by the same investigator. BCNI was induced by crushing both cavernous nerves with an ultra-fine hemostat (cat# 13006-12, Fine Science Tools) at a constant "two-click" pressure for two times 2 minutes per side. Sham surgeries were completed by exposing the cavernous nerves but not manipulating them. In-house produced bipolar platinum microelectrodes (Figures 4:1 A, B, and C) were placed on each CN at the site of injury or on the untouched nerve for the Sham group and maintained in place by a drop of veterinarian tissue adhesive (Vetbond Tissue Adhesive, cat# 35061000, WPI). The electrode wires were then wound up and placed in a subcutaneous pocket with or without an implantable pulse generator before closing the skin incision with staples. In the BCNI+LIES group, the electrode's cables were connected to a custom implantable pulse generator (Figure 4:1 A), delivering low-intensity and low-frequency electrical stimulation for 1h per day. The stimulation parameters were 1volt intensity, 0.1ms square-wave pulse duration, and 12Hz frequency for 1h per day for 7 days.

Penile erection studies.

Intracavernosal pressure (ICP) was measured in anesthetized rats (ketamine 100 mg/Kg, Xylazine 10 mg/Kg – Ketasol/Xylasol, Graeub, intraperitoneal injection), as described previously[18]. To induce penile erection, electrical stimulation was applied to the bilateral CN using the implanted microelectrodes. Electrode's cables were accessed by an abdominal midline incision, and the crura of the penis were identified by opening the penis foreskin. Stimulation parameters to induce erectile response were 2, 4, 6, and 8 Volts at a frequency of 18 Hz with square-wave pulses lasting 5 ms for 1 min. Maximum ICP (maximum pressure that is reached during CN electrical stimulation) and total ICP (ICP area under the curve, indicating the ICP response for the duration of CN electrical stimulation) were expressed per mean arterial pressures (MAP) as a difference from baseline. Response parameters were recorded using data acquisition (DI-190, Dataq Instruments), and results were analyzed using Matlab software (Mathworks).

Immunohistochemistry

Intra-cavernosal reactive oxygen species (ROS) content

To assess ROS content within rat corpus cavernosum, cryosections were stained with dihydroethidium (DHE; Cat# 37291, Sigma) as previously described[20]. Slides containing rat penis transversal cryosections of 8 µm were thawed at RT and washed with PBS. Sequentially, the sections were incubated with DHE at 15mM in PBS for 20 min in a dark humid chamber at 37 °C. After three washes, the slides were coverslip-sealed with DAPI containing mounting medium (Fluoroshield™, cat#F6057, Sigma) and examined under a confocal microscope (Carl Zeiss LSM 700, 20x magnification). DHE fluorescence intensity of acquired digital images was quantified by Image J software (NIH).

Intra-cavernosal total collagen content

Total intra-cavernosal collagen was assessed by Sirius red staining (Sigma Chemical Co), as previously reported[21]. Briefly, 8 µm rat penis cryosections were rinsed with water, and the nuclei stained with Weigert's hematoxylin for 10 min. After washing in tap water, the slides were incubated with 0.1% Sirius red in saturated picric acid for 60 min. Subsequently, the sections were rinsed twice with 5% acetic acid for 10 seconds, then immersed in absolute ethanol three times before clearing in xylene twice and cover-slipped. The sections were photographed using identical exposure settings under bright field light microscopy. Intra-cavernosal total collagen content was quantified by Image J software (NIH). Data were calculated as percentages of stained area to total area.

Intra-cavernosal smooth muscles content

The intracavernosal smooth muscle content was determined by immunostaining of the alpha-smooth muscle actin (α-SMA), as previously reported[22]. Briefly, slides containing rat penis transversal cryosections of 8 µm were thawed at RT and washed with PBS. Subsequently, tissue sections were fixed with ice-cold acetone for 10min, washed 2x with PBS before heat-induced epitope retrieval (HIER) in 10mM trisodium citrate buffer (pH 6.0) at 95°C for 20min. After cooling and rinsing the samples 2x with a washing solution containing 1% BSA (Sigma) + 0.025% Triton X-100 (Sigma) in PBS, the slides were blocked with a 3% BSA in PBS solution for 30min. After two additional rinses with the washing solution, tissue sections were incubated overnight at 4°C with a primary antibody mouse anti-α-SMA (dilution 1:800, cat# AB7817, Abcam) in a humidified chamber. The following day, tissues were rinsed 3x in washing solution before incubation with a fluorophore-conjugated secondary antibody donkey anti-mouse AF 488 (dilution 1:500, cat#

A21202, Thermofisher). After three washes, the slides were coverslip-sealed with DAPI containing mounting medium (Fluoroshield™, cat#F6057, Sigma) and examined under a confocal microscope (Carl Zeiss LSM 700, 20x magnification). Intra-cavernosal smooth muscle content was quantified by Image J software (NIH). Data were calculated as percentages of stained area to total area.

Cavernous nerve staining

The cavernous nerves between the crush injury and the MPG were stained for beta III tubulin (Tub-III), a nerve cell marker, S100 protein, a Schwann cell marker which is directly correlated to the nerve myelin sheet thickness[23] and neuronal nitric oxide synthase (nNOS) by immunostaining as previously described[24,25]. Briefly, slides containing paraffin-embedded prostate transversal section of 8 µm were dewaxed and washed with PBS before heat-induced epitope retrieval (HIER) in 10mM trisodium citrate buffer (pH 6.0) at 95°C for 20min. After cooling and rinsing the samples 2x with a washing solution containing 1% BSA (Sigma) + 0.025% Triton X-100 (Sigma) in PBS, the slides were blocked with a 3% BSA in PBS solution for 30min. After two additional rinses with the washing solution, tissue sections were incubated overnight at 4°C with a primary antibody rabbit anti-Tub-III (dilution 1:500, cat# AB52623, Abcam), mouse anti-S100 (dilution 1:500, cat# AB212816, Abcam) and/or goat anti-nNOS (dilution 1:500, cat# AB1376, Abcam) respectively in a humidified chamber. The following day, tissues were rinsed 3x in washing solution before incubation with a fluorophore-conjugated secondary antibody donkey anti-rabbit AF 555 (dilution 1:500, cat# A32794, Thermofisher), donkey anti-mouse AF 647 (dilution 1:500, cat# A31571, Thermofisher) and/or donkey anti-goat AF647 (dilution 1:500, cat# A21447, Thermofisher). After three washes, the slides were coverslip-sealed with DAPI containing mounting medium (Fluoroshield™, cat#F6057, Sigma) and examined under a confocal microscope (Carl Zeiss LSM 700, 40x magnification).

Cavernous nerve myelination was quantified by Image J software (NIH), and data were calculated as the percentage of the myelinated area (stained by S100) to percentage of nerve area (stained by Tub-III). The cavernous nerve nNOS-positive cells ratio was quantified by Image J software (NIH), and data were calculated as the number of nNOS-positive cells normalized by total nerve cells.

Apoptosis of penile dorsal nerve cells

The number of apoptotic nerve cells within the dorsal penile nerves was determined by Transferase-Mediated dUTP-biotin Nick End Labeling (TUNEL) staining (ApopTag® red *In Situ* apoptosis detection kit, cat# S7165, Millipore) following the manufacturer's protocol and recommendations. In brief, slides containing rat penis transversal cryosections of 8 µm were thawed at RT and washed with PBS. Sequentially, tissue sections were fixed with 1% paraformaldehyde in PBS solution for 10min, washed 2x with PBS and post-fixed in precooled ethanol and acetic acid solution (2:1) at -20°C for 5min. Following two additional washes with PBS, heat-induced epitope retrieval (HIER) in 10mM trisodium citrate buffer (pH 6.0) at 95°C for 20min was applied. After cooling and rinsing the samples 2x with PBS, the slides were incubated in a equilibration buffer for 1min at room temp. before incubation at 37°C in a humidified chamber with working strength TdT enzyme (70% reaction buffer and 30% TdT enzyme stock). The enzymatic reaction was stopped by placing the specimens in stop/wash buffer for 10min at room temp. before incubating the samples in working strength anti-digoxigenin rhodamine-conjugated antibody solution (53% blocking solution and 47% sheep anti-digoxigenin antibody stock) with rabbit anti-Tub-III (dilution 1:500, cat# AB52623, Abcam) primary antibody for 30min. Following three washes in PBS, samples were incubated with donkey anti-rabbit AF 647 (dilution 1:500, cat# A31573, Thermofisher) secondary antibody for 30min. After three washes, the slides were coverslip-sealed with DAPI containing mounting medium (Fluoroshield™, cat#F6057, Sigma) and examined under a confocal microscope (Carl Zeiss LSM 700, 40x magnification). The number of apoptotic nerve cells within the dorsal penile nerves was quantified by Image J software (NIH). Data were calculated as the percentage of TUNEL stained area to nerve area.

Western Blot

Protein expression levels of transforming growth factor-beta 1 (TGF-β1), interleukin 6 (IL-6), C reactive protein (CRP), collagen type I (Col-I), endothelial nitric oxide synthase (eNOS), total and phosphorylated extracellular signal-regulated kinases (ERK1/2 and P-ERK1/2 respectively, phosphorylation sites: ERK1-Thr185 and ERK2-Tyr187), total and phosphorylated protein kinase B (AKT and P-AKT respectively, phosphorylation site: Ser473), within the rat corpus cavernosum were assessed by Western Blotting.

After running 40 µg of rat penis protein extraction on a 10% SDS-PAGE gel, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilion™, cat# IPFL00010, Merck Millipore, 0.45 µm pore size). After blocking for 1h, the membranes were probed with one of the following primary antibodies: anti-TGF-β1 (dilution 1:500, cat# AB179695, Abcam, Inc.); anti-IL-6 (dilution 1:500, cat# AB65842, Abcam); anti-CRP (dilution 1:500, cat# AB9324, Abcam); anti-Col-I (dilution 1:1000, cat# AB765P, Merck); anti-eNOS (dilution 1:1000, cat# AB76198, Abcam); anti-ERK1/2 (dilution 1:1000, cat# AB54230, Abcam); anti-P-ERK1/2 (dilution 1:1000, cat# 700012, Thermofisher); anti-AKT (dilution 1:1000, cat# CST 4691-S, Cell Signaling Technology); or anti-P-AKT (dilution 1:1000, cat# CST S473, Cell Signaling Technology). Membranes were washed three times for 10 min with Tris-buffered saline-Tween (TBS-T) and incubated with the following secondary antibodies: anti-mouse IgG conjugated with IRDye 680RD (dilution 1:5000, cat#

926-68072, LiCor Biosciences), anti-Rabbit IgG conjugated with IRDye 680RD (dilution 1:5000, cat# 926-68073, LiCor Biosciences), anti-goat IgG conjugated with IRDye 800CW (dilution 1:5000, cat# 926-32212, LiCor Biosciences) or anti-rabbit IgG conjugated with IRDye 800CW (dilution 1:5000, cat# 926-32213, LiCor Biosciences) for 2h at RT. After three washes of 10 min with TBS-T, the fluorescence signal of the membranes was detected by an Odyssey CLx imaging system (LiCor Biosciences). The total amount of loaded protein and the membrane transfer quality were assessed by REVERT™ total protein stain kit (cat# 926-11015, LiCor Biosciences). Acquired images were analyzed similarly to the blot images using Image Studio Lite software (LiCor Biosciences). The relative protein expression level was determined by normalization, referring to the sham group.

Data Analysis

Statistical analyses for all measurements were performed using an unpaired Student t-test (one-tailed) or one-way/two-way analysis of variance (ANOVA), respectively comprising intergroup comparisons by Tukey's posthoc test. Statistical analysis was performed using Prism 6.0 software (GraphPad). A *p-value* <0.05 was considered significant, and the results are expressed as mean ± SEM.

4.4 Results

LIES enhanced the erectile function following CN injury

One week after nerve injury, the EF of each animal was assessed. As expected, the EF of BCNI animals was significantly impacted by the nerve crush damage, and only a minor response to CN stimulation at higher current intensity was observed. In contrast, the EF of Sham animals was unaffected and equivalent to a typical EF response as reported[24,26]. Interestingly, after one week of LIES treatment, a partially restored EF was observed (Figure 4:2 A). A significant increase of both the maximum ICP and total ICP (normalized ICP area above basal) was found in the BCNI+LIES group compared to the BCNI only group (5.9-fold increase, $p<0.01$ and 7-fold increase, $p<0.01$ respectively, n=6 animals per group). However, one week of LIES treatment was not able to induce a full recovery of the EF, as a significant difference with the Sham group was still observed (Figure 4:2 B, C, D, and E). The maximal difference between groups was seen at the stimulation of the CN at a sub-maximal intensity of 6 volts. A stimulation and contraction of the pelvic muscles were observed at an intensity higher than 8 volts. Only a negligible response was observed at 2 volts stimulation which differs from previously reported data[27], yet this slight difference could be attributed to the use of different electrodes for the stimulation or the fibrotic encapsulation of the implanted electrodes after one week, requiring a higher intensity to induce an erectile response. Nevertheless, these results also confirm that the LIES stimulation parameters chosen (1V) are far under the threshold required to induce an erectile response. Altogether, these results indicate a definite positive effect of LIES on EF recovery following CN injury.

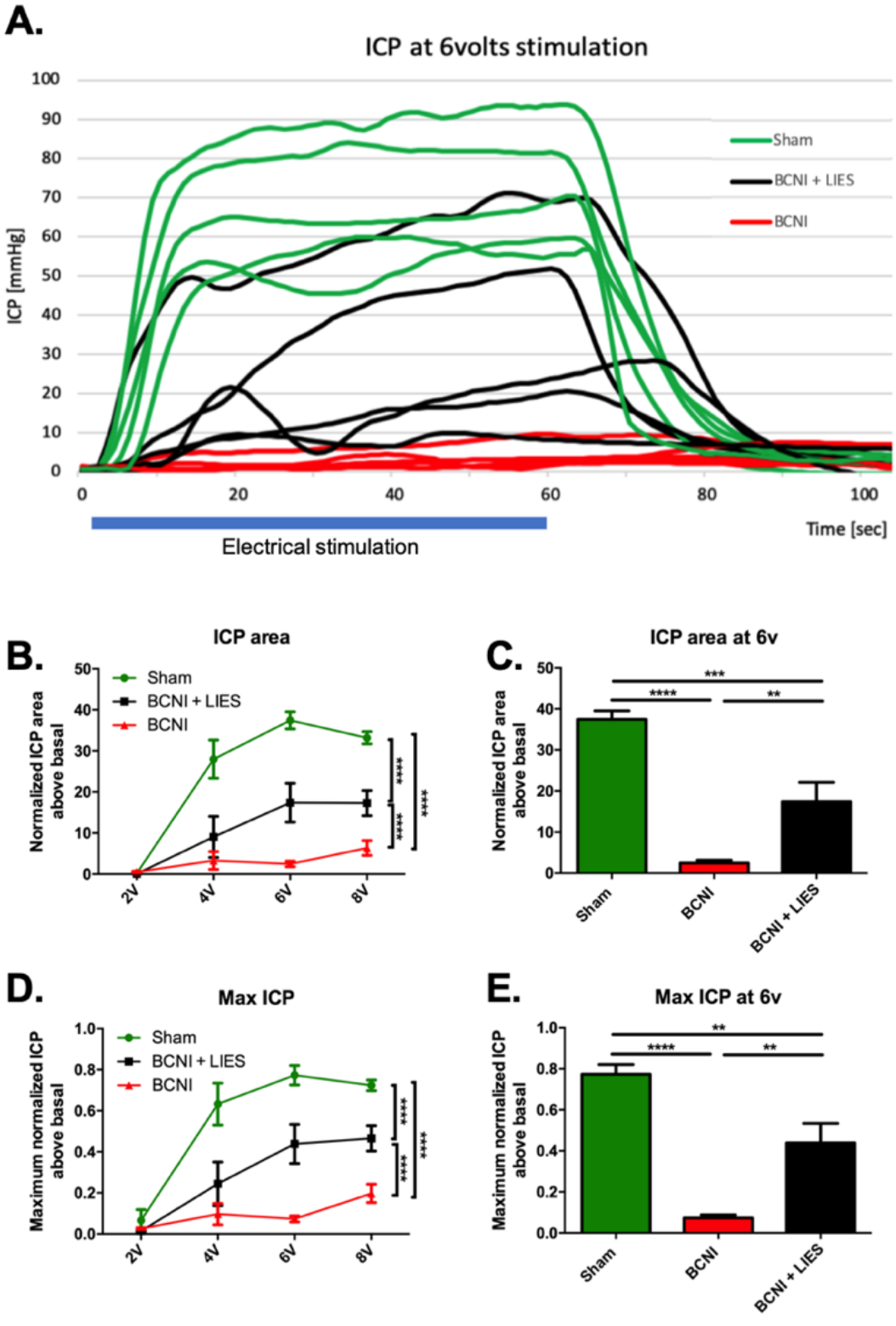

Figure 4:2 - LIES treatment enhances erectile function recovery after nerve injury. To assess EF, ICP and MAP were measured continuously while an erectile response was induced by electrically stimulating the CN for 1min. Maximal normalized ICP response was observed at 6-volt stimulation. A) Representative ICP response curve to 6V stimulation showing a normal response for Sham, strongly reduced response for BCNI animals and partially restored response for BCNI+LIES group. B and D) Normalized ICP area and maximum normalized ICP above basal at 2, 4, 6, and 8V stimulation for each group, indicating a maximal response at 6V stimulation, and significant difference between groups. C and E) Normalized ICP area and maximum normalized ICP above basal at 6V showing significant difference in EF. n=6 animals per group. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (Two-way ANOVA or Unpaired Student t test, with equal standard deviation (SD)). Each data point represents the mean ± SEM. Abbreviations: LIES: low-intensity electrostimulation, ICP: intra-cavernosal pressure, MAP: mean arterial pressure, CN: cavernous nerve, BCNI: bilateral cavernous nerve injury, SEM: standard error of the mean.

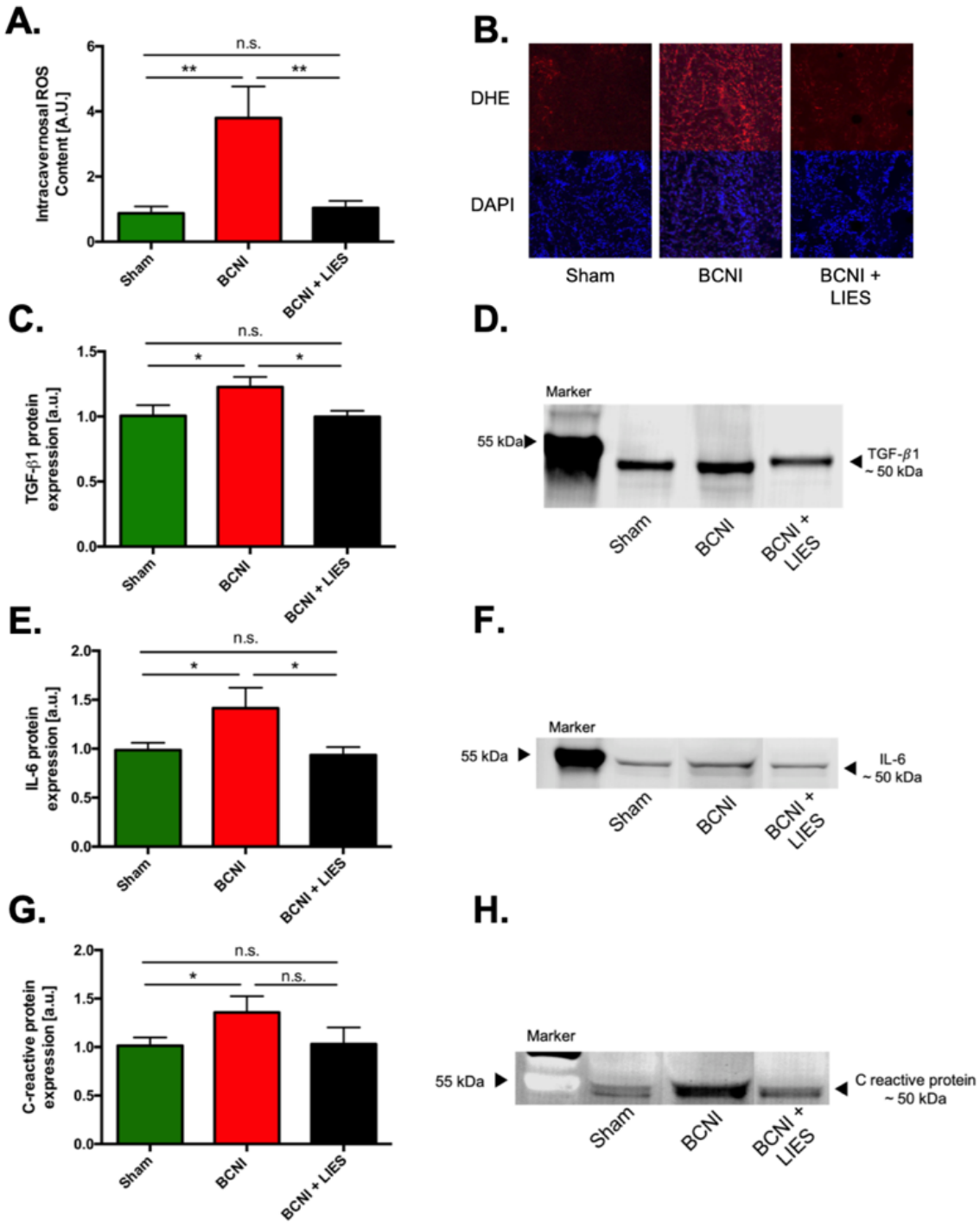

Figure 4:3 - LIES treatment reduces intra-cavernosal oxidative stress and inflammation following CN injury. To assess intra-cavernosal tissue health, ROS, pro-fibrotic and pro-inflammatory cytokines as well as inflammation marker protein expression have been measured. A) Quantification of intra-cavernosal ROS content measured by DHE staining (n=9 animals per group). B) Representative images of the CC slices stained by DHE (Top) and DAPI (Bottom), indicating oxidative stress level and staining the cell nucleus respectively at 10x magnification. C, E and G) Quantification of the intra-cavernosal protein expression of the pro-fibrotic cytokine TGF-β1, the pro-inflammatory cytokine IL-6 and the inflammation marker CRP respectively (n=9 animals per group). D, F and H) Representative images of the Western blotting of TGF-β1, IL-6 and CRP expression showing a specific single band at the expected molecular weight. * p<0.05, ** p<0.01 (One-tailed unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative protein expression or relative fluorescence in arbitrary unit (A.U.). Abbreviations: LIES: low-intensity electrostimulation, ROS: reactive oxygen species, DHE: dihydroethidium, CC: corpus cavernosum, DAPI: 4',6-diamidino-2-phenylindole, TGF-β1: transforming growth factor beta 1, IL-6: interleukin 6, CRP: C-reactive protein, CN: cavernous nerve, BCNI: bilateral cavernous nerve injury, SEM: standard error of the mean. Marker = molecular weight markers.

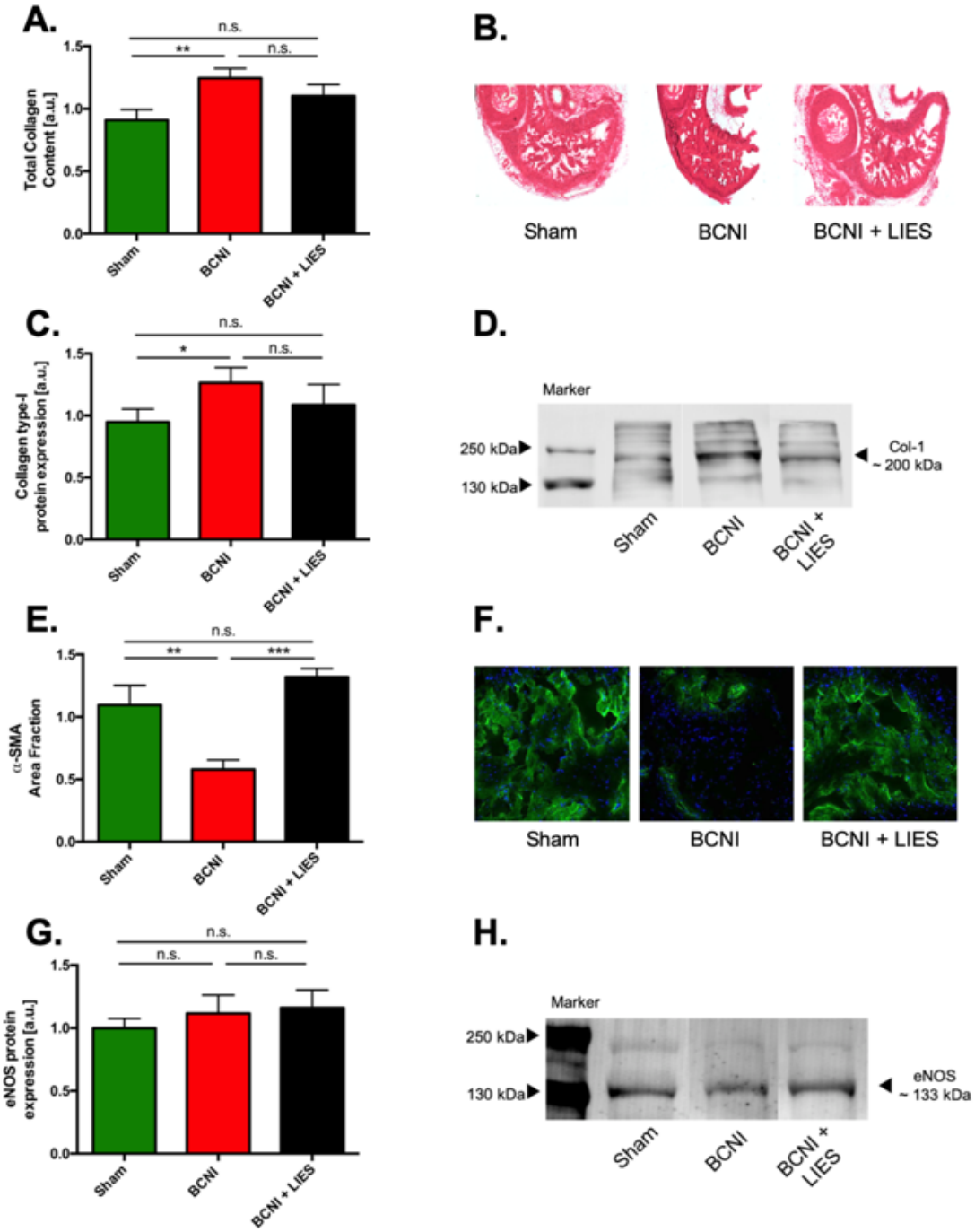

Figure 4:4 - LIES protect the CC against nerve-injury induced increase of fibrosis and smooth muscle loss. In order to assess the fibrosis and smooth muscle cells content within the CC, total collagen by Sirius Red staining, intra-cavernosal collagen type-I protein expression and α-SMA immunostaining, a marker for smooth muscle cells, were conducted. A) Quantification of total intra-cavernosal collagen content calculated from Sirius Red stained CC sections (n=9 animals per group). B) Representative image of CC sections stained with Sirius Red. Redder color indicates more collagen content. C and G) Quantification of Col-I and eNOS expression respectively, from penis tissue protein extracts (n=9 animals per group). D and H) Representative images of the Western blotting of Col-I and eNOS expression respectively, showing a specific single band at the expected molecular weight. E) Quantification of the area fraction positive to α-SMA from CC sections immuno-stained against this marker and indicating the amount of smooth muscle cells content (n=9 animals per group). F) Representative images of CC sections stained for α-SMA (a marker for smooth muscle cells) in green and DAPI in blue. * p<0.05, ** p<0.01, *** p<0.001 (One-tailed unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative protein expression or area fraction in arbitrary unit (A.U.). Abbreviations: LIES: low-intensity electrostimulation, CC: corpus cavernosum, α-SMA: alpha smooth muscle actin, Col-I: collagen type-I, eNOS: endothelial nitric-oxide synthase, DAPI: 4',6-diamidino-2-phenylindole, BCNI: bilateral cavernous nerve injury, SEM: standard error of the mean. Marker = molecular weight markers.

LIES protected the CC structure and health following CN injury

After one week of treatment, the rat penises were collected for histological and protein expression analyses. A significant increase of intra-cavernosal reactive oxygen species (ROS) content was observed in the BCNI group compared to the BCNI+LIES and Sham groups (2.9-fold increase, p=0.0065, n=9 animals per group), as shown by DHE staining (Figure 4:3 A and B). Following the same pattern, the pro-inflammatory and pro-fibrotic protein expression of TGF-β1 protein (Figure 4:3 C and D), the expression of pro-inflammatory cytokine IL-6 (Figure 4:3 E and F) and the expression of inflammation signaling cascade protein and inflammation marker CRP (Figure 4:3 G and H) were significantly increased in the BCNI group ,while maintained at a normal level in the treated and Sham group (22.2 $\pm$ 11.3% increase, p=0.034; 42.7 $\pm$ 23.6% increase, p=0.042; 34.2 $\pm$ 18.7% increase, p=0.042 respectively, n=9 animals per group). A significant increase in total and type-1 collagen expression was also observed in the BCNI group compared to Sham (33.7 $\pm$ 11.6% increase, p=0.01; 31.7 $\pm$ 16.1% increase, p=0.033 respectively, n=9 animals per group) (Figure 4:4 A, B, C, and D). A protective effect of LIES against intra-cavernosal collagen increase is possible as no significant difference was seen between Sham and BCNI+LIES group, however, no significant difference was observed between BCNI+LIES and BCNI groups either, potentially due to the natural high amount of collagen present in the CC tissues masking small differences in collagen content. On the other hand, the CN crush injury induced a significant decrease in intra-cavernosal smooth muscle content in the BCNI group compared to Sham (51.7 $\pm$ 7.7% decrease, p=0.008, n=9 animals per group). No decrease of smooth muscle content was observed in the LIES treatment group (Figure 4:4 E and F). Finally, no difference in intra-cavernosal eNOS protein expression was observed between groups (Figure 4:4 G and H), indicating that the reduction in smooth muscle cells is likely not associated with a significant decrease in endothelial cells following seven days after BCNI.

Those results indicate that one week of LIES treatment protects the CC structure and health by preventing intra-cavernosal oxidative stress, inflammation, fibrosis, and smooth muscle loss following CN injury.

To investigate the potential molecular mechanisms involved in the protective effects of LIES, the expression of ERK and AKT signaling cascades have been measured in the protein extract from the rat penis. A significant increase in both p-ERK/ERK and p-AKT/AKT ratio was observed after one week of LIES treatment compared to Sham and BCNI groups (103.4 $\pm$ 44.0% increase, p=0.002; 60.2 $\pm$ 30.7% increase, p=0.035 respectively, n=9 animals per group) (Figure 4:5), indicating that both signaling pathways have been activated by LIES treatment.

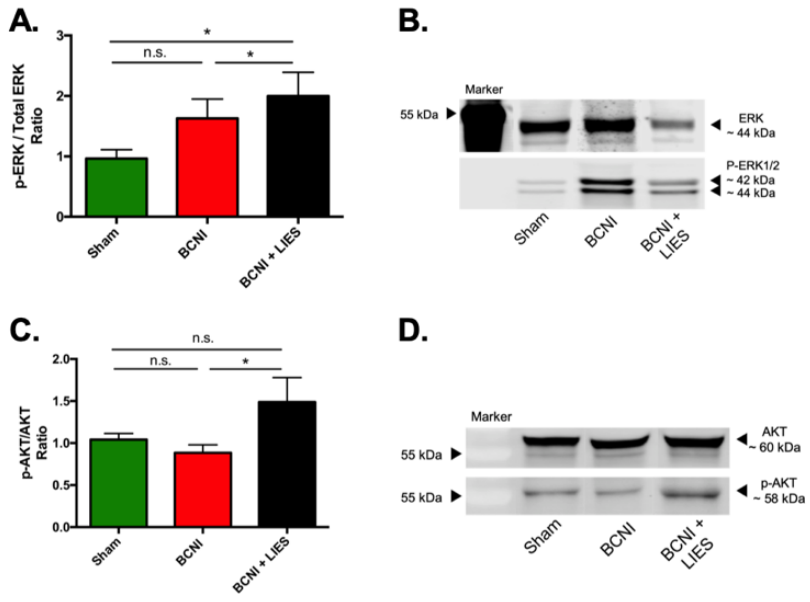

Figure 4:5 - LIES treatment activate ERK and AKT signaling pathways. To investigate the potential molecular mechanisms underlying the positive effects of LIES after BCNI, the total and phosphorylated form of ERK1/2 and AKT protein expression within the penis have been measured by Western Blot. A and C) Ratio of the phosphorylated ERK1/2 (phosphorylation site: ERK1-Thr185 and ERK2-Tyr187) over total ERK and phosphorylated AKT (phosphorylation site: Ser473) over AKT expression respectively, indicating an activation of both pathways by LIES treatment compared to controls. B and D) Representative images of the Western blotting of total ERK, p-ERK1/2, AKT and p-AKT expression respectively, showing a specific single band at the expected molecular weight (two distinct bands at 42 and 44 kDa for p-ERK1 and p-ERK2 respectively). n=9 animals per group. * p<0.05 (One-tailed unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative protein expression in arbitrary unit (A.U.). Abbreviations: LIES: low-intensity electrostimulation, CC: corpus cavernosum, ERK1/2: extracellular signal-regulated kinases 1 and 2, p-ERK: phosphorylated ERK, AKT = protein kinase B, p-AKT: phosphorylated AKT, BCNI: bilateral cavernous nerve injury, SEM: standard error of the mean. Marker = molecular weight markers.

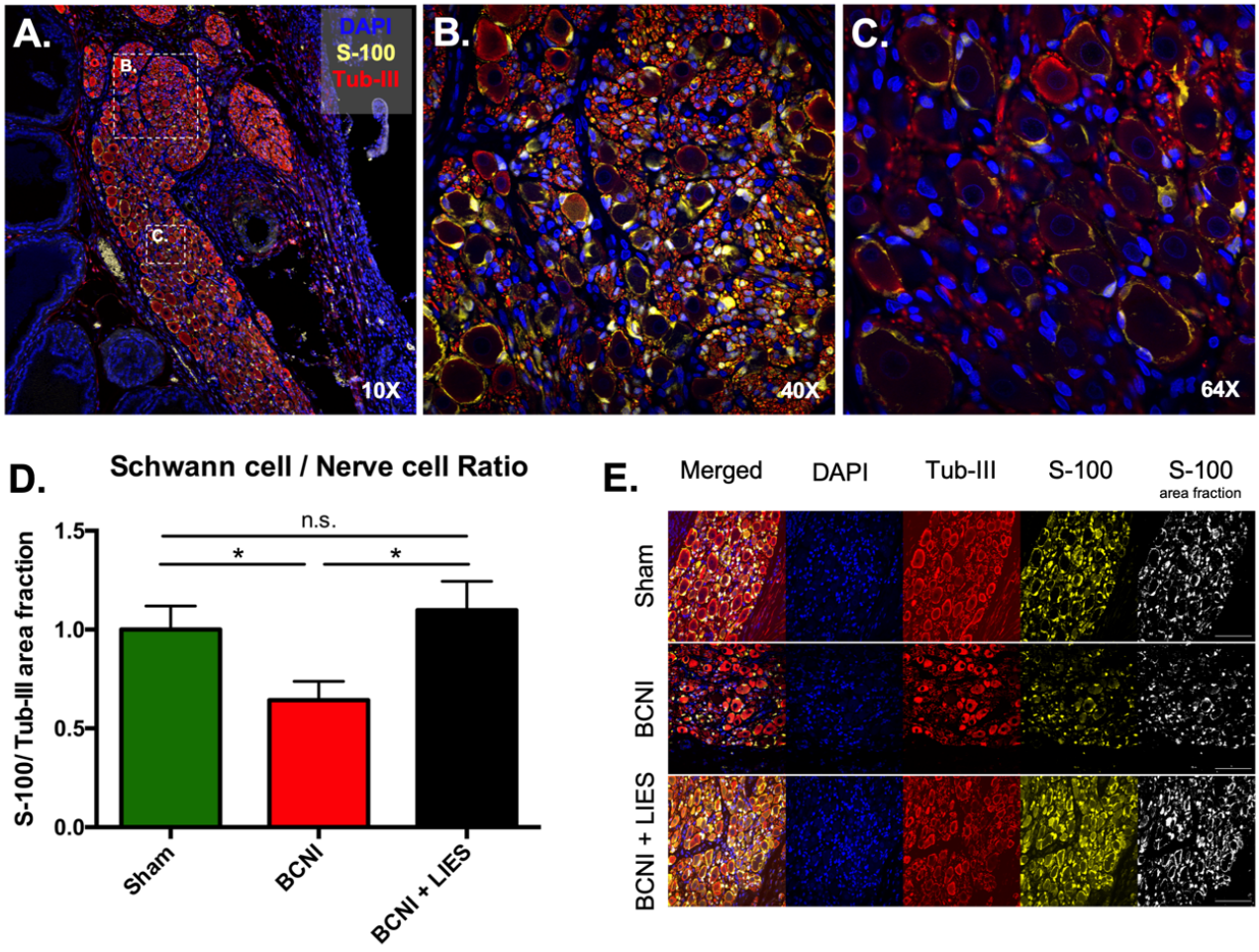

Figure 4:6 - LIES treatment preserve the Schwann cells and myelination of CN following injury. The S-100 protein, a marker for Schwann cells, for which expression is also directly related to the level of myelination of nerve fibers has been used to assess the CN structure following injury and LIES treatment. A, B and C) Representative images of the immunofluorescent staining of the CN bundle at the exit of the MPG at a 10x, 40x and 64x magnification. DAPI stains cell nucleus in blue, S-100 stains Schwann cells in yellow and Tub-III stains neural cells in red. D) Quantification of the area fraction stained by S-100 over the area fraction stained by Tub-III, representing the normalized amount of Schwann cells by the total number of neural cells. E) Representative images of the immunofluorescent stained CN sections at a 40x magnification used for the quantification of Schwann cells, showing each molecular marker channels, a merged image of all channels and the respective area fraction stained by S-100 for each group. Scale bar = 100μm. n=8 animals per group. * p<0.05, ** p<0.01, *** p<0.001 (One-tailed unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative area fraction ratio in arbitrary unit (A.U.). Abbreviations: LIES: low-intensity electrostimulation, CN: cavernous nerve, S-100: S100 protein, Tub-III: class III β-tubulin, DAPI: 4',6-diamidino-2-phenylindole, SEM: standard error of the mean.

LIES protected the CN structure following nerve crush injury

A prostate slide containing CN at the entry of the MPG level was stained for S-100 (Schwann cell marker), Tub-III (nerve cell marker), and/or nNOS. A significant decrease of the S-100 stained area (35.7 $\pm$ 17 % decrease, p=0.0283, n=8 animals per group) and nNOS positive nerve cells (37.9 $\pm$ 10 % decrease, p=0.0008, n=8 animals per group) within the CN section were observed following the nerve injury in BCNI group. The amount of S-100 protein is directly proportional to the myelin layer thickness of nerve cells[23], indicating here an impairment of the myelination following BCNI. Interestingly, one week of LIES treatment protected the myelination level and nNOS positive nerve cells in the BCNI+LIES group to the physiological Sham level (Figures 4:6 and 4:7). The apoptosis of DPN cells was also evaluated by TUNEL staining of CC slice. BCNI significantly increased the number of apoptotic nerve cells following nerve injury (2.3-fold increase, p=0.0064, n=5 animals per group). In comparison, LIES reduced the number of apoptotic cells in the BCNI+LIES group (1.6-fold decrease, p=0.025, n=5 animals per group) at a level slightly superior to the Sham group (1.7-fold increase, p=0.044, n=5 animals per group) (Figure 4:8).

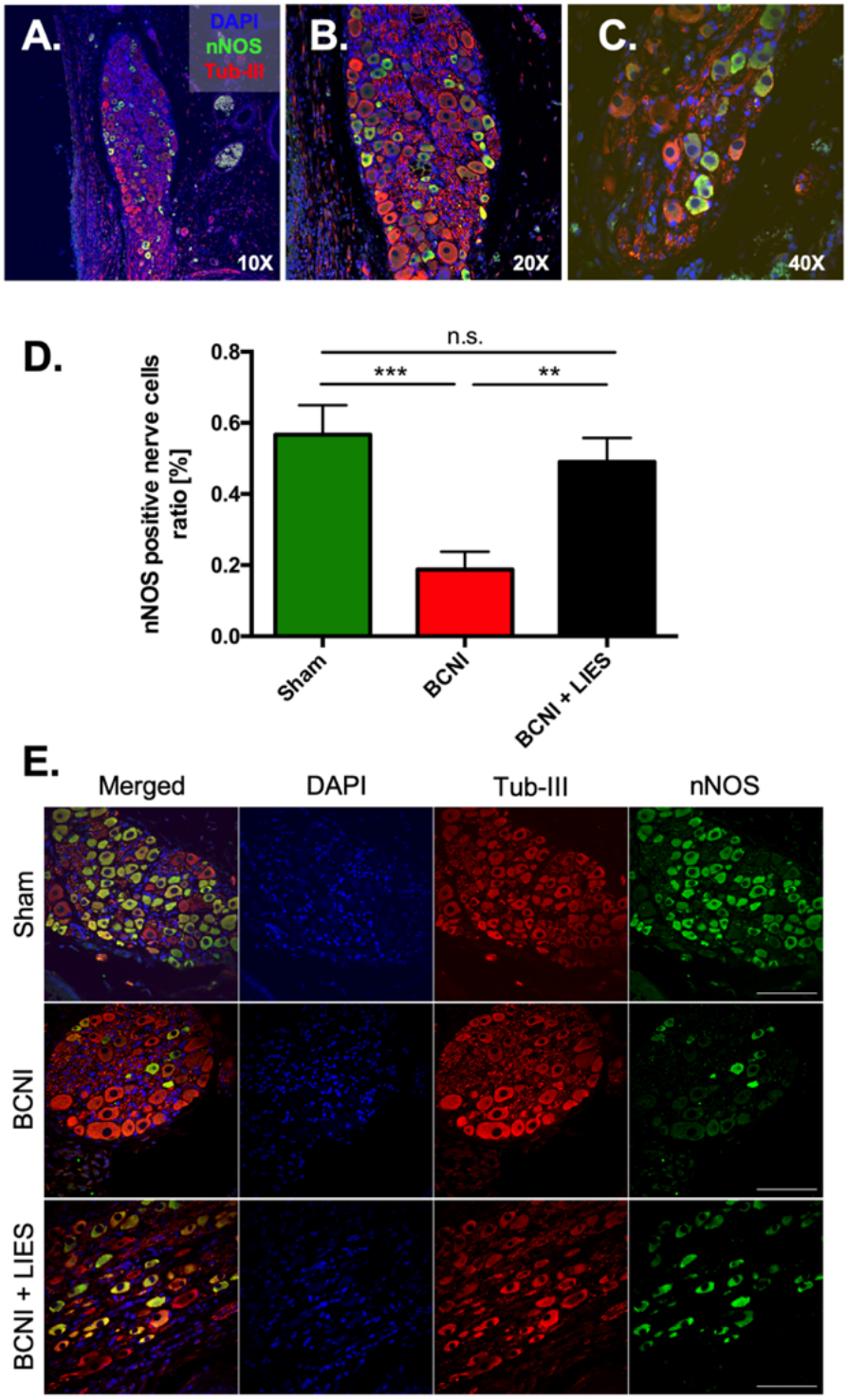

Figure 4:7 - LIES treatment prevent the loss of nitrergic nerve cells in the CN following nerve injury. The number of nNOS positive nerve fibers within the CN has been used to assess the CN structure following injury and LIES treatment. A, B and C) Representative images of the immunofluorescent staining of the CN bundle at the exit of the MPG at a 10x, 20x and 40x magnification. DAPI stains cell nucleus in blue, nNOS stains nitrergic nerve cells in green and Tub-III stains neural cells in red. D) Quantification of the cell count ratio of nNOS positive cells over the total number of nerve cells stained by Tub-III. E) Representative images of the immunofluorescent stained CN sections at a 20x magnification used for the quantification, showing each molecular marker channels and a merged image of all channels for each group. Scale bar = 200μm. n=8 animals per group. * p<0.05, ** p<0.01, *** p<0.001 (One-tailed unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative nNOS positive cell count ratio in arbitrary unit (A.U.). Abbreviations: LIES: low-intensity electrostimulation, CN: cavernous nerve, nNOS: neuronal nitric-oxide synthase, Tub-III: class III β-tubulin, DAPI: 4',6-diamidino-2-phenylindole, SEM: standard error of the mean.

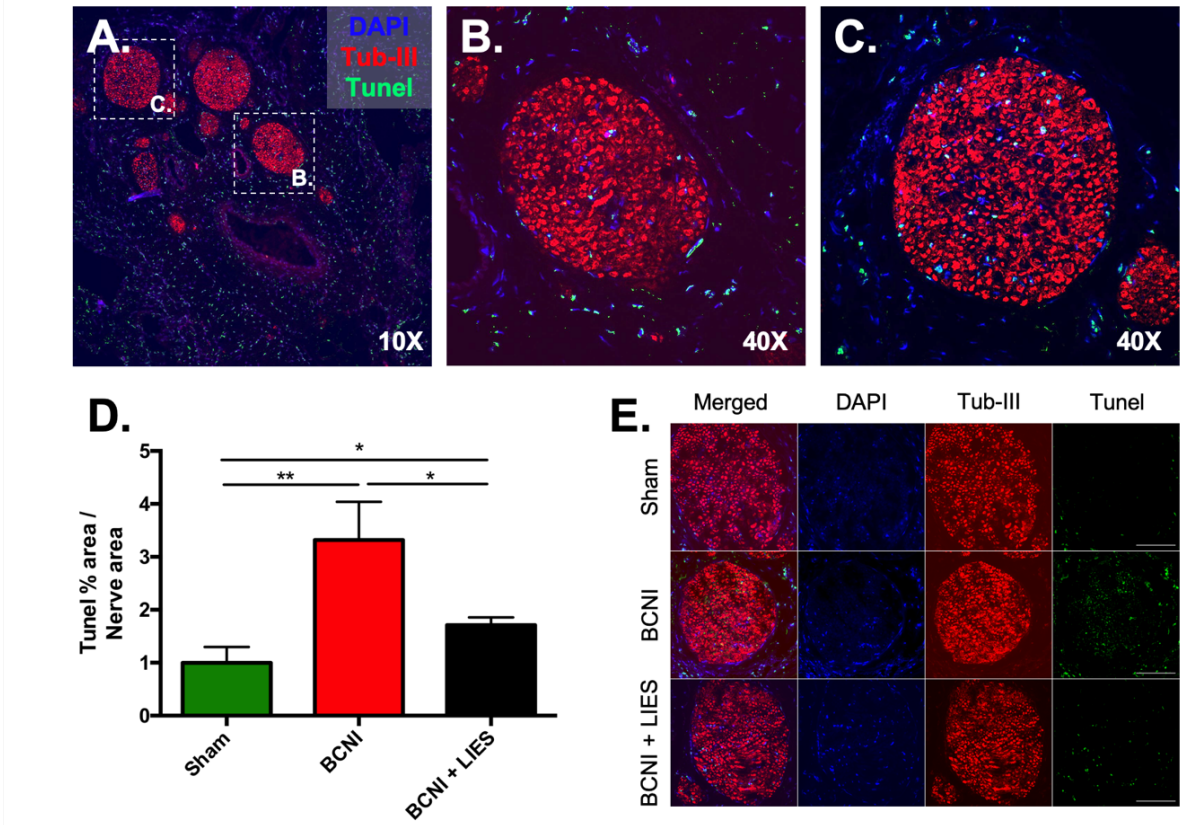

Figure 4:8 - LIES treatment diminish DPN nerve cells apoptosis following BCNI. The number of apoptotic nerve cells within the DPN, 7 days after CN injury, has been measured by TUNEL assay. A, B and C) Representative images of the immunofluorescent staining of the DPN fibers within the CC at a 10x and 40x magnification. DAPI stains cell nucleus in blue, Tub-III stains neural cells in red and TUNEL assay stains apoptotic cells in green. D) Quantification of the ratio of TUNEL stained area fraction over nerve area. E) Representative images of the immunofluorescent stained CC sections at a 40x magnification used for the quantification, showing each molecular marker channels and a merged image of all channels for each group. Scale bar = 100μm. n=5 animals per group. * p<0.05, ** p<0.01, *** p<0.001 (One-tailed unpaired Student t test, with equal standard deviation (SD)). Each column represents the mean ± SEM of relative TUNEL stained area ratio normalized by nerve area in arbitrary unit (A.U.). Abbreviations: LIES: low-intensity electrostimulation, BCNI: bilateral cavernous nerve injury, DPN: dorsal penile nerve, CN: cavernous nerve, TUNEL: transferase-mediated dUTP-biotin nick end labeling, Tub-III: class III β-tubulin, DAPI: 4',6-diamidino-2-phenylindole, SEM: standard error of the mean.

4.5 Discussion

This study shows, for the first time, that LIES improves EF recovery by protecting erectile tissue and CN structures and functions from the harmful effect of BCNI. It indicates that LIES can be a promising tool to promote penile rehabilitation and diminish the burden of post-prostatectomy ED. Despite the fact that prostate cancer remains the most common cancer among men[28], the survival rate is nearly 100 % for this locally restricted disease, due to the excellent cancer control provided by radical prostatectomy. Unfortunately, even with nerve-sparing techniques and technological improvements, some degree of nerve damage is inevitable due to the proximity of the CN with the prostate gland and can lead to temporary or permanent ED in the majority of cases[29]. CN and peripheral nerves are generally known to have the capability to regenerate[30], however, the period of time for nerve regeneration is considerably long, which is detrimental for penile tissue structure[3]. Now that prostate cancer management has reached such a high survival rate, it points out the need for better strategies to manage surgical complications such as post-prostatectomy ED to increase the quality of life of these patients. The impact of penile rehabilitation on humans is not fully validated as, collectively, clinical trials have reported minor to no improvement using current tools[31–36], indicating that existing penile rehabilitation therapies are insufficient to prevent detrimental effects of nerve injury on penis and nerve structure. Hence, a combination of therapies or strategies that would take in account both penile tissue perfusion and nerve regeneration may provide the highest chances of erectile function recovery following injury or trauma[37]. Therefore, LIES appears as an optimal strategy by inducing nerve regeneration and penile erection to maintain normal tissue perfusion, protecting the penile tissue from the negative effect of neurapraxia.

This study indicates that LIES treatment protects the CN structures as a decrease in apoptosis within the CN was observed compared to control. However, it cannot be determined whether or not LIES improves CN regeneration and nerve fiber regrowth by itself, as

shown for other peripheral nerves[30]. Nonetheless, this study indicates that the beneficial effect of LIES may involve the activation of the cell growth and survival signaling pathways, as we observed increased phosphorylation of ERK1/2 and AKT kinases. Indeed, Akt, also referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis[38], while the extracellular signal-related kinases (ERK1/2) regulate cell proliferation[39]. In accordance with these results, activation of ERK and AKT has been observed in rat peripheral nerve regeneration[40], while the inhibition of RhoA/ROCK signaling cascade, which inhibits AKT, prevented CN apoptosis and nNOS uncoupling, preserving neurogenic mediated nitrergic relaxation of the CC[24]. In addition, ERK phosphorylation has also shown an anti-apoptotic effect in neural cells[41,42]. It is then tempting to hypothesize that LIES protects the CN structure and function through the activation of ERK and AKT signaling; however, further studies are required to confirm this assumption. Further evaluation of alternative pathways such as cAMP/PKA, JAK/STAT, RhoA/ROCK or the production of neurotrophins such as BDNF may also shed more light on the actions of molecular mechanisms of LIES.

The difficulty to recover EF following RP is attributed to slow CN regeneration and deleterious penile tissue remodeling[3]. However, it may also be due to a loss of sensitivity to NO. Indeed, following the crushing of both penile nerves, excellent regeneration of nitrergic axons occurred after 10–12 weeks in rats, but neurogenic relaxation of CC muscles was shown to remain relatively poor. This was at least partly due to attenuated tissue response to nitric oxide 3 weeks after injury[37], showing a clear disparity between regeneration of nitrergic axons and functional recovery. In the same study, retaining normal tissue perfusion contributed to maintaining nitric oxide signaling and tissue response to NO, therefore exposing the importance of continued erectile function for the CC to retain responsiveness to nitric oxide[37]. Although LIES intensity is much lower than the CN activation threshold necessary to induce penile erection, it is possible that it still induces an undetected erectile response or allows maintainability of the NO responsiveness by activating some nerve fibers, while still not inducing enough NO release to provoke a penile erection. It is also feasible that LIES protects the cavernous nerve terminals, as it has been postulated that the CN terminals may generate survival signals to penile tissues[27].

A clear beneficial effect on the CC structure following LIES treatment has also been observed in this study, however, it is unknown whether LIES has an effect on CN by protecting the CC from harmful oxidative stress conseculatively with inflammation damage, or if there is a direct effect of LIES on the stimulation and survival of smooth muscle cells and penile tissue oxygenation. The results of this study show an increased Akt and ERK phosphorylation in the CC following LIES treatment. The beneficial effects of LIES may then be due to the protection of smooth muscle cells through the pro-survival signaling of AKT and ERK pathways. Alternatively It has been shown that the activation of cavernous eNOS via stimulation of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway is required for the maintenance of penile erection following initiation of penile erection by neural-derived nitric oxide[43]. A direct activation of smooth muscle cells by LIES may then activate AKT pathway, which in turn activates eNOS and prevents eNOS uncoupling and superoxide generation[44,45]. Another possible mechanism could be that LIES may prevent nNOS uncoupling and ROS production, maintaining NO bioavailability, and reducing oxidative stress and subsequent inflammation[24,46,47]. Although the present study does not allow us to determine with certainty the complete underlying mechanisms of action of LIES on the protection of EF and erectile tissue structure, it indicates interesting possible mechanisms that further research may discover.

It is important to point out several limitations of the current study. Primarily, the use of an animal model. The use of a well-defined animal model is desired to allow the comparison of the actual data presented here with previously published research, however, animal models are not perfect in representing human pathophysiology and discoveries made in animals may not be transferrable to humans. It has also been shown that in rats, that CN regenerates quickly after crush injuries (10-12 weeks)[37], while in humans, the process may take more than 18 months following RP[3]. In addition, the CN in rats appear as an easily identifiable nerve bundle exiting the MPG, while in humans, although it is commonly accepted that the CN fibers are passing along the posterolateral side of the prostate within the so-called neurovascular bundles[2,6], there is rising evidence that proximally to the prostate, some fibers present more variability in their course and show a more “spray-like” distribution on the posterolateral until the anterolateral surface of the prostate gland, up to the level of the 2 o’clock and 10 o’clock positions. Most of the CN fibers are also not macroscopically visible in humans, increasing the probability of nerve damage during RP[48–50]. Nonetheless, this study uses a well-established model of post-prostatectomy neurogenic ED, first developed by Quinlan et al.[51]. This model was then optimized by Martinez et al.[52] and Mills et al.[53] by introducing the ICP measurement and the normalization of the ICP measurement by the MAP in order to generate reproducible data and taking into account animal inter-variability and effects of blood pressure on the EF. Different nerve injury techniques have previously been investigated, such as nerve transection, freezing, crushing with a bulldog clamp or a fine hemostat, with all showing reproducible and consistent damage leading to ED[16,26]. However, bilateral CN crush injury is considered to best represent nerve-sparing RP, inducing neuropraxia and ED, while not being as severe as nerve transection which better represents a non-nerve-sparing surgery[16]. BCNI has been shown to induce neuropraxia, causing CC hypoxia and inflammation, finally leading to erectile tissue modifications such as fibrosis and loss of smooth muscle cells in rats[54–57]. CN injury was also seen to induce apoptosis of nNOS positive ganglion in rat MPG[24]. In accordance, similar histological changes were observed in the human penis after RP[55], which is consistent with the results of this study where BCNI induced an increase of intra-cavernosal ROS content, an increase of inflammation as shown

by a rise in TGF-β1, IL-6 and CRP protein expression, an increased collagen content, a reduction of smooth muscle cells, an increased apoptosis in DPN and ultimately, a decrease of erectile function, endorsing the reliability of the animal model selected.

Another limitation is that the CN are peripheral nerves, and the parasympathetic and nitrergic status of those nerves may be responsible for different molecular and physiological response to injury than other peripheral nerves. This possibility should be kept in mind while comparing CN and other peripheral nerves' physiology and regeneration mechanisms. Finally, this study only shows the effects of LIES treatment after 7 days of nerve injury. The long-time effect of this treatment remains unknown. Nonetheless, this study presents promising results that may be translatable to human use in order to improve post RP ED management and diminish the unwanted effects of this essential surgical procedure.

4.6 Conclusion

Altogether, the results of this study are showing for the first time the beneficial effects of LIES treatment following CN injury. One week of treatment not only enhanced the EF of animals following BCNI, but also showed a protective effect on penile and nerve tissues, including the prevention of oxidative stress, inflammation, loss of smooth muscle cells, as well as increased fibrosis of the erectile tissue, while preventing apoptosis of the nitrergic CN fibers and maintaining nerve fiber myelination. This indicates LIES as a promising tool to reduce the burden of post-prostatectomy ED and propose a novel regiment to the principle of penile rehabilitation.

4.7 Conflict of Interest

None to be declared.

4.8 Acknowledgements

This research was funded by the Swiss National Science Foundation [DocMobility-Grant P1ELP3_178215 to M. Sturny] and the National Institutes of Health NIH [NIH-Grant to S. Karakus]. We thank the histology core facility of the EPFL Faculty of Life Science for helping with the histological sectioning and Sirius red staining. We also warmly thank Biljana Musicki for her help, support and advices during this study.

4.9 References

(1) Gratzke, C.; Angulo, J.; Chitaley, K.; Dai, Y.; Kim, N. N.; Paick, J.-S.; Simonsen, U.; Ückert, S.; Wespes, E.; Andersson, K. E.; others. Anatomy, Physiology, and Pathophysiology of Erectile Dysfunction. J. Sex. Med. 2010, 7 (1pt2), 445–475.

(2) Weyne, E.; Castiglione, F.; Van der Aa, F.; Bivalacqua, T. J.; Albersen, M. Landmarks in Erectile Function Recovery after Radical Prostatectomy. Nat. Rev. Urol. 2015, 12 (5), 289–297. https://doi.org/10.1038/nrurol.2015.72.

(3) Capogrosso, P.; Salonia, A.; Briganti, A.; Montorsi, F. Postprostatectomy Erectile Dysfunction: A Review. World J. Mens Health 2016, 34 (2), 73–88. https://doi.org/10.5534/wjmh.2016.34.2.73.

(4) McCullough, A. R. Sexual Dysfunction after Radical Prostatectomy. Rev. Urol. 2005, 7 (Suppl 2), S3–S10.

(5) Weyne, E.; Mulhall, J.; Albersen, M. Molecular Pathophysiology of Cavernous Nerve Injury and Identification of Strategies for Nerve Function Recovery after Radical Prostatectomy. Curr. Drug Targets 2015, 16 (5), 459–473.

(6) Sopko, N. A.; Burnett, A. L. Erection Rehabilitation Following Prostatectomy--Current Strategies and Future Directions. Nat. Rev. Urol. 2016, 13 (4), 216–225. https://doi.org/10.1038/nrurol.2016.47.

(7) Skoufias, S.; Sturny, M.; Fraga-Silva, R.; Papaioannou, T. G.; Stergiopoulos, N.; Adamakis, I.; Constantinides, C. A. Novel Concept Enabling an Old Idea: A Flexible Electrode Array to Treat Neurogenic Erectile Dysfunction. J. Sex. Med. 2018, 15 (11), 1558–1569. https://doi.org/10.1016/j.jsxm.2018.09.003.

(8) Burnett, A. L.; Teloken, P. E.; Briganti, A.; Whitehurst, T.; Montorsi, F. Intraoperative Assessment of an Implantable Electrode Array for Cavernous Nerve Stimulation. J. Sex. Med. 2008, 5 (8), 1949–1954. https://doi.org/10.1111/j.1743-6109.2008.00865.x.

(9) Huang, J.; Zhang, Y.; Lu, L.; Hu, X.; Luo, Z. Electrical Stimulation Accelerates Nerve Regeneration and Functional Recovery in Delayed Peripheral Nerve Injury in Rats. Eur. J. Neurosci. 2013, 38 (12), 3691–3701. https://doi.org/10.1111/ejn.12370.

(10) Willand, M. P.; Nguyen, M.-A.; Borschel, G. H.; Gordon, T. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 2016, 30 (5), 490–496. https://doi.org/10.1177/1545968315604399.

(11) Chan, K. M.; Curran, M. W. T.; Gordon, T. The Use of Brief Post-surgical Low Frequency Electrical Stimulation to Enhance Nerve Regeneration in Clinical Practice. J. Physiol. 2016, 594 (13), 3553–3559. https://doi.org/10.1113/JP270892.

(12) Gordon, T. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 2016, 13 (2), 295–310. https://doi.org/10.1007/s13311-015-0415-1.

(13) Gu, X.; Fu, J.; Bai, J.; Wang, J.; Pan, W.; Zhang, C. Low-Frequency Electrical Stimulation Induces the Proliferation and Differentiation of Peripheral Blood Stem Cells Into Schwann Cells. Am. J. Med. Sci. 2015, 349 (2), 157–161. https://doi.org/10.1097/MAJ.0000000000000385.

(14) Wang, L.; Sanford, M. T.; Xin, Z.; Lin, G.; Lue, T. F. Role of Schwann Cells in the Regeneration of Penile and Peripheral Nerves. Asian J. Androl. 2015, 17 (5), 776–782. https://doi.org/10.4103/1008-682X.154306.

(15) Haan, N.; Song, B. Therapeutic Application of Electric Fields in the Injured Nervous System. Adv. Wound Care 2014, 3 (2), 156–165. https://doi.org/10.1089/wound.2013.0450.

(16) Canguven, O.; Burnett, A. Laboratory Forum: Cavernous Nerve Injury Using Rodent Animal Models. J. Sex. Med. 2008, 5 (8), 1776–1785. https://doi.org/10.1111/j.1743-6109.2008.00955.x.

(17) Haney, N. M.; Nguyen, H. M. T.; Honda, M.; Abdel-Mageed, A. B.; Hellstrom, W. J. G. Bilateral Cavernous Nerve Crush Injury in the Rat Model: A Comparative Review of Pharmacologic Interventions. Sex. Med. Rev. 2018, 6 (2), 234–241. https://doi.org/10.1016/j.sxmr.2017.07.007.

(18) Burnett, A. L.; Sezen, S. F.; Hoke, A.; Caggiano, A. O.; Iaci, J.; Lagoda, G.; Musicki, B.; Bella, A. J. GGF2 Is Neuroprotective in a Rat Model of Cavernous Nerve Injury-Induced Erectile Dysfunction. J. Sex. Med. 2015, 12 (4), 897–905. https://doi.org/10.1111/jsm.12834.

(19) Musicki, B.; Bhunia, A. K.; Karakus, S.; Burnett, A. L. S-Nitrosylation of NOS Pathway Mediators in the Penis Contributes to Cavernous Nerve Injury-Induced Erectile Dysfunction. Int. J. Impot. Res. 2018, 30 (3), 108–116. https://doi.org/10.1038/s41443-018-0021-y.

(20) Fraga-Silva, R. A.; Costa-Fraga, F. P.; Faye, Y.; Sturny, M.; Santos, R. A. S.; da Silva, R. F.; Stergiopulos, N. An Increased Arginase Activity Is Associated with Corpus Cavernosum Impairment Induced by Hypercholesterolemia. J. Sex. Med. 2014, 11 (5), 1173–1181. https://doi.org/10.1111/jsm.12482.

(21) Fraga-Silva, R. A.; Costa-Fraga, F. P.; Savergnini, S. Q.; De Sousa, F. B.; Montecucco, F.; da Silva, D.; Sinisterra, R. D.; Mach, F.; Stergiopulos, N.; da Silva, R. F.; Santos, R. A. S. An Oral Formulation of Angiotensin-(1-7) Reverses Corpus Cavernosum Damages Induced by Hypercholesterolemia. J. Sex. Med. 2013, 10 (10), 2430–2442. https://doi.org/10.1111/jsm.12262.

(22) Li, H.; Chen, L.; Wang, T.; Wang, S.; Liu, J. The Effect of Cavernous Nerve Traction on Erectile Function in Rats. PLOS ONE 2017, 12 (10), e0186077. https://doi.org/10.1371/journal.pone.0186077.

(23) Mata, M.; Alessi, D.; Fink, D. J. S100 Is Preferentially Distributed in Myelin-Forming Schwann Cells. J. Neurocytol. 1990, 19 (3), 432–442. https://doi.org/10.1007/BF01188409.

(24) Hannan, J. L.; Matsui, H.; Sopko, N. A.; Liu, X.; Weyne, E.; Albersen, M.; Watson, J. W.; Hoke, A.; Burnett, A. L.; Bivalacqua, T. J. Caspase-3 Dependent Nitrergic Neuronal Apoptosis Following Cavernous Nerve Injury Is Mediated via RhoA and ROCK Activation in Major Pelvic Ganglion. Sci. Rep. 2016, 6. https://doi.org/10.1038/srep29416.

(25) Albersen, M.; Fandel, T. M.; Lin, G.; Wang, G.; Banie, L.; Lin, C.-S.; Lue, T. F. Injections of Adipose Tissue-Derived Stem Cells and Stem Cell Lysate Improve Recovery of Erectile Function in a Rat Model of Cavernous Nerve Injury. J. Sex. Med. 2010, 7 (10), 3331–3340. https://doi.org/10.1111/j.1743-6109.2010.01875.x.

(26) Mullerad, M.; Donohue, J. F.; Li, P. S.; Scardino, P. T.; Mulhall, J. P. Functional Sequelae of Cavernous Nerve Injury in the Rat: Is There Model Dependency. J. Sex. Med. 2006, 3 (1), 77–83. https://doi.org/10.1111/j.1743-6109.2005.00158.x.

(27) Lagoda, G.; Jin, L.; Lehrfeld, T. J.; Liu, T.; Burnett, A. L. FK506 and Sildenafil Promote Erectile Function Recovery after Cavernous Nerve Injury Through Antioxidative Mechanisms. J. Sex. Med. 2007, 4 (4, Part 1), 908–916. https://doi.org/10.1111/j.1743-6109.2007.00519.x.

(28) Haas, G. P.; Delongchamps, N.; Brawley, O. W.; Wang, C. Y.; de la Roza, G. The Worldwide Epidemiology of Prostate Cancer: Perspectives from Autopsy Studies. Can. J. Urol. 2008, 15 (1), 3866–3871.

(29) Wang, R. Penile Rehabilitation after Radical Prostatectomy: Where Do We Stand and Where Are We Going? J. Sex. Med. 2007, 4 (4, Part 2), 1085–1097. https://doi.org/10.1111/j.1743-6109.2007.00482.x.

(30) Zuo, K. J.; Gordon, T.; Chan, K. M.; Borschel, G. H. Electrical Stimulation to Enhance Peripheral Nerve Regeneration: Update in Molecular Investigations and Clinical Translation. Exp. Neurol. 2020, 332, 113397. https://doi.org/10.1016/j.expneurol.2020.113397.

(31) Montorsi, F.; Guazzoni, G.; Strambi, L. F.; Da Pozzo, L. F.; Nava, L.; Barbieri, L.; Rigatti, P.; Pizzini, G.; Miani, A. Recovery of Spontaneous Erectile Function after Nerve-Sparing Radical Retropubic Prostatectomy with and without Early Intracavernous Injections of Alprostadil: Results of a Prospective, Randomized Trial. J. Urol. 1997, 158 (4), 1408–1410.

(32) Padma-Nathan, H.; McCullough, A. R.; Levine, L. A.; Lipshultz, L. I.; Siegel, R.; Montorsi, F.; Giuliano, F.; Brock, G.; Study Group. Randomized, Double-Blind, Placebo-Controlled Study of Postoperative Nightly Sildenafil Citrate for the Prevention of Erectile Dysfunction after Bilateral Nerve-Sparing Radical Prostatectomy. Int. J. Impot. Res. 2008, 20 (5), 479–486. https://doi.org/10.1038/ijir.2008.33.

(33) Novara, G.; Ficarra, V. Re: Francesco Montorsi, Gerald Brock, Jay Lee, et al. Effect of Nightly versus on-Demand Vardenafil on Recovery of Erectile Function in Men Following Bilateral Nerve-Sparing Radical Prostatectomy. Eur Urol 2008;54:924-31. Eur. Urol. 2009, 55 (5), e95-96; author reply e97-98. https://doi.org/10.1016/j.eururo.2008.11.055.

(34) Pavlovich, C. P.; Levinson, A. W.; Su, L.-M.; Mettee, L. Z.; Feng, Z.; Bivalacqua, T. J.; Trock, B. J. Nightly vs On-Demand Sildenafil for Penile Rehabilitation after Minimally Invasive Nerve-Sparing Radical Prostatectomy: Results of a Randomized Double-Blind Trial with Placebo. BJU Int. 2013, 112 (6), 844–851. https://doi.org/10.1111/bju.12253.

(35) Montorsi, F.; Brock, G.; Stolzenburg, J.-U.; Mulhall, J.; Moncada, I.; Patel, H. R. H.; Chevallier, D.; Krajka, K.; Henneges, C.; Dickson, R.; Büttner, H. Effects of Tadalafil Treatment on Erectile Function Recovery Following Bilateral Nerve-Sparing Radical Prostatectomy: A Randomised Placebo-Controlled Study (REACTT). Eur. Urol. 2014, 65 (3), 587–596. https://doi.org/10.1016/j.eururo.2013.09.051.

(36) Mulhall, J. P.; Burnett, A. L.; Wang, R.; McVary, K. T.; Moul, J. W.; Bowden, C. H.; DiDonato, K.; Shih, W.; Day, W. W. A Phase 3, Placebo Controlled Study of the Safety and Efficacy of Avanafil for the Treatment of Erectile Dysfunction after Nerve Sparing Radical Prostatectomy. J. Urol. 2013, 189 (6), 2229–2236. https://doi.org/10.1016/j.juro.2012.11.177.

(37) Nangle, M. R.; Keast, J. R. Reduced Efficacy of Nitrergic Neurotransmission Exacerbates Erectile Dysfunction after Penile Nerve Injury despite Axonal Regeneration. Exp. Neurol. 2007, 207 (1), 30–41. https://doi.org/10.1016/j.expneurol.2007.05.011.

(38) Franke, T. F.; Kaplan, D. R.; Cantley, L. C. PI3K: Downstream AKTion Blocks Apoptosis. Cell 1997, 88 (4), 435–437. https://doi.org/10.1016/s0092-8674(00)81883-8.

(39) Sun, Y.; Liu, W.-Z.; Liu, T.; Feng, X.; Yang, N.; Zhou, H.-F. Signaling Pathway of MAPK/ERK in Cell Proliferation, Differentiation, Migration, Senescence and Apoptosis. J. Recept. Signal Transduct. Res. 2015, 35 (6), 600–604. https://doi.org/10.3109/10799893.2015.1030412.

(40) Auer, M.; Schweigreiter, R.; Hausott, B.; Thongrong, S.; Höltje, M.; Just, I.; Bandtlow, C.; Klimaschewski, L. Rho-Independent Stimulation of Axon Outgrowth and Activation of the ERK and Akt Signaling Pathways by C3 Transferase in Sensory Neurons. Front. Cell. Neurosci. 2012, 6. https://doi.org/10.3389/fncel.2012.00043.

(41) Jiang, H.; Zhang, L.; Koubi, D.; Kuo, J.; Groc, L.; Rodriguez, A. I.; Hunter, T. J.; Tang, S.; Lazarovici, P.; Gautam, S. C.; Levine, R. A. Roles of Ras-Erk in Apoptosis of PC12 Cells Induced by Trophic Factor Withdrawal or Oxidative Stress. J. Mol. Neurosci. MN 2005, 25 (2), 133–140. https://doi.org/10.1385/JMN:25:2:133.

(42) Cheung, E. C. C.; Slack, R. S. Emerging Role for ERK as a Key Regulator of Neuronal Apoptosis. Sci. STKE Signal Transduct. Knowl. Environ. 2004, 2004 (251), PE45. https://doi.org/10.1126/stke.2512004pe45.

(43) Musicki, B.; Burnett, A. L. ENOS Function and Dysfunction in the Penis. Exp. Biol. Med. Maywood NJ 2006, 231 (2), 154–165. https://doi.org/10.1177/153537020623100205.

(44) Hurt, K. J.; Musicki, B.; Palese, M. A.; Crone, J. K.; Becker, R. E.; Moriarity, J. L.; Snyder, S. H.; Burnett, A. L. Akt-Dependent Phosphorylation of Endothelial Nitric-Oxide Synthase Mediates Penile Erection. Proc. Natl. Acad. Sci. U. S. A. 2002, 99 (6), 4061–4066. https://doi.org/10.1073/pnas.052712499.

(45) Musicki, B.; Liu, T.; Lagoda, G. A.; Strong, T. D.; Sezen, S. F.; Johnson, J. M.; Burnett, A. L. Hypercholesterolemia-Induced Erectile Dysfunction: Endothelial Nitric Oxide Synthase (ENOS) Uncoupling in the Mouse Penis by NAD(P)H Oxidase. J. Sex. Med. 2010, 7 (9), 3023–3032. https://doi.org/10.1111/j.1743-6109.2010.01880.x.

(46) Sánchez, A.; Contreras, C.; Martínez, M. P.; Climent, B.; Benedito, S.; García-Sacristán, A.; Hernández, M.; Prieto, D. Role of Neural NO Synthase (NNOS) Uncoupling in the Dysfunctional Nitrergic Vasorelaxation of Penile Arteries from Insulin-Resistant Obese Zucker Rats. PLOS ONE 2012, 7 (4), e36027. https://doi.org/10.1371/journal.pone.0036027.

(47) Karakus, S.; Musicki, B.; La Favor, J. D.; Burnett, A. L. CAMP-Dependent Post-Translational Modification of Neuronal Nitric Oxide Synthase Neuroprotects Penile Erection in Rats. BJU Int. 2017, 120 (6), 861–872. https://doi.org/10.1111/bju.13981.

(48) Kaiho, Y.; Nakagawa, H.; Saito, H.; Ito, A.; Ishidoya, S.; Saito, S.; Arai, Y. Nerves at the Ventral Prostatic Capsule Contribute to Erectile Function: Initial Electrophysiological Assessment in Humans. Eur. Urol. 2009, 55 (1), 148–155. https://doi.org/10.1016/j.eururo.2008.09.022.

(49) Reeves, F.; Battye, S.; Borin, J. F.; Corcoran, N. M.; Costello, A. J. High-Resolution Map of Somatic Periprostatic Nerves. Urology 2016, 97, 160–165. https://doi.org/10.1016/j.urology.2016.08.027.

(50) Costello, A. J.; Dowdle, B. W.; Namdarian, B.; Pedersen, J.; Murphy, D. G. Immunohistochemical Study of the Cavernous Nerves in the Periprostatic Region. BJU Int. 2011, 107 (8), 1210–1215. https://doi.org/10.1111/j.1464-410X.2010.09711.x.

(51) Quinlan, D. M.; Nelson, R. J.; Partin, A. W.; Mostwin, J. L.; Walsh, P. C. The Rat as a Model for the Study of Penile Erection. J. Urol. 1989, 141 (3), 656–661. https://doi.org/10.1016/s0022-5347(17)40926-8.

(52) Martínez-Piñeiro, L.; Brock, G.; Trigo-Rocha, F.; Hsu, G. L.; Lue, T. F.; Tanagho, E. A. Rat Model for the Study of Penile Erection: Pharmacologic and Electrical-Stimulation Parameters. Eur. Urol. 1994, 25 (1), 62–70.

(53) Mills, T. M.; Stopper, V. S.; Wiedmeier, V. T. Effects of Castration and Androgen Replacement on the Hemodynamics of Penile Erection in the Rat. Biol. Reprod. 1994, 51 (2), 234–238. https://doi.org/10.1095/biolreprod51.2.234.

(54) Mulhall, J. P.; Müller, A.; Donohue, J. F.; Mullerad, M.; Kobylarz, K.; Paduch, D. A.; Tal, R.; Li, P. S.; Cohen-Gould, L.; Scardino, P. T. The Functional and Structural Consequences of Cavernous Nerve Injury Are Ameliorated by Sildenafil Citrate. J. Sex. Med. 2008, 5 (5), 1126–1136. https://doi.org/10.1111/j.1743-6109.2008.00794.x.

(55) Iacono, F.; Giannella, R.; Somma, P.; Manno, G.; Fusco, F.; Mirone, V. Histological Alterations in Cavernous Tissue after Radical Prostatectomy. J. Urol. 2005, 173 (5), 1673–1676. https://doi.org/10.1097/01.ju.0000154356.76027.4f.

(56) Vignozzi, L.; Filippi, S.; Morelli, A.; Ambrosini, S.; Luconi, M.; Vannelli, G. B.; Donati, S.; Crescioli, C.; Zhang, X.-H.; Mirone, V.; Forti, G.; Maggi, M. Effect of Chronic Tadalafil Administration on Penile Hypoxia Induced by Cavernous Neurotomy in the Rat. J. Sex. Med. 2006, 3 (3), 419–431. https://doi.org/10.1111/j.1743-6109.2006.00208.x.

(57) Ferrini, M. G.; Kovanecz, I.; Sanchez, S.; Umeh, C.; Rajfer, J.; Gonzalez-Cadavid, N. F. FIBROSIS AND LOSS OF SMOOTH MUSCLE IN THE CORPORA CAVERNOSA PRECEDE CORPORAL VENO-OCCLUSIVE DYSFUNCTION (CVOD) INDUCED BY EXPERIMENTAL CAVERNOSAL NERVE DAMAGE IN THE RAT. J. Sex. Med. 2009, 6 (2), 415–428. https://doi.org/10.1111/j.1743-6109.2008.01105.x.

Chapter 5 : Conclusion

5.1 Achieved Results

In an attempt to investigate and develop new strategies to treat and alleviate ED using a multidisciplinary and multicausal approach, we have brought the first evidence of a chronic positive effect of Apelin-13, which represent an additional benefical effect than the acute and transcient effects shown previously[1]. Our findings indicate that long-term treatment with Apelin-13 produces significant beneficial effects against the deleterious hypercholesterolemia-induced corpus cavernosum fibrosis. Apelin-13 behaves as a potent suppressor of increased collagen deposition within the cavernosal tissues through the increase of MMPs expression and activity and potentially the reduction of fibroblast to myofibroblast differentiation. Altogether, it suggests an essential role of the Apelin system in modulating cavernosal structure, indicating the Apelin/APJ system as a promising target for the development of new treatment against fibrosis-associated ED. Further research may lead to the development of a treatment that would not only treat the symptoms of ED (i.e. inducing erection) as the currently available therapies but might prevent and possibly reverse the root cause of a frequent vascular ED that fibrosis represents.

The use of electro-stimulation to induce penile erection is a long known concept that appears very attractive in the treatment of neurogenic ED[2–5]. Indeed, it would basically allow to bypass any neurological injury that had happen on the CN course such as surgical-induced impairment or spinal cord injury. However, the difficulty to identify the CN location for electrode placement has blocked further development and clinical use of CN electromodulation. In the second work of this thesis, we investigated a new concept, a flat and flexible electrode array to guarantee the blind identification of the cavernosal nerve path within the pelvic plexus, without the need of extensive intraoperative burden. The proposed flexible electrode array was able to induce penile response in all patients, providing substantial evidence that our approach can guarantee the nerve activation, without requirement of precise identification of the nerve fiber. Importantly, this study open avenue for further development of a novel implantable medical device to alleviate ED, particularly in cases associated with mechanical neuronal damage, such as post-prostatectomy and spinal cord injury patients, providing a more comfortable and less problematic solution.

Finally, in addition of being able to activate the CN and induce on demand erection as shown in this thesis, recent studies also indicate that lower intensity stimulation, inferior to the nerve activation threshold, accelerate and enhance peripheral nerve regeneration[6–9]. Our findings in this third work of the thesis are showing for the first time the beneficial effects of LIES treatment following CN injury. One week of treatment not only enhanced the EF of animals following BCNI but showed a protective effect on penile and nerve tissues, preventing oxidative stress, inflammation, loss of smooth muscle cells and increased fibrosis of the erectile tissue, while preventing apoptosis of the nitrergic CN fibers and maintaining nerve fiber myelination, indicating LIES as a promising tool to reduce the burden of post-prostatectomy ED and propose a novel regiment for penile rehabilitation. Similarly to Apelin-13 treatment development, such strategy may also prevent and reverse ED, improving the quality of life of patients and reducing the burden and cost this disease is placeing on medical and public health systems.

5.2 Limitations and Future Development

In brief, this thesis work has provided new therapeutic opportunities for the management of ED, with the potential of overcoming the drawbacks of currently available treatments. However, although showing promising outcomes, it is important to point out that the results obtained here present several limitations. Studies in animal or in small patient cohort need to be confirmed in larger and well controlled clinical trials in order to guarantee the safety and efficacy of such novel treatments before they could be used for the clinical management of ED. In the case of Apelin-13, additional functional and toxicological study addressing erectile response *in vivo* would bring extended validation regarding the relevance of such beneficial effect and toxicology at long-term treatment, before being investigated on humans. The investigation of this system in other pathophysiological conditions of ED such as diabetes should also be considered. To confirm the efficacy of Apelin-13, comparator studies with alternative apelin isoforms and the use of an APJ inhibitor should be investigated.

In the case of electrostimulation of the CN to induce erection, a fully implantable medical device needs to be developed in order to investigate whether it is still feasible to induce penile erection after tissue encapsulation of the electrodes has happen, and whether

the injured nerves keep the capability to be stimulated in a chronic scenario. Furthermore, one important aspect to consider in order to bring the present concept into the clinical practice is the available biomedical technology. The device envisioned here presents standard features and similar stimulation parameters of existing implantable neuromodulators used today for treating chronic pain and Parkinson[10]. Therefore, no major innovation is required in terms of implantable pulse generator or implantable electrodes array for the clinical translation of this concept into a potential medical device. Nontheless, the road to develop an active implantable medical device is long and challenging. The development of such device is currently ongoing and could represent a great tool to confirm the feasibility of chronic CN neuromodulation in humans as well as to investigate whether the protectives effects of LIES are translatable into humans. And hopefully, as quoted by the physician Edward Teller, "*The science of today is the technology of tomorrow.*" will appear to be true in this case in a close future.

5.3 References

(1) Kwon, M.-H.; Tuvshintur, B.; Kim, W. J.; Jin, H.-R.; Yin, G. N.; Song, K.-M.; Choi, M. J.; Kwon, K.-D.; Batbold, D.; Ryu, J.-K.; Suh, J.-K. Expression of the Apelin–APJ Pathway and Effects on Erectile Function in a Mouse Model of Vasculogenic Erectile Dysfunction. J. Sex. Med. 2013, 10 (12), 2928–2941. https://doi.org/10.1111/jsm.12158.

(2) Lue, T. F.; Gleason, C. A.; Brock, G. B.; Carroll, P. R.; Tanagho, E. A. Intraoperative Electrostimulation of the Cavernous Nerve: Technique, Results and Limitations. J. Urol. 1995, 154 (4), 1426–1428.

(3) Burnett, A. L.; Lue, T. F. Neuromodulatory Therapy to Improve Erectile Function Recovery Outcomes After Pelvic Surgery. J. Urol. 2006, 176 (3), 882–887. https://doi.org/10.1016/j.juro.2006.04.020.

(4) Burnett, A. L.; Teloken, P. E.; Briganti, A.; Whitehurst, T.; Montorsi, F. Intraoperative Assessment of an Implantable Electrode Array for Cavernous Nerve Stimulation. J. Sex. Med. 2008, 5 (8), 1949–1954. https://doi.org/10.1111/j.1743-6109.2008.00865.x.

(5) Facio, F.; Burnett, A. L. Penile Rehabilitation and Neuromodulation https://www.hindawi.com/journals/tswj/2009/474587/abs/ (accessed Sep 21, 2017). https://doi.org/10.1100/tsw.2009.86.

(6) Huang, J.; Zhang, Y.; Lu, L.; Hu, X.; Luo, Z. Electrical Stimulation Accelerates Nerve Regeneration and Functional Recovery in Delayed Peripheral Nerve Injury in Rats. Eur. J. Neurosci. 2013, 38 (12), 3691–3701. https://doi.org/10.1111/ejn.12370.

(7) Willand, M. P.; Nguyen, M.-A.; Borschel, G. H.; Gordon, T. Electrical Stimulation to Promote Peripheral Nerve Regeneration. Neurorehabil. Neural Repair 2016, 30 (5), 490–496. https://doi.org/10.1177/1545968315604399.

(8) Chan, K. M.; Curran, M. W. T.; Gordon, T. The Use of Brief Post-surgical Low Frequency Electrical Stimulation to Enhance Nerve Regeneration in Clinical Practice. J. Physiol. 2016, 594 (13), 3553–3559. https://doi.org/10.1113/JP270892.

(9) Gordon, T. Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans. Neurotherapeutics 2016, 13 (2), 295–310. https://doi.org/10.1007/s13311-015-0415-1.

(10) Hunter Peckham, P.; Michael Ackermann, D. Chapter 18 - Implantable Neural Stimulators. In Neuromodulation; Academic Press: San Diego, 2009; pp 215–228. https://doi.org/10.1016/B978-0-12-374248-3.00019-7.

Curriculum Vitae: Mikaël Sturny

PhD assistant
E-mail: mikael.sturny@epfl.ch
Additional e-mail: mikael.sturny@gmail.com
OrcID number: 0000-0001-8360-6048
Bibliographic citation: Sturny, M.

Home address
Ave. Du Censuy 5
1020 Renens, VD
Switzerland
Phone: +41768225259

Work address
EPFL STI-IBI-LHTC
MED 3-2925 - Station 9
1015 Lausanne, Switzerland
Phone: +41216938120

Academic Education

2009-2013	Federal Institute of Technology of Lausanne (EPFL), Bachelor's Degree in Life Sciences & Technology
2013-2015	Federal Institute of Technology of Lausanne (EPFL), Master Degree in Life Sciences & Technology (Master Thesis), *Specialization in Molecular Medicine*
2015-Actually	Federal Institute of Technology of Lausanne (EPFL), PhD in Bioengineering and Biotechnology (EDBB), *PhD advisor: Prof. Nikolaos Stergiopulos*
2018 (March-September)	Johns Hopkins School of Medicine, Baltimore, USA. SNF-funded PhD mobility exchange *Under the supervision of Dr. A.L. Burnett*

Academic & Working Experience

2013-2014 (February-May)	Bachelor and Semester's projects in the Laboratory of Hemodynamics and Cardiovascular Technology (LHTC), Prof. Stergiopulos, EPFL. *Arginase activity measurement and its role in modulating NO and ROS production within the corpus cavernosum of hypercholesterolemic mice*
2014 (August-December)	Laboratory Immersion in the Laboratory of Hemodynamics and Cardiovascular Technology (LHTC), Prof. Stergiopulos, EPFL. *- Evaluation of key proteins in the RAS during ageing process.* *- Evaluation of different methods for circuit deposition on silicon substrate.* *- Arginase activity in the Corpus Cavernosum of hypercholoesterolemic mice.*
2015 (February-June)	Master Thesis in the Laboratory of Hemodynamics and Cardiovascular Technology (LHTC), Prof. Stergiopulos, EPFL. *Development of a Flexible Multi-Electrode Array for an Electro-Stimulator Device.*
2006-2015 (Week-ends)	Employment in a supermarket (COOP) in Romont, Switzerland.
2013-2015 (Nights/Week-ends)	Home helper for a quadraplegic person in Saint-Sulpice, Switzerland.
2017	Co-founder of Comphya SA.

Teaching activities

2015-2019	Teaching assistant for the classes of "fluid dynamics" and "system physiology II" at EPFL. 320 hours of teaching certified (Doctoral program's required amount: 300 hrs) *Hours include preparation, exercise solving, organization of simulation labs and seminars, answering students' questions, holding revision sessions*

Skills

Molecular biology (western-blot, immunostaining, PCR, animal care, histology), Acute clinical trial management, Engineering (prototyping, mold for silicone injection, micro machining, biomedical safety analysis), Computer science (Programming C++, Matlab, Java, Microsoft Office, Photoshop, CAD), Languages (French, English, German).

Publications

[1] **An Increased Arginase Activity Is Associated with Corpus Cavernosum Impairment Induced by Hypercholesterolemia,** *J Sex Med.* ***2014*** *May;11(5):1173-81.* Fraga-Silva R., Costa-Fraga F., Faye Y., **Sturny M.**, Santos R., da Silva R., Stergiopulos N.

[2] **Diminazene Protects Corpus Cavernosum Against Hypercholesterolemia-Induced Injury,** *J Sex Med.* ***2015*** *Feb;12(2):289-302.* Fraga-Silva R., Costa-Fraga F., Montecucco F., **Sturny M.**, Faye Y., Mach F., Pelli G., Shenoy V., da Silva R., Raizada M., Santos R., Stergiopulos N.

[3] **Novel Concept Enabling an Old Idea: A Flexible Electrode Array to Treat Neurogenic Erectile Dysfunction.** *J Sex Med.* ***2018****;15(11):1558-1569. doi:10.1016/j.jsxm.2018.09.003.* Skoufias S*, **Sturny M***, Fraga-Silva R, et al.

[4] **Performance and safety of treatment options for erectile dysfunction in patients with spinal cord injury: a review of the literature** [published online ahead of print, **2020** Aug 2]. Andrology. 2020;10.1111/andr.12878. doi:10.1111/andr.12878. Afferi L, Pannek J, Burnett AL, Razaname C, Tzanoulinou S, Bobela W, Fraga-Silva R, **Sturny M**, Stergiopulos N, Cornelius J, Moschini M, Iselin C, Salonia A, Mattei A, Mordasini L.

[5] **Characterization of the renin-angiotensin system in aged cavernosal tissue and its role in penile fibrosis.** [Accepted for publication, **2020** Aug 3], *J Sex Med.* Bragina M, Costa-Fraga F, **Sturny M**, Ebadi B, Ruoccolo R, Santos R, Fraga-Silva R, Stergiopulos N.

[6] **Apelin-13 Protects Corpus Cavernosum Against Fibrosis Induced by High-Fat Diet in an MMP-Dependent Mechanism.** [Submitted in J Sex Med, **2020** May 8]. **Sturny M**, Anguenot L, Costa-Fraga F, Bragina M, Martins Lima A, da Silva R, Fraga-Silva R, Stergiopulos N.

[7] **Low-intensity electrostimulation enhances neuroregeneration and improves erectile function in a rat model of cavernous nerve injury.** [Article in preparation for submission, **2020** Aug]. **Sturny M***, Karakus S*, Fraga-Silva R, Stergiopulos N, Burnett A.L.

Conferences

[1] **Apelin-13 Protects Corpus Cavernosum Against Hypercholesterolemia-Induced Fibrosis**, *20th World Meeting on Sexual Medicine, Beijing, China, September 22-25* ***2016****, Podium presentation.*

[2] **Implantable Neurostimulator for the Treatment of Erectile Dysfunction.** *Swiss Medtech Day, June 13* ***2017****, Bern, Switzerland. Poster presentation.*

[3] **Protective Effect of Apelin-13 Against Hypercholesterolemia-Induced Fibrosis of the Corpus Cavernosum.** *World Meeting on Sexual Medicine, Lisbon, Portugal, February 28 - March 3* ***2018****, Poster presentation.*

[4] **Novel Concept to Enable an Old Idea: A Flexible Electrode Array to Recover Neurogenic Erectile Dysfunction.** *World Meeting on Sexual Medicine, Lisbon, Portugal, February 28 - March 3* ***2018****, Podium presentation.*

[5] **Low-intensity electrostimulation of injured cavernosal nerve improves erectile function recovery in a rat model of post-prostatectomy erectile dysfunction.** *Annual Congress of the European Society for Sexual Medicine (ESSM) 2019, Ljubljana, Slovenia, February 17-19* ***2019****. Podium presentation.*

[6] **Low-intensity electrostimulation of injured cavernous nerves ameliorates erectile dysfunction and promotes penile rehabilitation in rats.** *Annual Congress of the European Society for Sexual Medicine (ESSM) 2020, Prague, Czech Republic, January 23-25* ***2020****. Podium presentation.*

.