Comorbidity of inflammatory diseases in the lower urinary tract

– the link between chronic prostatitis and bladder dysfunction

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To my grandfather

Abstract

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a common disease in men that currently lacks satisfactory pharmacological treatment alternatives. The aim of this thesis was to investigate the effects of CP/CPPS on lower urinary tract (LUT) function. Further, we aimed to investigate the effects of treatment with a soluble guanylate cyclase (sGC) activator, BAY 60-2770, on possible alterations in bladder function caused by CP/CPPS.

The effects of CP/CPPS on LUT function were evaluated both *in vivo* (Paper I-III) and *in vitro* (Paper IV). To create a functional animal model for CP/CPPS, rats were intraprostatically injected with either zymosan (Paper I, III, IV) or lipopolysaccharide (LPS, paper II). The effects of BAY 60-2770 on bladder function after induction of chronic prostatitis were examined *in vivo* (Paper III) and *in vitro* (Paper IV). Micturition parameters were investigated in a metabolic cage and alterations in bladder function were assessed with cystometry during *in vivo* rat studies (Paper I, II, and III). The prostate (Paper I-III) and bladder (Paper I-IV) were examined histopathologically. To investigate how the innate bladder contractility and receptor expression were affected by induction of chronic prostatitis, an *in vitro* organ bath set-up was utilized (Paper IV).

The findings in this thesis showed that induction of CP/CPPS led to bladder dysfunction, mainly overactivity. Bladder overactivity was observed regardless of if the prostate inflammation was chemically induced (with zymosan) or induced by LPS (mimicking an infectious focus in the prostate). The data show that the functional changes in the bladder were partly caused by altered afferent signalling. Our findings thus indicated that induction of chronic prostate inflammation could lead to bladder dysfunction via cross-organ sensitization. Cystometry and organ bath experiments showed that induced prostatitis also led to local alterations in the bladder as well as on efferent signalling. Further, our findings showed that treatment with a sGC activator had a dramatic ameliorative effect on functional bladder alterations caused by CP/CPPS.

In conclusion, the findings in this thesis support the hypothesis that cross-organ sensitization between the prostate and bladder can be triggered by

chronic inflammation. This complex physiological process may be the reason for the unsuccessful treatment of chronic prostatitis. Targeting the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway, *i.e.,* with sGC activators, could be a promising pharmacological treatment option to alleviate the symptoms of men with CP/CPPS.

Keywords
Chronic pelvic pain syndrome, chronic prostatitis, LUTS, guanylate cyclase activator, BAY 60-2770, NO/cGMP

Sammanfattning på svenska

Kronisk prostatit/kroniskt bäckensmärtsyndrom är en vanlig sjukdom hos män som idag saknar tillfredsställande farmakologiska behandlingsalternativ. Syftet med denna avhandling var att undersöka effekterna av kroniskt bäckensmärtsyndrom på de nedre urinvägarnas funktion. Vidare ämnade vi undersöka effekterna av behandling med en guanylatcyklasaktiverare, BAY 60-2770, på möjliga förändringar i blåsfunktionen orsakade av kroniskt bäckensmärtsyndrom.

Effekterna av kroniskt bäckensmärtsyndrom på de nedre urinvägarnas funktion utvärderades både *in vivo* (delarbete I-III) och *in vitro* (delarbete IV). För att skapa en funktionell djurmodell för kroniskt bäckensmärtsyndrom injicerades råttor i prostatan med antingen zymosan (I, III, IV) eller lipopolysackarid (LPS; II). Effekterna av BAY 60-2770 på blåsfunktionen efter induktion av kronisk prostatit undersöktes *in vivo* (III) och *in vitro* (IV). Miktionsparametrar undersöktes i en metabolismbur och förändringar i blåsfunktionen utvärderades med *in vivo* cystometri (I-III). Prostata (I-III) och urinblåsa (I-IV) undersöktes histopatologiskt. För att undersöka hur blåsans kontraktilitet och receptoruttryck påverkades av induktion av kronisk prostatit användes en *in vitro* organbaduppställning (IV).

Fynden i denna avhandling visade att induktion av kroniskt bäckensmärtsyndrom ledde till blåsdysfunktion, främst överaktivitet. Blåsöveraktivitet observerades oavsett om prostatainflammationen var kemiskt inducerad (med zymosan) eller inducerad av LPS (som efterliknar infektiöst inducerad prostatit). Data visar att de funktionella förändringarna i urinblåsan delvis orsakades av förändrad afferent signalering. Våra resultat indikerade således att induktion av kronisk prostatainflammation kan leda till urinblåsdysfunktion via korsorgansensitisering. Cystometri- och organbadsexperiment visade att inducerad prostatit också ledde till lokala förändringar i urinblåsan samt på efferent signalering. Vidare visade våra resultat att behandling med en sGC-aktiverare hade en dramatisk förbättrande effekt på funktionella blåsförändringar orsakade av kroniskt bäckensmärtsyndrom.

Sammanfattningsvis stöder resultaten i denna avhandling hypotesen att korsorgansensitisering mellan prostata och urinblåsa kan uppkomma som ett

resultat av kronisk inflammation. Denna komplexa fysiologiska process kan vara orsaken till den hittills misslyckade behandlingen av kronisk prostatit. Att rikta in sig på kvävemonoxid/cycliskt guanylatmonofosfat (NO/cGMP), till exempel med guanylatcyklasaktiverare, kan vara ett lovande farmakologiskt behandlingsalternativ för att lindra symtomen hos män med kroniskt bäckensmärtsyndrom.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Aydogdu, O., Gocun, P.U., Aronsson, P., Carlsson, T., Winder, M.

Prostate-to-bladder cross-sensitization in a model of zymosan-induced chronic pelvic pain syndrome in rats.

Prostate 2021 Mar;81(4):252-260. doi: 10.1002/pros.24101.

II. Aydogdu, O., Gocun, P.U., Aronsson, P., Carlsson, T., Winder, M.

Cross-organ sensitization between the prostate and bladder in an experimental rat model of lipopolysaccharide (LPS)-induced chronic pelvic pain syndrome.

BMC Urology 2021 Aug 21;21(1):113. doi: 10.1186/s12894-021-00882-9.

III. Aydogdu, O., Perez, F., Aronsson, P., Gocun, P.U., Carlsson, T., Sandner, P., Patel, B., Winder, M.

Treatment with the soluble guanylate cyclase activator BAY 60-2770 normalizes bladder function in an in vivo rat model of chronic prostatitis.

European Journal of Pharmacology 2022 Jul 15;927:175052. doi: 10.1016/j.ejphar.2022.175052.

IV. Aydogdu, O., Perez, F., Aronsson, P., Carlsson, T., Sandner, P., Patel, B., Winder, M.

Effects of the soluble guanylate cyclase activator BAY 60-2770 on in vitro bladder contractile responses and receptor expression in a rat model of chronic prostatitis.

Submitted manuscript

Content

Abbreviations

5-ARI	5-alpha reductase inhibitors
ACh	Acetylcholine
ATP	Adenosine-5′-triphosphate
BPH	Benign prostate hyperplasia
COX-2	Cyclooxygenase-2
CP/CPPS	Chronic prostatitis/chronic pelvic pain syndrome
CRP	C-reactive protein
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DRE	Digital rectal examination
DRG	Dorsal root ganglia
EFS	Electrical field stimulation
ESWL	Extracorporeal shock wave lithotripsy
GTP	Guanosine triphosphate
GUPI	Genitourinary pain index
HE	Haematoxylin-eosin
IC/BPS	Interstitial cystitis/bladder pain syndrome
IL-1α	Interleukin-1α
IL-6	Interleukin-6
IL-8	Interleukin-8
IPSS	International prostate symptom score
LP	Lamina propria
LPS	Lipopolysaccharide
LUT	Lower urinary tract
LUTS	Lower urinary tract symptoms
MeCh	Methacholine
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
NA	Noradrenaline
NGAL	Neutrophil gelatinase-associated lipocalin
NGS	Normal goat serum
NIH	National Institutes of Health
NIH-CPSI	National Institutes of Health Chronic Prostatitis Symptom Index
NO/cGMP	Nitric oxide/cyclic guanosine monophosphate

NSAIDs	Non-steroidal anti-inflammatory drugs
NVCs	Non-voiding contractions
OAB-V8	Overactive Bladder-Validated 8 questionnaire
PAP	Prostatic acid phosphatase
PBS	Phosphate buffered saline
PDEI	Phosphodiesterase inhibitors
PDE5	Phosphodiesterase type 5
PE	Phenylephrine
PFA	Paraformaldehyde
PSA	Prostate specific antigen
QOL	Quality of life
s.c.	Subcutaneous
sGC	Soluble guanylate cyclase
TLR2	Toll-like receptor 2
TURP	Transurethral resection of the prostate
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1

1. Introduction

1.1 General anatomy and physiology of the lower urinary tract

1.1.1. Urinary bladder

The urinary bladder can be divided into three main parts including the corpus, fundus and trigone (Andersson, 2020). The bladder wall is composed of the serosa, muscularis propria and the mucosa layers (Birder & Andersson, 2013). The innermost layer of the bladder wall (mucosa) can further be divided into lamina propria (LP), basal membrane and uroepithelium (urothelium). There is also an additional layer in the lamina propria (LP) that contains smooth muscle, this layer is known as the muscularis mucosae (Andersson, 2020).

The urothelium is found in the innermost layer of the bladder, the superior part of the urethra and glandular structures in the prostate (Andersson, 2020; Winder et al., 2014). Beginning with the proximal part of the urethra, the urothelium changes from a transitional epithelial tissue to a stratified epithelium. The urothelium serves as a barrier separating the underlying tissues in the bladder wall and the space in the bladder (Birder & Andersson, 2013) and consists of three main layers - the basal, intermediate, and apical layers. The apical layer contains umbrella cells (Apodaca, 2004; Khandelwal et al., 2009). Umbrella cells are bonded with tight junctions consisting of occludin and claudin proteins that have an important role in the barrier function of the urothelium (Andersson, 2020). Further, the glycosaminoglycan layer covering the umbrella cells has a critical role in the barrier function of the urothelium (Klingler, 2016).

The lamina propria (LP) consists of vascular tissues, elastic fibers, muscularis mucosae, fibroblasts, interstitial cells, and sensory nerve endings (Andersson & McCloskey, 2014). The function of the lamina propria (LP) has not been exactly understood yet but it seems to be important for bladder compliance (Andersson et al., 2017).

The muscle fibers that make up the detrusor have a random orientation in the bladder (Figure 1), (Elbadawi, 1996; Shah et al., 2014). However, in the close vicinity

of the bladder neck these fibers are oriented as three separate muscular layers, forming the proximal urethral sphincter (Andersson, 2020). The superior, middle, and inferior vesical arteries arising from the hypogastric branch of the internal iliac artery are the main arteries supplying the bladder. The venous plexus draining the bladder is Santorini´s plexus, which is located between the prostate and the bladder. Micturition can be started and stopped voluntarily and the coordination between the somatic and autonomic efferent mechanisms coordinating the bladder function and urethral smooth muscle activity is critical (Andersson & Arner, 2004; Andersson & Wein, 2004; Andersson, 2020; De Groat et al., 2015).

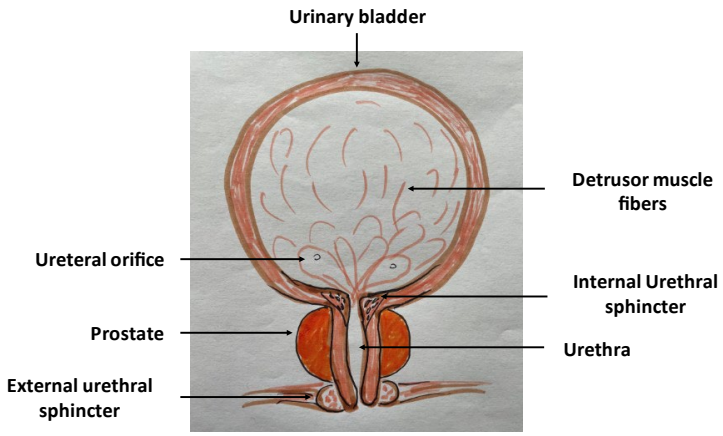

Figure 1. Schematic drawing of the lower urinary tract (LUT) anatomy.

The lower urinary tract (LUT) consists of the urinary bladder, internal urethral sphincter, urethra, prostate, external urethral sphincter, and pelvic floor muscles. Micturition and LUT function are controlled by a complex neural system. The urinary bladder has two main parts including a corpus and base. The corpus of the bladder lies above the ureteral orifices, and the base consists of the trigone and the bladder neck. Muscle fibers that constitute the detrusor branch randomly in the bladder. These fibers form three different muscular layers close to the bladder neck, forming the internal urethral sphincter. The urethra consists of striated and smooth muscle fibers. The outer layer of striated muscle in the urethra extends throughout the prostate and the external urethral sphincter originates from these muscle fibers.

1.1.2. Urethra

The male urethra consists of four main parts, from distal to proximal: penile, membranous, prostatic, and pre-prostatic urethra. The stratified columnar epithelial cells change to stratified squamous cells close to the distal part of the urethra. The

internal urethral sphincter is a continuation of the detrusor muscle fibers and encloses the proximal urethra (Andersson, 2020). Smooth muscle fibers at the level of the internal urethral sphincter are lined up in a horseshoe shaped manner. However, at the superior part of the urethra, the smooth muscle fibers are arranged circularly (Andersson, 2020; Wallner et al., 2009). The striated muscle fibers originating from levator ani have a circular arrangement and encircle the smooth muscle in the membranous urethra (Jung et al., 2012). The proximal part of the urethra is supplied by the inferior vesical artery while the distal part is supplied by the bulbourethral artery, a branch of the internal pudendal artery (Amend, 2020). The external urethral sphincter is a skeletal muscle in the membranous urethra and provides voluntary urethral closure, which is controlled by the pudendal nerve (Jung et al., 2012; Yucel & Baskin, 2004).

Adequate relaxation and contraction of the smooth muscle in the urethra is critical for a successful micturition cycle. During the voiding phase of the micturition cycle, the relaxation of the bladder outlet and the urethra is followed by the detrusor contraction (Andersson, 2020). Therefore, a typical finding during the voiding phase is an initial decrease in the urethral pressure followed by a robust increase in intravesical pressure (Anderson, 1993). Smooth muscle tone and LP are important factors in terms of sufficient urethral contraction and relaxation (Andersson, 2001; Canda et al., 2008).

Noradrenaline (NA) is the main neurotransmitter regulating urethral contraction (Andersson, 2001, 2020). Several mechanisms have been suggested to explain urethral relaxation upon activation of adrenergic receptors. These mechanisms include co-stimulation of muscarinic receptors on adrenergic nerves, release of acetylcholine (ACh), and nitric oxide (NO) dependent pathways (Anderson, 1993; Andersson, 2020; Canda et al., 2008).

1.1.3. Prostate

The human prostate can be divided into four main parts - the peripheral zone, central zone, transitional zone, and fibromuscular stroma (Aaron et al., 2016; McNeal, 1984). The fibromuscular stroma is a non-glandular structure surrounding the prostate. The peripheral, central, and transitional zones contain glandular structures and ductal systems (Figure 2).

The central zone encircles the ejaculatory ducts, and the peripheral zone encloses the central zone and then extends inferiorly and surrounds the distal urethra. The transitional zone is a small glandular area making up approximately 5 % of the organ and surrounds the prostatic urethra.

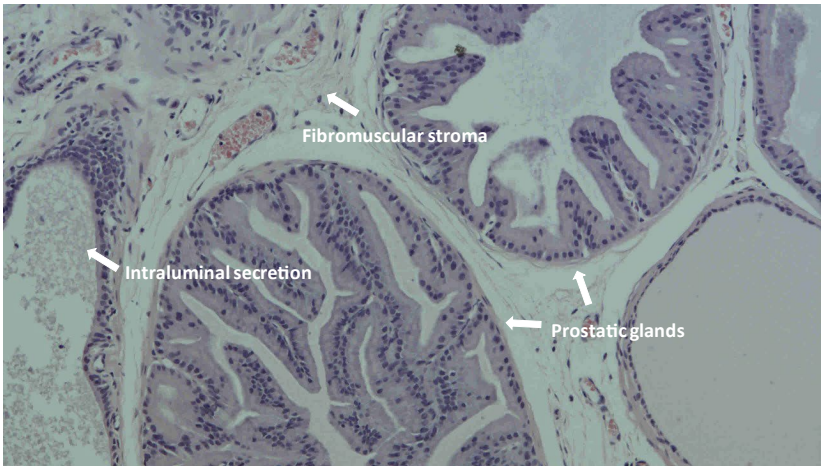

Figure 2. Histopathological view of the prostate.
The fibromuscular stroma is a non-glandular structure surrounding the prostate. The peripheral, central, and transitional zones of the prostate contain glandular structures and ductal systems containing intraluminal secretion.

The acini in the peripheral zone empty into the ducts, which are surrounded by a loose stroma. Both ductal structures and acini are covered with a simple columnar epithelium. Of interest in the current thesis, the peripheral zone is the main region involved in chronic prostatitis (Aaron et al., 2016). Contrarily, the likelihood of observing prostatitis in the central zone is very low. The ducts in the central zone run close to the ejaculatory ducts. Also, the muscular stroma around the ducts is stronger than in the peripheral zone.
The rat prostate has some anatomical differences compared to human prostate. The rat prostate does not have as compact anatomical structure as in humans. Also, the rat prostate is divided into four lobular regions including the anterior, dorsal, ventral, and lateral lobes (Aaron et al., 2016; Hayward et al., 1996; Marker et al., 2003). Approximately 30% of the human ejaculate volume comes from the prostate (Oelke., 2020). The main function of the prostate secretion in the ejaculate is to protect the spermatozoa. In addition, the contents of the prostate secretion such as fructose, glucose, proteins, and minerals are critical in terms of nourishing spermatozoa (Frick & Aulitzky, 1991). About 1% of prostate secretion is composed of proteins, including prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and various proteolytic enzymes (Oelke., 2020). Although seminal fluid and prostate secretion composition can be different in different species, a functional prostate is mandatory for fertility in both rats and humans (Hayward &

Cunha, 2000). The prostate is dependent on androgens, mainly testosterone, and the conversion of testosterone to its active form dihydrotestosterone (DHT) by 5α-reductase enzyme is necessary for normal prostate function.

1.2 Innervation of the lower urinary tract

1.2.1. Central mechanisms

The coordination between the bladder, urethra, and pelvic floor, which is controlled by a complex communication between the central and peripheral nervous system, is critical for a satisfactory micturition cycle (De Groat, 2015; Fry, 2005; Morrison, 2005). The micturition cycle is controlled by a spino-bulbospinal pathway and central mechanisms work like on-off circuits (Andersson, 2020; Fowler et al., 2008; Griffiths & Fowler, 2013). Previous studies have showed that three supraspinal areas in the central nervous system are involved (Andersson, 2020; Arya & Weissbart, 2017; Fowler et al., 2008; Griffiths & Fowler, 2013). The pontine micturition centre is responsible for the excitation of bladder motor neurons and inhibition of urethral motor neurons. Meanwhile, the afferent signalling from the bladder which occurs continuously during bladder filling is processed by the periaqueductal grey. Upon reaching the innate activation threshold, micturition is initiated in the pre-optic area in the hypothalamus.

1.2.2. Efferent innervation

Parasympathetic neurons located at the S2-S4 spinal cord level are responsible for detrusor contraction and bladder outlet relaxation (Figure 3) (Andersson, 2020; De Groat, 1993). Parasympathetic impulses are carried by the pelvic nerve to postganglionic nerves in the pelvic plexus, vesical ganglia, and intramural ganglia (Andersson, 2020). Acetylcholine is the main neurotransmitter mediating preganglionic neurotransmission and acts on post-ganglionic nicotinic ACh receptors. The postganglionic neurotransmitter responsible for excitatory parasympathetic effects in the bladder is also ACh, acting on muscarinic receptors (Anderson, 1993; De Groat, 2015). Although both M2 and M3 muscarinic receptors are found in the detrusor, M3 is the main receptor subtype responsible for the excitatory effects

(Andersson & Arner, 2004; Chess-Williams, 2002; Chess-Williams et al., 2001; Giglio & Tobin, 2009).
Parasympathetic stimulation can also cause a non-cholinergic detrusor contraction mediated by adenosine-5´-triphosphate (ATP) that acts on P2X receptors (De Groat, 2015). The most important subtype of P2X receptors in the human and rat detrusor is $P2X_1$ (Andersson & Arner, 2004; Burnstock, 2001; De Groat, 2015; Ralevic & Burnstock, 1998). Purinergic bladder excitation plays an important role in healthy rat detrusor contraction but seems to be of little importance in the healthy human bladder. However, in a state of chronic bladder inflammation, purinergic contraction has been shown to be of importance also in humans (Andersson, 2020; Burnstock, 2001; Palea et al., 1993; Zhong et al., 2003). Even though ATP can have direct effects on the detrusor, its contractile effects are partly mediated via induced release of ACh, mainly from the urothelium (Chess-Williams, 2002; Stenqvist et al., 2020). Thus, urothelial purinergic activation is also of importance for bladder contraction, at least in rodents (Vesela et al., 2011).
The parasympathetic input carried by the pelvic nerve to the bladder outlet, prostate and urethra is not mainly mediated by ACh but instead by NO, leading to relaxation (Andersson & Persson, 1995; De Groat et al., 2015; Dey et al., 2012). However, the neurotransmitters responsible for urethral contraction are ACh and ATP (De Groat, 2015; Zoubek et al., 1993). The role of NO in the lower urinary tract will be discussed in detail in a later paragraph.
Sympathetic nerves innervating the lower urinary tract (LUT) mainly originate from the T10-L2 spinal cord level (Figure 3), (Andersson, 2020). The preganglionic axons are carried by the hypogastric and pelvic nerves (De Groat, 2015). Postganglionic sympathetic nerves release NA causing contraction of the bladder base and urethra as well as relaxation of the detrusor in the bladder corpus (De Groat, 2015; DeLancey, 2002). The bladder base and proximal urethra are rich in terms of α-adrenergic receptors, while β-adrenergic receptors are mainly concentrated in the bladder corpus (Andersson & Arner, 2004). The stimulation of β_3-adrenergic receptors causes bladder relaxation whereas α_1-adrenergic receptor stimulation causes contraction (Andersson & Arner, 2004; De Groat, 2015). The most prominent α-adrenergic receptor subtype in the bladder and prostate is α_{1A} (De Groat et al., 2015; Moro et al., 2013; Oelke., 2020). Functional adrenergic receptors are also expressed in the urothelium and lamina propria (Moro et al., 2013).
The somatic innervation of the external urethral sphincter originates from Onuf´s nucleus located at the S1-S3 spinal cord level and is transmitted via the pudendal nerve (Figure 3), (Beckel & Holstege, 2011; Fowler et al., 2008). The main neurotransmitter is ACh which acts on postganglionic nicotinic receptors. The external

urethral sphincter is also innervated by adrenergic nerves, making it a unique structure since it has both somatic and autonomic innervation (Andersson, 2020).

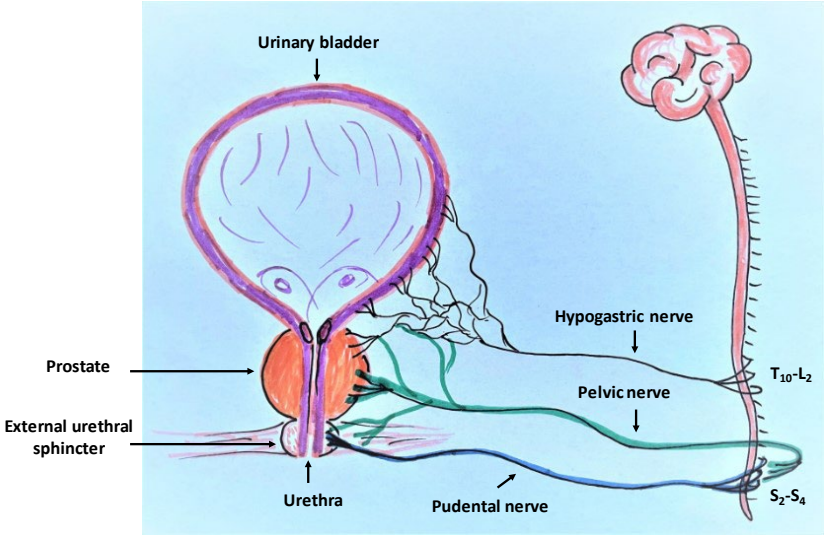

Figure 3. Schematic drawing of the lower urinary tract (LUT) innervation.

Sympathetic nerves innervating the lower urinary tract (LUT) mainly originate from the T10-L2 spinal cord level. The preganglionic axons are carried by the hypogastric and pelvic nerves. Post-ganglionic sympathetic nerves release NA, causing contraction of the bladder base and urethra as well as relaxation of the detrusor in the bladder corpus. Parasympathetic neurons located at the S2-S4 spinal cord level are responsible for detrusor contraction and bladder outlet relaxation.

1.2.3. Afferent innervation

The afferent innervation from the LUT to the lumbosacral spinal cord is transmitted by the pelvic, hypogastric, and pudendal nerves (Andersson, 2020; De Groat, 2015; Kanai & Andersson, 2010). There are two different types of afferent nerves innervating the urethra and bladder; myelinated Aδ and unmyelinated C-fibers (De Groat, 2015). Unmyelinated C-fibers are found in the detrusor and LP and are responsible for nociceptive sensation (Andersson, 2020). Myelinated Aδ fibers are mainly located in the detrusor and sensitive to tension (Rong et al., 2002). Previous

studies showed that the micturition cycle is initiated by myelinated Aδ fibres in the healthy bladder (De Groat, 2015; Habler et al., 1990). In rats, it has been shown that unmyelinated C-fibers are less responsive to bladder contractions than myelinated Aδ fibres (De Groat, 2015; Dmitrieva & McMahon, 1996; Morrison, 1997; Sengupta & Gebhart, 1994). High intraurethral pressure can also activate afferent innervation, transmitted by the pelvic nerve (De Groat, 2015; Feber et al., 1998). It was shown that afferent pathways in the pelvic and hypogastric nerves respond to intraurethral pressure changes more rapidly than afferent pathways in the pudendal nerve (De Groat, 2015).

1.3 Pelvic organ cross sensitization

Pelvic organ cross sensitization is defined as a diseased organ in the pelvic region having negative effects on the function of a another, previously healthy, organ in the pelvic region (Malykhina, 2007; Zhang et al., 2019). Previous studies have shown that pelvic pain in men with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) could be due to pelvic organ cross sensitization (Chen et al., 2005; Zhang et al., 2019). Detailed studies on urinary frequency, urgency, and pain radiating to the abdomen and back also suggest that cross sensitization between pelvic organs can be the reason for the broad symptom profile in men with CP/CPPS (Schwartz et al., 2016).

Some central mechanisms, including the convergence of neural inputs onto second order neurons in the spinal cord, have been suggested to relay pelvic organ cross sensitization (Brumovsky & Gebhart, 2010; Pezzone et al., 2005). More specifically, the branching axons originating from the dorsal root ganglia (DRG) which innervate pelvic organs were proposed as the peripheral mechanism for pelvic organ cross sensitization (Chen et al., 2005; Schwartz et al., 2016; Zhang et al., 2019). In a previous animal study, Schwartz et al. aimed to investigate the extent of alterations in immunomodulatory mediators in the prostate and bladder after chronic prostatitis (Schwartz et al., 2016). The authors also aimed to examine possible changes in bladder function and afferent sensitization. They showed an afferent contribution to CP/CPPS and a potential cross sensitization between the prostate and bladder. The authors also speculated that the changes in the bladder could be due to dorsal root reflexes and potential neurogenic inflammation in the bladder caused by the induction of chronic prostatitis. Similarly, Song et al. examined the potential role of DRG neurons that innervate the bladder and prostate (Song et al., 2009). The authors proposed a neural reflex between the prostate and bladder as an explanation for the voiding dysfunction often caused by CP/CPPS. In another

study, Chen et al. examined the distribution of dichotomizing afferents innervating both the bladder and prostate (Chen et al., 2010). The authors speculated that the origin of pelvic pain in men with CP/CPPS could be the bladder. They concluded that convergent DRG neurons might have an important role in cross organ sensitization between the prostate and bladder. Lastly, in a recent study, Funahashi et al. examined the potential effects of prostate inflammation on bladder function in a rat model (Funahashi et al., 2019). The authors concluded that cross organ sensitization between the prostate and bladder via pelvic afferent pathways containing dichotomized afferents might have a critical role in the possible changes in bladder function that arise after prostate inflammation.

1.4 Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS)

1.4.1. Definition and aetiology

According to epidemiological studies, the incidence of prostatitis is between 4.5% and 9% (Clemens et al., 2007; Khattak et al., 2021; Polackwich & Shoskes, 2016). According to the National Institutes of Health (NIH) classification system, prostatitis can be divided into four categories: acute bacterial prostatitis, chronic bacterial prostatitis, CP/CPPS, and asymptomatic inflammatory prostatitis (Doiron, 2020; Polackwich & Shoskes, 2016). Almost 95% of men with prostatitis have category 3 prostatitis or CP/CPPS (Doiron, 2020). The main symptoms are chronic pelvic pain and lower urinary tract symptoms (LUTS) with no identifiable bacterial infection (Polackwich & Shoskes, 2016).

Chronic prostatitis/chronic pelvic pain syndrome is a multifactorial disease, and the pathogenesis is not clear. Although it is widely accepted that CP/CPPS does not have any infectious causative agent, in individual cases it is impossible to rule out the possibility that an initial bacterial infection might have started the inflammatory process in the prostate. Recently, some have speculated that the reason for not identifying any bacterial infection in men with CP/CPPS is simply due to limited microbiological techniques (Doiron, 2020). Thus, proposing that there may be some bacteria species that cannot be detected with current methods since they grow in a biofilm in the prostate, urethra and/or bladder (Doiron, 2020; Jamal et al., 2018; Wolcott, 2017; Wolcott & Ehrlich, 2008). In a previous study, Shoskes et al. compared urinary microbiomes of CP/CPPS patients with healthy controls (Shoskes, Altemus, et al., 2016). The authors showed a unique microbiome and significantly higher number of Clostridia species in the urinary tract of men with

CP/CPPS compared to controls. In another study, a lower count of *Prevotella* species was shown in the intestinal microbiome of patients with CP/CPPS (Shoskes, Wang, et al., 2016). These finding show that a potential deterioration of the bacterial microbiome in the urinary tract may have an important role in CP/CPPS.
It has previously been shown that anatomical defects in the prostate, bladder or urethra might also have an important role in CP/CPPS pathogenesis (Blacklock, 1991; Doiron, 2020). Immunological changes can also have a role in the pathogenesis of CP/CPPS, since men with CP/CPPS have an increased immune sensitivity (Breser et al., 2017; Doiron, 2020; Kouiavskaia et al., 2009). Similarly, patients with CP/CPPS have different autonomic nervous activity, indicating that a central pathway and neural dysfunction may be important in CP/CPPS (Yilmaz et al., 2010; Yilmaz et al., 2007). As described previously, pelvic organ cross sensitization is another potential mechanism for the broad symptomatology of CP/CPPS (Schwartz et al., 2016). Psychosocial factors are also important in terms of perception of physical symptoms caused by CP/CPPS and different patients with different psychosocial backgrounds can experience the disease completely differently (Doiron, 2020).

1.4.2. Clinical evaluation

A thorough anamnesis ruling out other possible diseases that can lead to similar symptoms is the first step for the management of patients with CP/CPPS (Doiron, 2020; Khattak et al., 2021). National Institutes of Health Chronic prostatitis Symptom Index (NIH-CPSI) is a validated and simple tool that can be used in the diagnosis and clinical evaluation of men with CP/CPPS (Propert et al., 2006; Wagenlehner et al., 2013). A careful pelvic and digital rectal examination (DRE) is also mandatory in the clinical evaluation of men with suspected CP/CPPS (Doiron, 2020; Polackwich & Shoskes, 2016). Sometimes it is better to perform the pelvic examination prior to DRE since the patient may have a tender prostate gland. It is especially important to examine trigger points, possible spasticity in the pelvic region and muscle tenderness during pelvic examination of men with CP/CPPS (Doiron, 2020). A urine test is also generally used as a part of the clinical evaluation, to identify a possible bacterial infection or presence of white blood cells in the urine (Doiron, 2020; Polackwich & Shoskes, 2016). Although its clinical usefulness has been questioned by some clinicians because of practical difficulties, the so called four-glass test is still the golden standard urine test to examine bacterial localisation and cytological investigation (McNaughton Collins et al., 2000; Meares & Stamey, 1968). In a previous study, Nickel et al. compared the two-glass test to the four-glass test and this study showed no significant difference

between the two tests (Nickel et al., 2006). Currently, imaging methods have no important role in the diagnosis of CP/CPPS, but they can be used to rule out other potential diseases like prostatic abscess, calculi, and bladder tumours (Nickel, 2011; Rees et al., 2015).

The UPOINT phenotyping has been widely used for the clinical evaluation and management of men with CP/CPPS (Nickel & Shoskes, 2010; Shoskes et al., 2009; Shoskes et al., 2010). This phenotyping has been used to provide more individualized and patient-centred assessment (Doiron, 2020; Shoskes & Nickel, 2013). The UPOINT phenotyping can divide the patients` symptoms into six different domains including urinary, psychosocial, organ specific, infectious, neurologic and pelvic floor tenderness (Doiron, 2020). It has been proven that clinical evaluation of patients with CP/CPPS using UPOINT phenotyping improves management outcomes (Shoskes et al., 2010).

Various inflammatory markers including tumour necrosis factor-α (TNF-α), interleukin-1α (IL-1α), IL-6, and IL-8 have been suggested for the diagnosis of CP/CPPS and follow-up of management (Doiron, 2020; Orhan et al., 2001; Penna et al., 2007). Other biomarkers that have been investigated for the diagnosis of CP/CPPS include vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2), MMP-9, neutrophil gelatinase-associated lipocalin (NGAL), VEGF receptor-1 (VEGFR-1), and MMP9-NGAL complex (Clemens et al., 2019). In a recent study, significantly higher levels of VEGF, MMP-9, and VEGFR-1 were shown in the patients with CP/CPPS compared to healthy controls (Dagher et al., 2017). This study also showed that the patients with higher levels of MMP-9, MMP-9/NGAL complex, and VEGFR-1 had more severe symptoms. Further studies are required to investigate the potential role of various biomarkers in the diagnosis and management of CP/CPPS.

1.4.3. Experimental CP/CPPS models in rats

Many researchers have successfully used rodents to create experimental animal models for CP/CPPS (Bjorling et al., 2011; Dos Santos Gomes et al., 2018; Kwon et al., 2001; Vykhovanets et al., 2007; Wang et al., 2018). However, there is no universally accepted standard experimental model for CP/CPPS, and each model has some limitations (Wang et al., 2018). Zhou et al. used prostate homogenate protein and complete Freund`s adjuvant to create an experimental autoimmune CP/CPPS model in rats (Qi et al., 2012; Wang et al., 2018). Spontaneous autoimmune prostatitis is another method that can be used to create a CP/CPPS model (Bjorling et al., 2011; Vykhovanets et al., 2007; Wang et al., 2018). Moderate CD4+ T cell infiltration and chronic inflammation in the interstitial tissues around the

acinar lobes are the main histological findings in spontaneous autoimmune prostatitis (Keith et al., 2001; Vykhovanets et al., 2005; Wang et al., 2018). Previous studies showed that approximately 70% of Lewis rats have spontaneous prostate inflammation at 12 weeks (Vykhovanets et al., 2007; Wang et al., 2018).

Hormone induced prostatitis can also be used as a model for CP/CPPS. In this model, the aim is to deteriorate the androgen balance to cause non-bacterial chronic inflammatory changes in the prostate gland (Wang et al., 2018). The induction of prostate inflammation using estradiol-17β followed by DHT administration was shown to be an effective method causing inflammatory changes in the prostate of castrated Wistar`s rats (Robinette, 1988; Wang et al., 2018). The most prominent histopathological changes in the prostate were lymphocyte and mononuclear cell infiltration, severe oedema and fibrotic changes (Robinette, 1988). Similarly, a soy-extracted isoflavone mixture with weak estrogenic activity can be used orally to create CP/CPPS and to avoid possible intra-prostatic morphological changes due to subcutaneous (s.c.) administration of estradiol-17β (Kwon et al., 2001).

Various chemical substances like carrageenan, formaldehyde-croton oil, amidraphane and glycerin can be injected into the prostate to create a CP/CPPS model in rats (Ihsan et al., 2017; Radhakrishnan & Nallu, 2009; Wang et al., 2018). Carrageenan preparations seem to be the most advantageous since they cause less tissue damage in the prostate gland and the model has more similarities with CP/CPPS in humans (Radhakrishnan & Nallu, 2009; Wang et al., 2018). In a previous study, Radhakrishnan and Nallu showed that interstitial hyperemia, monocyte and lymphocyte infiltration, fibrous connective tissue hyperplasia, and oedema were the most prominent histopathological changes in the prostate tissues in a rat model of CP/CPPS created by injection of 3% carrageenan (Radhakrishnan & Nallu, 2009).

Partial urethral obstruction in rats was proposed as an alternative method to create prostate inflammation with lymphocyte infiltration and interstitial oedema (Takechi et al., 1999), suggesting that intraprostatic reflux of urine could be the responsible mechanism for the chronic inflammatory changes in the prostate in this animal model.

In a recent study, Dos Santos Gomes et al. aimed to propose an animal model of benign prostate hyperplasia (BPH) and prostatitis induced by intraurethral lipopolysaccharide (LPS) administration (Dos Santos Gomes et al., 2018). The authors speculated that LPS, which is a component of the gram-negative bacteria cell wall and has the potential to induce the release of TNF-α, IL-1, and IL-6 could be used to induce chronic prostate inflammation without the presence of an infectious component. This study showed that intraurethral administration of LPS could be used as an effective experimental animal model of chronic prostate inflammation.

Recently, zymosan, acting via toll-like receptor 2 (TLR2), has been established as a substance that can be used to induce CP/CPPS in rodents (Schwartz et al., 2016). Zymosan-induced prostatitis shares many prominent similarities with CP/CPPS in humans. The main drawbacks of this method are (a) a minimal risk of causing pelvic floor injury and (b) leakage of zymosan into surrounding tissues. However, with a meticulous injection technique it is possible to safely avoid diffusion of the substance into the bladder.

1.4.4. Management of CP/CPPS

1.4.4.1 Non-pharmacological

Management of patients with CP/CPPS requires an individualized and multimodal approach (Shoskes & Nickel, 2013; Shoskes et al., 2009). The UPOINT clinical phenotyping is a very helpful method to understand a patient's clinical phenotype and can be used to propose personalized management of CP/CPPS (Doiron, 2020; Shoskes & Nickel, 2013).

The management of men with CP/CPPS should begin with lifestyle modifications. Some simple lifestyle modifications like local heat treatment, testicular support for patients with scrotal pain, and using more comfortable seats for patients who like to cycle can help improve the symptoms (Kelly, 2018). Moderate physical exercise like swimming, aerobic, walking, or running can also help improve NIH-CPSI scores (Giubilei et al., 2007). It is very important to recognize that complete cure is rarely possible and the management should always focus on the most prominent symptoms, to improve the functional status of the patient (Doiron, 2020).

Pelvic floor physiotherapy is a non-pharmacological treatment alternative that can be used in CP/CPPS patients with pelvic floor dysfunction. Anderson et al. showed an improvement rate of 72% in pelvic floor dysfunction symptoms in men with CP/CPPS after at least 1 month of treatment with pelvic floor physiotherapy (Anderson et al., 2005). Acupuncture is another non-pharmacological treatment alternative with modest improvement in CP/CPPS symptoms in properly selected patients (Lee & Lee, 2009; Lee et al., 2008; Sahin et al., 2015). Extracorporeal shock wave lithotripsy (ESWL) has also been proposed as a non-pharmacological treatment method for CP/CPPS, showing some improvement in symptoms (Pajovic et al., 2016; Vahdatpour et al., 2013; Zeng et al., 2012). However, further

research is required to understand the efficacy of ESWL treatment in men with CP/CPPS.

Psychological intervention is also an important part of the non-pharmacological management of CP/CPPS since the psychological background of the patient can affect treatment outcomes and has an impact on quality of life (QOL) (Nickel et al., 2008). Although some minimally invasive treatment methods such as transrectal thermotherapy have previously been used, they are not included in the current treatment guidelines (Gao et al., 2012; Rees et al., 2015). Surgical treatment can only be used when pharmacological or other non-pharmacological treatment alternatives are not sufficient. Surgical treatment can be an option in case of ejaculatory duct obstruction, urethral stricture, bladder neck obstruction, and other potential pathologies that can be associated with CP/CPPS symptoms (Doiron, 2020). The diagnosis of CP/CPPS alone is never an indication for transurethral resection of the prostate (TURP) or radical prostatectomy.

1.4.4.2 Pharmacological

Although the role of antibiotics has not been clearly understood, they are still used as a treatment option for almost all types of prostatitis, including CP/CPPS (Khattak et al., 2021; Kulovac et al., 2007). It is widely accepted that the efficacy of antibiotics not only depends on their anti-microbial effects but also on their anti-inflammatory effects (Khattak et al., 2021). Previous studies showed reduced expression of inflammatory markers IL-6 and IL-8 with ciprofloxacin and levofloxacin treatment in patients with CP/CPPS (Alexander et al., 2004; Kulovac et al., 2007; Wang et al., 2016). Most previous studies on the use of antibiotics in the treatment of patients with CP/CPPS included combination drug treatment, mostly with alpha-blockers, and the treatment almost always resulted in a significant improvement in NIH-CPSI scores (Alexander et al., 2004; Khattak et al., 2021; Kulovac et al., 2007; Nickel, Downey, et al., 2003; Wang et al., 2016). These studies suggested antibiotics as an important part of a multidrug therapy, but the authors also mentioned a potential risk for antibiotic resistance (Alexander et al., 2004; Kim et al., 2011; Nickel, Downey, et al., 2003).

Alpha-blockers are another pharmacological treatment alternative for CP/CPPS, mostly due to their positive effects on LUTS (Lee et al., 2017; Nickel & Touma, 2012). The efficacy of alpha-blockers in the treatment of CP/CPPS partly depends on their anti-inflammatory effect since they can reduce neurogenic inflammation (Khattak et al., 2021). Previous studies on the use of alpha-blockers in patients with CP/CPPS showed that tamsulosin gave the best improvement in NIH-CPSI

scores with acceptable side effects (Chen et al., 2011; Wang et al., 2016). However, the best improvement in LUT function was observed with terazosin (Chen et al., 2011; Franco et al., 2020; Khattak et al., 2021; Wang et al., 2016).

The use of 5-alpha reductase inhibitors (5-ARI) can reduce intraprostatic ductal reflux and decrease intraprostatic pressure in men with CP/CPPS (Khattak et al., 2021). They can also suppress angiogenesis and reduce chronic inflammation in the prostate (Leskinen et al., 1999; Nickel et al., 2004). Previous studies have shown that 5-ARI cannot be used as monotherapy (Khattak et al., 2021; Nickel et al., 2004). However, they can be used as part of a multimodal pharmacological therapy.

Some symptoms of CP/CPPS can be explained by the chronic inflammation in the prostate (Polackwich & Shoskes, 2016). Previous studies showed that anti-inflammatory drugs can decrease CP/CPPS symptoms without significant side effects (Franco et al., 2020). Various anti-inflammatory drugs including cyclooxygenase-2 (COX-2) inhibitors, corticosteroids, tanezumab, and zafirlukast have been investigated for the treatment of CP/CPPS (Bates et al., 2007; Goldmeier et al., 2005; Nickel et al., 2012; Nickel, Pontari, et al., 2003; Zhao et al., 2009). Only COX-2 inhibitors have shown significant improvement in CP/CPPS symptoms. However, the clinical improvement with COX-2 inhibitors was modest.

Phosphodiesterase inhibitors (PDEI) may also have role in the treatment of CP/CPPS by increasing blood flow to the pelvic floor and improving LUTS (Chakrabarty et al., 2019; Khattak et al., 2021). Previous studies, even though small and with a short follow-up period, investigated the use of PDEI in the treatment of CP/CPPS and showed a significant improvement in NIH-CPSI scores and reduction in pain which was comparable to non-steroidal anti-inflammatory drugs (NSAIDs) (Benelli et al., 2018; Cantoro et al., 2013; Kong do et al., 2014). There are also some previous studies on the use of anti-depressants in the treatment of CP/CPPS (Giannantoni et al., 2014; Zhang et al., 2017). These studies showed that anti-depressants might have a place in the treatment of CP/CPPS and sertraline could be tolerated better than duloxetine (Zhang et al., 2017).

Various other drugs have been studied as potential pharmacological treatment alternatives for CP/CPPS (Franco et al., 2020; Khattak et al., 2021; Polackwich & Shoskes, 2016). Although there is still no effective pharmacological treatment alternative for patients with CP/CPPS, a systematic review by Franco et al. showed that anti-inflammatory drugs demonstrated the greatest symptom improvement (Franco et al., 2020). It has also been advocated that the UPOINT system could be used both to identify the clinical phenotype of each patient with CP/CPPS and to guide individualized treatment (Polackwich & Shoskes, 2016). By using the UPOINT phenotyping, LUTS can be treated with alpha-blockers, psychological symptoms can be treated with anti-depressants, and neurologic symptoms can be

treated with amitriptyline or gabapentin (Polackwich & Shoskes, 2016; Shoskes et al., 2009; Shoskes et al., 2010). A systematic review of the usefulness of UPOINT phenotyping, comparing patients who took part in phenotyping compared to those who did not, remains to be conducted.

1.5 Nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway

It has recently been shown that the NO/cGMP pathway has a critical, protective role in various diseases including fibrotic diseases, congestive heart failure, pulmonary hypertension, angina pectoris (Beyer et al., 2012; Follmann et al., 2017; Halank et al., 2017; Sandner et al., 2017). Nitric oxide has been indicated to be part of the inflammatory response in various tissues and various diseases including LUT diseases such as cystitis (Aronsson, Vesela, et al., 2014; Vesela et al., 2012). Previous studies have shown that NO is an important non-adrenergic, non-cholinergic neurotransmitter for the LUT to function properly (Gotoh et al., 2021). Nitric oxide, formed by NO synthases (eNOS, iNOS, nNOS), is an inhibitory neurotransmitter that has an important role in regulating smooth muscle relaxation and blood flow to the LUT (Fullhase et al., 2015; Leiria et al., 2014). Nitric oxide triggers formation of cGMP from guanosine-5'-triphosphate (GTP) through binding to the Fe^{2+} containing heme group of the enzyme soluble guanylate cyclase (sGC) (Fullhase et al., 2015; Lasker et al., 2013). Phosphodiesterase type 5 (PDE5) is responsible for the degradation of cGMP to GMP and PDE5-inhibitors like sildenafil, vardenafil, and tadalafil can decrease cGMP degradation (Estancial et al., 2015; Fullhase et al., 2015). However, PDE5 inhibitors are dependent on sufficient endogenous NO to perform their function (Fullhase et al., 2015). Pathological conditions that lead to oxidative stress can potentially block NO binding to the heme group of the sGC enzyme and thus block cGMP formation (Bau et al., 2010; Fullhase et al., 2015; Sommer et al., 2018).
Previous studies have shown that the NO/cGMP pathway has an important role in improving smooth muscle relaxation by increasing intracellular cGMP production and subsequently decreasing intracellular calcium concentration (Gotoh et al., 2021). Drugs affecting the NO/cGMP pathway have a great potential to be used in several urological diseases caused by defective smooth muscle relaxation in the bladder or urethra. In addition, there is a dense nitrergic innervation in the prostate and drugs targeting NO/cGMP can also be used to treat various prostate pathologies (Calmasini et al., 2016).

1.5.1. Soluble guanylate cyclase (sGC) modulators

Soluble guanylate cyclase becomes insensitive to NO if the heme group is removed or oxidized (Calmasini et al., 2016; Thoonen et al., 2015). Soluble guanylate cyclase modulators can enhance cGMP formation even if endogenous NO is not sufficient (Fullhase et al., 2015; Lasker et al., 2013). There are two types of sGC modulators: sGC stimulators and sGC activators. Both drugs can trigger sGC enzyme independently of NO stimulation. However, only sGC activators can trigger the heme free and/or oxidized form of the enzyme (Fullhase et al., 2015) (Figure 4).

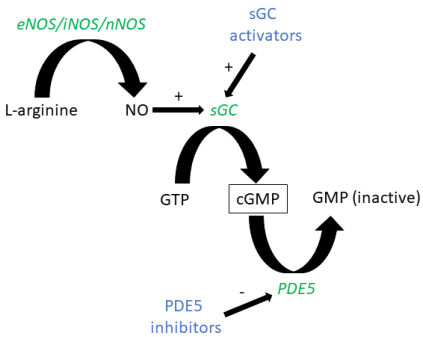

Figure 4. Nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway.
Formation of nitric oxide (NO) is catalysed by nitric oxide synthase (NOS). Nitric oxide binds to the soluble guanylate cyclase (sGC) enzyme, leading to formation of cyclic GMP (cGMP).
Upon production, cGMP leads to several physiological responses. The same effect is achieved by sGC activators, which can trigger the heme free and/or oxidized form of sGC enzyme. The breakdown of cGMP is catalysed by phosphodiesterase-5 (PDE5).

In recent years there has been an increasing interest in the use of sGC stimulators and activators in various urological diseases (Alexandre et al., 2014; Calmasini et al., 2016; Fullhase et al., 2015; Lasker et al., 2013; Leiria et al., 2014). Da Silva et al. aimed to investigate the relaxing responses of vas deferens to the sGC stimulator BAY 41-2272 in an *in vitro* animal study (da Silva et al., 2012). The authors concluded that BAY 41-2272 could be used in the treatment of premature

ejaculation since it increased cGMP levels and relaxed vas deferens smooth muscle. In another study, Bau et al. examined the potential effects of BAY 41-2272 on smooth muscle relaxation in rat, rabbit, and mouse bladder (Bau et al., 2010). This study showed that BAY 41-2272 caused concentration-dependent bladder smooth muscle relaxation and had great potential to be used in the management of bladder disorders.

Estancial et al. aimed to characterise the relaxatory responses in the rabbit corpus cavernosum to the sGC activator BAY 60-2770 (Estancial et al., 2015). The study showed that BAY 60-2770 increased relaxation in the corpus cavernosum independently of NO and that the relaxation was further enhanced by the oxidation of the sGC enzyme. Thus, the authors speculated that sGC activators such as BAY 60-2770 might be superior to PDE5 inhibitors and sGC stimulators. Similarly, Gotoh et al. investigated the effects of BAY 60-2770 and low dose insulin on LUT dysfunction in a rat model of diabetes mellitus (Gotoh et al., 2021). This study demonstrated that BAY 60-2770 might be an effective pharmacological alternative in the treatment of overactive bladder and dysfunctional voiding caused by diabetes mellitus. Similar studies supported the idea of targeting the NO/cGMP pathway, especially using sGC activators, as a novel treatment alternative to improve functional bladder abnormalities (Fullhase et al., 2015; Leiria et al., 2014).

The potential effects of sGC modulators on prostate contractility and urethral function has also been examined in a few previous studies (Alexandre et al., 2014; Calmasini et al., 2016). Alexandre et al. investigated the relaxatory responses in the urethral smooth muscle of obese mice and possible effects of BAY 60-2770 on muscle relaxation (Alexandre et al., 2014). This study showed that deteriorated relaxation of the smooth muscle in the urethra of obese mice was due to the increased production of radical oxygen species and an impaired NO/cGMP pathway. Treatment with BAY 60-2770 improved the urethral relaxatory responses by activating sGC. Calmasini et al. evaluated the effects of the sGC stimulator BAY 41-2272 and the sGC activator BAY 60-2770 on smooth muscle contractility in rabbit and human prostate (Calmasini et al., 2016). This study showed the expression of the sGC-α_1 subunit in the transition zone of the human prostate. In addition, the authors concluded that BAY 60-2770 was a better alternative since it enhanced cGMP levels even if sGC was oxidized.

2. Aims

The overall objective of this thesis was to identify pathophysiological alterations underlying LUT comorbidity caused by CP/CPPS and examine if these could be alleviated by pharmacological treatment with a new class of drugs, sGC activators. In a broader perspective, the findings could be used as a guide for the design of future clinical studies aimed at improving the management of male patients with CP/CPPS and its frequent comorbidities.

This thesis addresses the following research issues:

1. Does CP/CPPS have any negative effects on bladder function and histopathology? What are the potential underlying mechanisms causing these alterations? (Paper I and Paper II)
2. Can an intraprostatic infectious focus act as a possible inducing factor for CP/CPPS, leading to cross-organ sensitization between the prostate and bladder? (Paper II)
3. Does treatment with a sGC activator ameliorate CP/CPPS-induced functional and immunohistopathological alterations in the bladder? (Paper III and Paper IV).
4. How is innate contractility and receptor expression in the bladder affected by CP/CPPS? How is this affected by treatment with a sGC activator? (Paper IV)

2.1 Significance

CP/CPPS affects 3-10% of all males and the associated pain can be debilitating (Krieger et al., 2003; Krieger et al., 2002). Apart from pelvic pain, most patients suffer from LUTS, most commonly overactive bladder. Patients with CP/CPPS are currently significantly undertreated due to lack of effective pharmacological treatment options and a complicated diagnostic procedure (Zhang et al., 2020). Previous studies have shown that CP/CPPS has a dramatic negative impact on the patients' quality of life (Polackwich & Shoskes, 2016). Even if correctly diagnosed, the treatment options for CP/CPPS mainly focus on lifestyle adjustments.

By identifying a potential link between CP/CPPS and bladder dysfunction and showing the usefulness of a sGC activator for treating LUT comorbidities, the current thesis has the potential to advance the scientific field. Ultimately, this could enable a greater objective - to introduce a new pharmacological treatment option for patients with CP/CPPS, thereby significantly improving their quality of life.

3. Materials and Methods

All experiments performed in this thesis were approved by the local ethics committee at the University of Gothenburg, Sweden (approval number: 1794/2018). Male Sprague-Dawley rats that were phylogenetically relevant for human comparisons were used in the experiments. During and after all experiments, every effort has been made to minimize animal suffering. An attempt was made to use the least possible number of the rats, which could obtain statistically reliable results for the experiments.

3.1 Zymosan-induced chronic prostatitis

The dose of zymosan used to induce chronic prostate inflammation was 0.1mg in 10 µl sterile saline. Zymosan was injected into the dorsal lobe of the rat prostate with laparotomy under deep anaesthesia using 3% isoflurane (Paper I, III, and IV). In all intraprostatic injections, a 1 ml insulin injector was used, and the needle was tunnelled subcapsularly and held in the same site for about 20-30 seconds. By this way, possible leakage from the injection site in the dorsal prostate was avoided. Postoperative analgesia was ensured using a single s.c. dose of 0.1 $mg^{*}kg^{-1}$ buprenorphine.

During intraprostatic injections the injection site was wiped with cotton swabs to control possible drug leakage. Although no evident drug leakage was observed during the intraprostatic injection procedure, confirmatory intraprostatic injections were performed to be sure about the safety of the procedure in terms of a potential diffusion of the drug from the prostate into the bladder. In these confirmatory experiments, a 10 µl volume of cresyl violet and zymosan was injected into the dorsal prostate. A possible leakage from the injection site or diffusion into the bladder could be excluded since, even though the dorsal prostate turned blue, no colour change could be observed in the bladder one hour after the intraprostatic injection (Figure 5). This finding was consistent after 72 hours.

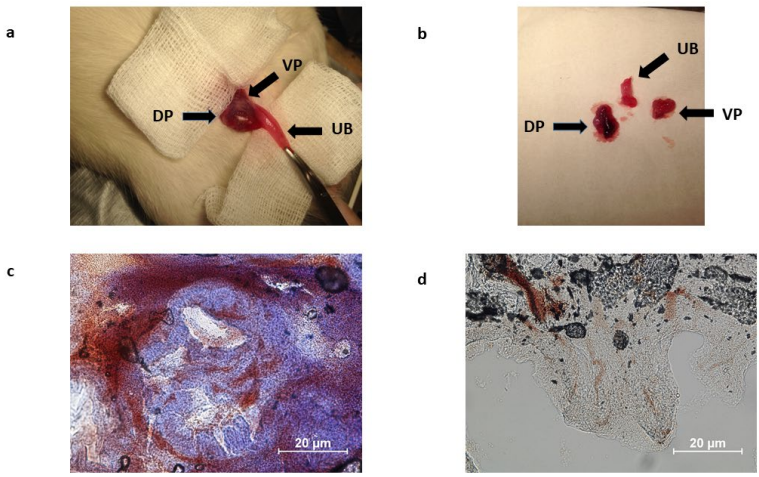

Figure 5. Colour change in the prostate and bladder one hour after cresyl violet and zymosan injection into the dorsal prostate.

Blue colour change was observed in the dorsal prostate both macroscopically (a and b) and microscopically (c) one hour after intraprostatic injection of cresyl violet and zymosan. No colour change was observed in the urinary bladder (a, b, and d).

DP: Dorsal prostate, VP: Ventral prostate, UB: Urinary bladder.

3.2 Lipopolysaccharide (LPS)-induced chronic prostatitis

In paper II, 50 µl LPS (100 $\mu g^{*}kg^{-1}$) was injected into both the dorsal and ventral lobes of the prostate, to create a functional model for chronic inflammation in the prostate. All intraprostatic injections with LPS were performed with laparotomy under deep anaesthesia using 3% isoflurane and, as in the zymosan injections, buprenorphine was used for postoperative analgesia. The same injection technique as in zymosan injections was used to avoid possible drug leakage from the prostate.

3.3 Subcutaneous treatments

The rats were treated daily with s.c. injections of BAY 60-2770 (Paper III & IV), celecoxib (Paper III) or a combination of celecoxib and BAY 60-2770 (Paper III) on days 8-20 after intraprostatic injection of zymosan. In the control groups, 0.05

$ml^{*}kg^{-1*}day^{-1}$ dimethyl sulfoxide (DMSO) was used as vehicle. The doses used for s.c. treatments were 0.5 and 20 $mg^{*}kg^{-1*}day^{-1}$ of BAY 60-2770 and celecoxib, respectively. All drugs were dissolved in 99.5% DMSO. Drug dosages and preparations were chosen based on previous similar studies (Fan et al., 2013; Fullhase et al., 2015; Lasker et al., 2013).

3.4 Metabolic cage investigations

Rats were placed individually in metabolic cages with full freedom to move around within the cage. The rats had free access to water, but not food, and voided urine was gathered for a period of 16 hours (Figure 6). The total amount of water intake and expelled urine was noted during the time period. The number of micturitions were registered using a laser doppler (WFL30-40B416; SICK, Richmond Hill, Canada). From these recordings, micturition frequency and voided volume per micturition were calculated. The data was recorded and analysed with a MP150WSW data acquisition system and the AcqKnowledge 3.8.1 software (BioPac Systems, Goleta, USA).

3.5 *In vivo* cystometry

In vivo cystometry was performed under deep anaesthesia with 3% isoflurane immediately after the metabolic cage investigations. During the cystometry experiments, the femoral artery was catheterized to monitor possible changes in blood pressure (Figure 7). Methacholine (MeCh; 1, 2, and 5 $\mu g^{*}kg^{-1}$) and ATP (5, 10, and 100 $\mu g^{*}kg^{-1}$) were administered via a catheter in the femoral vein. The bladder dome was also catheterized using two different catheters to stimulate micturition cycles with saline infusion and to register intravesical pressure changes respectively. Alterations in intravesical pressure, volume changes, voiding time, and non-voiding contractions (NVCs) were recorded with a MP150WSW data acquisition system and the AcqKnowledge 3.8.1 software (BioPac Systems, Goleta, USA). Bladder compliance was calculated by dividing intravesical volume change by pressure change during bladder filling. The rats were euthanized, and the prostate and bladder were excised after the cystometry experiments.

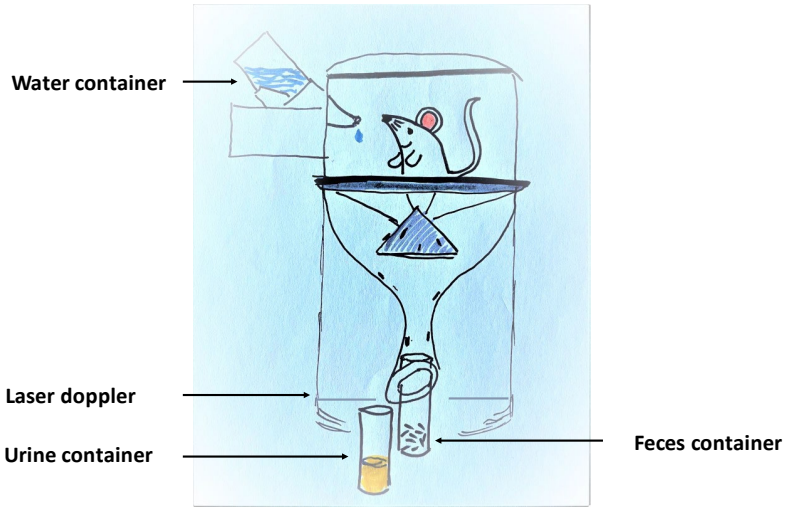

Figure 6. Schematic drawing of metabolic cage experiments.

Voided urine was gathered for a period of 16 hours during which the rats had free access to water. The total amount of water intake and expelled urine was noted. The number of micturitions were registered using a laser doppler.

3.6 *In vitro* organ bath functional studies

For all *in vitro* experiments (Paper IV), the rats were euthanized by an intraperitoneal injection with an overdose of pentobarbital (>60 mg/kg) followed by a heart incision. Subsequently, the urinary bladder was excised and kept in Krebs solution containing $CaCl_2$ (1.25 mM), glucose (5.5 mM), KCl (4.6 mM), KH_2PO_4 (1.15 mM), $MgSO_4$ (1.15 mM), NaCl (118 mM), and $NaHCO_3$ (25 mM). From each bladder, two full-thickness detrusor strips (6x2 mm) were excised from the area between the ureters and above the trigone. The bladder tissues were mounted in 20 ml organ baths that were filled with Krebs solution and kept at 37°C (Figure 8). The organ bath was continuously gassed with 5% CO_2 and 95% O_2, maintaining the pH at 7.4. The tissues were pre-stretched to approximately 10 mN and left to equilibrate for 45 minutes, and thereafter stretched to a baseline tension of 5 mN. Electrical field stimulation (EFS) was applied at 2, 5, 10, 20, and 40 Hz, respectively, as square wave pulses with a duration of 0.8 ms delivered at a supramaximal voltage of 50 V. All agonists and antagonists used during the organ bath

experiments were administered in a volume of 100 µl, yielding a dilution of 1:200 in the organ bath. The bladder preparations were weighed after each experiment. The pure NO in aqueous solution (2 mM) that was used in the organ bath experiments was added at increasing volumes of 40, 100, 200, and 400 µl, thus yielding final concentrations of 4, 10, 20, and 40 µM, respectively, in the bath.

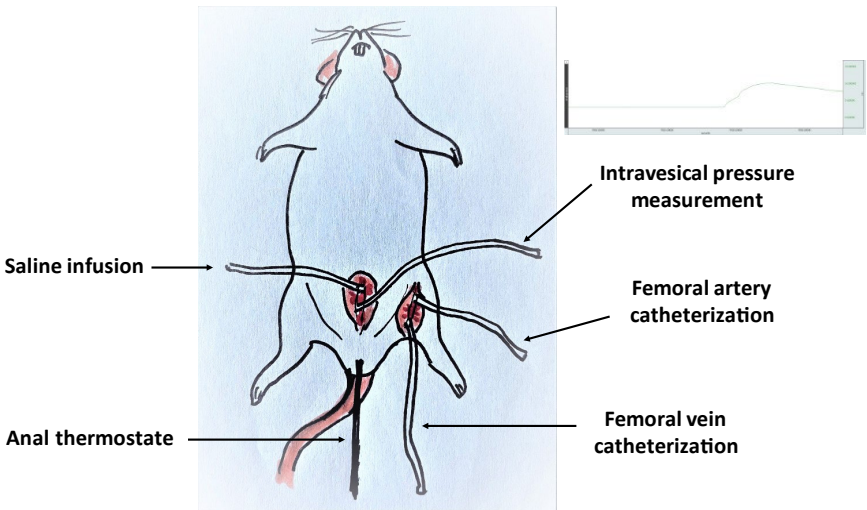

Figure 7. Schematic drawing of cystometry experiments.

Cystometry was performed under deep anaesthesia with 3% isoflurane. During cystometry experiments, the femoral artery was catheterized to monitor possible changes in blood pressure. Methacholine and ATP were given via a catheter in the femoral vein. The bladder dome was catheterized using two different catheters to stimulate micturition cycles with saline infusion and to register intravesical pressure changes.

3.7 Urine analyses of ATP and NO levels

Urine samples from the metabolic cage experiments were stored at -80°C and sent to the University of Brighton, United Kingdom on dry ice for urine analyses. All samples were thawed, gently mixed and then vortexed before the measurements. The measurements were done blindly with a conventional three electrode system, a 842d potentiostat, and a faraday cage (CH Instruments, Bee Cave, USA). A CH Instruments software was used to analyse the data.

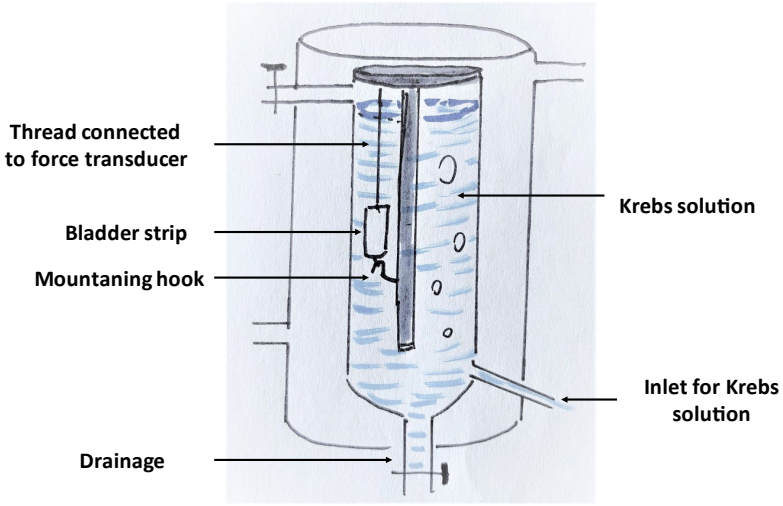

Figure 8. Schematic drawing of organ bath setup.

During organ bath experiments, the bladder strips were mounted in 20 ml organ baths that were filled with Krebs solution and kept at 37°C. The organ bath was continuously gassed with 5% CO_2 and 95% O_2 and the pH was always kept at 7.4.

3.8 Grading of inflammation in the prostate and bladder

The dorsal prostate, ventral prostate and bladder tissues were fixed in paraformaldehyde (PFA, 4% in 0.1 M phosphate buffer solution) prior to histopathological analysis and grading of inflammation. After the tissues were embedded in paraffin and sectioned into 8 µm thin sections (Histolab Products AB, Gothenburg, Sweden), counterstaining with haematoxylin-eosin (HE) was performed. The grading of inflammation in the prostate and bladder was done blindly following a similar procedure as a recent study (Inamura et al., 2018). The grade of inflammation was scored between 0 and 3 (0: no inflammation and 3: severe inflammation) where the extent of inflammation was scored as focal, multifocal, or diffuse.

3.9 Immunohistochemistry

Immediately following each organ bath experiment (Paper IV), the bladder tissues were fixed in PFA (4% in 0.1 M phosphate buffer solution) for

immunohistochemical analysis. After fixation, all tissues were embedded in paraffin and prepared as 8 µm thin sections (Histolab Products AB, Gothenburg, Sweden). In the smooth muscle and urothelium of the bladder, protein expression of sGC, muscarinic M3, and purinoceptor $P2X_1$ and $P2X_3$ was examined. All tissue sections were deparaffinized in xylene, rehydrated and incubated with Blocking solution (1% normal goat serum, phosphate buffered saline, 0.1% Triton X-100) to reduce nonspecific background signal. After overnight incubation with primary antibody in a dark chamber at 4°C, the sections were incubated with secondary antibody. The tissue sections were also incubated overnight without primary antibody to generate negative controls. The tissue sections were mounted using Prolong Gold anti-fade reagent with DAPI (P36931; Thermo Fisher Scientific).

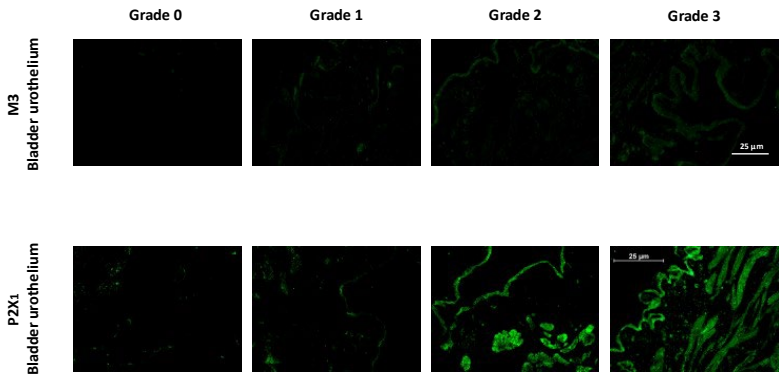

Figure 9. Representative micrographs for grading of receptor expression.
Grade 0: no receptor expression and Grade 3: very strong receptor expression.

The sections were then viewed and analysed using a DS-Fi camera and NIS Element 4.40 software (Nikon Corporation, Tokyo, Japan). The grade of receptor expression was scored between 0 and 3 (0: no receptor expression and 3: very strong receptor expression; Figure 9).

3.10 Materials

All drugs were purchased from Sigma Aldrich, St Louis, USA unless otherwise stated. Isoflurane (Attene Vet 1000 mg/g, Piramal Healthcare UK Limited, UK), KCl, KH_2PO_4, $MgSO_4$, NaCl, $NaHCO_3$, pentobarbitone sodium (Pentobarbitalnatrium vet.; APL, Stockholm, Sweden), sucrose, MeCh, ATP (Tocris Bioscience, Bristol, United Kingdom), $CaCl_2$ (Invitrogen, Paisley; United Kingdom), DMSO, ethanol (Kemetyl, Stockholm, Sweden), xylene (Kemetyl), glucose (Leo Pharma, Ballerup, Denmark), DAPI, phosphate buffered saline (PBS), cresyl violet acetate, PFA, Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific, Rockford, IL, USA), normal goat serum (NGS; Vector Lab, Burlingame, CA, USA), ammonium acetate (VWR International, Radnor, PA, USA).

3.11 Statistical analyses

Statistical calculations were performed by GraphPad Prism (GraphPad Software inc., San Diego, USA). One-way or two-way ANOVA followed by Tukey`s or Bonferroni`s correction was used for multiple comparisons of functional data. Kruskal-Wallis test and subsequent comparisons of mean ranks with Dunn`s test were used for non-parametric multiple comparisons.
Functional data and urine sample measurements were presented as mean ± SEM while immunohistopathological data were presented as median with range. Statistical significance was regarded for p values < 0.05.

4. Results

4.1 The effects of chronic prostatitis on the bladder

Metabolic cage experiments (Paper I-III) showed that induction of chronic inflammation in the prostate led to increased urinary frequency and decreased urine volume per micturition (Table 1).
Cystometry experiments (Paper I-III) showed that induction of CP/CPPS led to significantly increased total number of NVCs, decreased bladder compliance, and increased voiding time. Bladder contractions as a response to agonist stimulation with either MeCh or ATP were lower after induction of chronic prostatitis, especially at higher doses (Table 2).
Organ bath experiments (Paper IV) showed that induction of CP/CPPS did not significantly change the innate bladder contractile responses to EFS or MeCh. However, bladder contractile responses to higher concentrations of ATP were significantly lower after induction of CP/CPPS as compared to controls (Table 3). Induction of CP/CPPS did not lead to any significant changes in relaxatory responses to NO in the bladder (Table 3).
Induction of prostatitis with zymosan did not lead to any significant changes in terms of urine nitrite or ATP levels, as compared to controls. Histopathological examinations showed that induction of prostatitis with zymosan (Paper I) led to a higher grade of inflammation in the bladder on the 14th and 21st day post intraprostatic zymosan injection. In addition, a trend towards an increased degree of inflammation over time was observed. However, this finding was not consistent in paper III since the induction of prostatitis did not lead to any significant change in terms of grade or extent of inflammation in the bladder on day 14 after intraprostatic zymosan injection. Similarly, induction of prostatitis with LPS (Paper II) did not cause any significant changes regarding grade or extent of inflammation in the bladder, as compared to controls.
Induction of prostatitis caused no significant changes in terms of purinergic (P2X1 & P2X3), cholinergic (M3) or nitrergic receptor (sGC) expression in the smooth muscle or urothelium of the bladder, as compared to controls.

4.2 The effects of chronic prostatitis on the prostate

Findings from paper I, II, and III showed that induction of prostatitis with both zymosan and LPS did not lead to any significant changes in terms of prostate weight compared to controls. Both grade and extent of inflammation were significantly higher compared to controls in both ventral and dorsal prostate after induction of prostatitis with LPS or zymosan. Combination treatment with BAY 60-2770 and celecoxib restored the alterations in grade and extent of inflammation caused by the induction of prostatitis by zymosan in both dorsal and ventral prostate (Paper III).

4.3 The effects of BAY 60-2770 and celecoxib on the bladder after induction of chronic prostatitis

Metabolic cage experiments (Paper III) showed that treatment with the soluble guanylate cyclase activator BAY 60-2770 alone or in combination with the COX-2 inhibitor celecoxib normalized all parameters examined after induction of chronic prostatitis with zymosan (Table 1).

	After induction of prostatitis	BAY-60-2770	BAY 60-2770 & celecoxib
Total water intake	↔	↔	↔
Total urine output	↔	↔	↔
N of micturitions	↑	↓	↓
Micturitions/h	↑	↓	↓
V/micturition	↓	↑	↑

Table 1. Summary of changes observed during metabolic cage experiments in paper I, II, and III.
N: number, h: hour, V: volume

Cystometry experiments (Paper III) showed that treatment with BAY 60-2770 alone or in combination with celecoxib significantly decreased the number of NVCs and improved bladder compliance after induction of chronic prostatitis. Regarding voiding time, complete improvement could be achieved by the combination treatment (BAY 60-2770 + celecoxib). The muscarinic and purinergic contractile responses in chronic prostatitis-induced rats were significantly

improved by treatment with BAY 60-2770 alone and completely normalized by the combination treatment (Table 2).

	Induction of prostatitis	BAY 60-2770	BAY 60-2770 & celecoxib
NVCs	↑	↓	↓
Compliance	↓	↑	↑
Voiding time	↑	↓	↓↓
Muscarinic contraction	↓	↑	↑↑
Purinergic contraction	↓	↑	↑↑

Table 2. Summary of data from cystometry experiments in paper I, II, and III.

Organ bath experiments (Paper IV) showed that treatment with BAY 60-2770 led to significantly increased bladder contractile responses to EFS after chronic prostatitis induction with zymosan, as compared to controls. Contractile responses to high concentrations of muscarinic and purinergic agonists were significantly higher in tissues from rats treated with BAY 60-2770 after induction of chronic prostatitis, as compared to the controls. The relaxatory responses to NO were also significantly higher in BAY 60-2770 treated animals, as compared to controls (Table 3).

	Induction of prostatitis	BAY 60-2770 treatment
Contractile responses		
EFS	↔	↑
MeCh	↔	↑
ATP	↓	↑
Relaxatory responses		
NO	↔	↑

Table 3. Summary of data from organ bath experiments in paper IV.
EFS: Electrical field stimulation, MeCh: Metacholine, ATP: adenosine-5′-triphosphate, NO: Nitric oxide.

Although induction of chronic prostatitis did not lead to any significant changes in urine ATP levels compared to the controls, treatment with BAY 60-2770

(intraprostatic zymosan + s.c. BAY 60-2770) led to significantly lower urine ATP levels compared to controls (intraprostatic zymosan + s.c. DMSO).
Treatment with BAY 60-2770 didn`t change the muscarinic (M_3) or purinergic (P_2X_1 & P_2X_3) receptor expression in the urothelium or detrusor. Expression of sGC was significantly decreased in the bladder urothelium, but not the detrusor, in rats treated with BAY 60-2770.

5. Discussion

This thesis project has demonstrated that induction of experimental chronic prostatitis in rats leads to functional alterations in the bladder and supports the idea of prostate-to-bladder cross-organ sensitization, which can possibly be an important cause for the unsatisfactory treatment of patients with CP/CPPS. The current findings are consistent with previous studies (Funahashi et al., 2019; Schwartz et al., 2016) and significantly advance the understanding of the underlying causes of CP/CPPS. Further, this thesis suggests that a new class of drugs, sGC activators, should be tested for the treatment of CP/CPPS.

5.1 Changes in the lower urinary tract due to CP/CPPS

Metabolic cage experiments showed that induction of chronic inflammation in the prostate led to bladder overactivity (Paper I, II, and III). This finding was further supported by the cystometry experiments that showed significant negative effects of chronic prostatitis induction on both afferent (NVCs, bladder compliance) and efferent (voiding time, purinergic and cholinergic contractile responses) signalling in the bladder. The reduced cholinergic contractile bladder responses that arose after induction of chronic prostatitis, identified during cystometry (Paper I, II, and III), were in concordance with previous animal models of interstitial cystitis/bladder pain syndrome (IC/BPS) (Andersson et al., 2008; Barut et al., 2019; Giglio et al., 2007; Stenqvist et al., 2017). This finding showed that cross-organ sensitization between the prostate and bladder after the induction of chronic prostatitis could potentially cause functional alterations in the bladder that mimic IC/BPS. Contrarily, the reduced purinergic contractile bladder responses that arose after the induction of chronic prostatitis (Paper I, II, and III) were opposite to previous studies on IC/BPS that showed that purinergic activity in the bladder was increased during bladder inflammation (Burnstock, 2001; Kumar et al., 2007; Smith et al., 2005; Sun & Chai, 2006). The difference in contractile alterations between IC/BPS animal models and CP/CPPS animal models could potentially be explained by the fact that a high degree of bladder inflammation is the causative agent in IC/BPS while a low or non-existent degree of bladder inflammation is present in animal models of CP/CPPS. Nevertheless, significantly decreased purinoceptor and muscarinic receptor induced contractile bladder responses arose in CP/CPPS animals, despite increased afferent signalling. This shows that cross-organ sensitization in the

pelvic region, induced by CP/CPPS, can lead to alterations in the smooth muscle, urothelium and/or efferent innervation of the bladder.

In vitro organ bath experiments (Paper IV) also showed reduced purinoceptor induced contractile bladder responses after induction of chronic prostatitis. This finding supported the idea of a potential prostate-to-bladder cross-organ sensitization triggered by chronic prostatitis. Previous studies have shown that purinergic contractile bladder responses largely depend on a cholinergic component (Birder et al., 2010; Stenqvist et al., 2017). However, it was not possible to explain the current findings by transmitter interplay since no significant changes in terms of cholinergic contractile bladder responses after induction of chronic prostatitis could be observed. This discordance can of course be explained by the difference between the studies regarding their study design. In addition, urine analyses (Paper IV) showed that induction of chronic prostatitis did not alter ATP production in the bladder. Furthermore, no significant differences were noted in CP/CPPS rats compared to controls in terms of purinergic $P2X_1$ or $P2X_3$ receptor expression in the bladder. Taken together, these results can be interpreted as potential effect of CP/CPPS solely on purinoceptor sensitivity, affinity, or activation in the bladder. These findings also support previous studies regarding the possible role of ATP in the release of various neurotransmitters that are important for bladder contractility (Hanna-Mitchell et al., 2007; Silva et al., 2015).

Previous studies have speculated about bacterial colonization in the prostate as a potential cause of unsatisfactory treatment of men with CP/CPPS (Nickel, Alexander, et al., 2003; Rudick et al., 2011). The current thesis demonstrates that induction of chronic prostatitis with LPS (Paper II) leads to bladder overactivity and significant negative alterations in both afferent and efferent bladder signalling. This supports the idea that a primary intraprostatic infectious focus, via cross-organ sensitization, may be a cause of ineffective management of LUTS in CP/CPPS, at least in some patients.

Longer voiding times after induction of chronic prostatitis induction was identified in cystometry experiments (Paper I, II, and III). This was interpreted as a negative effect of chronic prostatitis on efferent signalling in the bladder. This finding could tentatively be due to infravesical obstruction caused by BPH or attenuated contractile activity in the urethra. However, there were no significant differences between the groups in terms of prostate weight (Paper I, II, and III).

In paper I, induction of chronic prostatitis with zymosan led to signs of inflammation in the bladder after both 14 and 21 days. However, no significant changes in bladder inflammation scores were observed in paper II after induction of prostatitis with LPS. Like paper II, the histopathological examination in paper III revealed no signs of bladder inflammation on day 21 after induction of chronic prostatitis with zymosan. The current literature regarding possible inflammatory changes in

the bladder following induction of prostatitis is controversial (Funahashi et al., 2019; Schwartz et al., 2016). Discordance between different studies could be explained by different substances used to induce prostatitis, differences in study design, and differences in evaluation method of inflammatory changes in the bladder. Although we had the same study design and used the same substance to induce chronic prostatitis, as well as the same inflammation evaluation method, we obtained different results in terms of bladder inflammation in paper I and paper III. Previous studies have showed that bladder inflammation could induce nitrite production in the bladder (Andersson et al., 2008; Hosseini et al., 2004; Logadottir et al., 2004; Patel et al., 2020). Thus, the urine measurements in paper III which showed no significant increase in nitrite levels after the induction of prostatitis strengthen the finding of no bladder inflammation after induction of chronic prostatitis with zymosan.

5.2 Targeting the NO/cGMP pathway to treat CP/CPPS

Recently there has been an increasing interest in targeting the NO/cGMP pathway to treat various urological pathologies (Alexandre et al., 2014; Calmasini et al., 2016; Estancial et al., 2015; Fullhase et al., 2015; Leiria et al., 2014; Sommer et al., 2018). Phosphodiesterase 5 (PDE5) inhibitors are mainly used in men with erectile dysfunction but can also be used in the treatment of LUTS in men with BPH or overactive bladder (Calmasini et al., 2016; Lee et al., 2017). However, PDE5 inhibitors are dependent on NO to exert their effects, which appears to be their main disadvantage (Gotoh et al., 2021; Morelli et al., 2011). Thus, sGC stimulators and activators seem to be more advantageous compared to PDE5 inhibitors since they function in a NO independent manner (Calmasini et al., 2016; Fullhase et al., 2015; Sommer et al., 2018). Further, sGC activators like BAY 60-2770 offer an additional advantage in that they can exert their effect even when the heme group of sGC enzyme is oxidized, for instance due to oxidative stress (Calmasini et al., 2016; Hu et al., 2016; Ihsan et al., 2017).

Paper III demonstrated that BAY 60-2770 counteracted negative effects of chronic prostatitis on bladder function. While monotherapy with BAY 60-2770 could effectively normalize the induced changes in bladder afferent signalling, combination therapy with celecoxib lead to additionally better outcomes in terms of improved efferent bladder signalling. In addition, combination therapy with BAY 60-2770 and celecoxib displayed the best anti-inflammatory effects in the prostate. The reason for specifically choosing celecoxib were data from previous studies which showed its potential effect on afferent bladder signalling and high degree of intraprostatic retention (Angelico et al., 2006; Yellepeddi et al., 2018). Although the findings in Paper III showed that celecoxib had limited use as monotherapy in

the treatment of CP/CPPS, it could effectively be used in combination treatment with BAY 60-2770.

The study in Paper IV was designed as an *in vitro* study to investigate how bladder smooth muscle *per se* responded to chronic prostatitis and treatment with BAY 60-2770. Increased detrusor contractile responses to EFS were observed in bladder tissues from animals treated with BAY60-2770. Considering the concomitantly observed increase in methacholine-induced contractile responses in the same tissues, the increased responses to EFS were assumed to be mainly due to an increase in the cholinergic component. Tentatively, the increased cholinergic responses could be due to an increase in muscarinic receptor expression or increased muscarinic receptor sensitivity. Immunostainings of muscarinic M3 receptors contradicted the possibility that the increase in contractile responses was due to increased muscarinic receptor expression.

Similarly, innate relaxatory detrusor responses to nitric oxide were increased by BAY 60-2770 treatment. This finding cannot be explained by an increased expression of sGC since the expression in the detrusor was not changed by induction of prostatitis or treatment with BAY 60-2770. Further, BAY 60-2770 treatment caused significantly lower expression of sGC in the urothelium, indicating that the urothelium is not directly involved in the relaxatory responses to NO. However, like increased muscarinic receptor sensitivity being a possible reason for the observed increased cholinergic contractile responses, long-term exposure to BAY 60-2770 may possibly increase sGC sensitivity.

In addition, in tissues from animals with zymosan-induced prostatitis, the contractile responses to the purinergic P2X agonist ATP were improved by treatment with BAY 60-2770, despite no observed alterations in the expression of $P2X_1$ or $P2X_3$ purinoceptors. Further, urine ATP production was decreased after treatment with BAY 60-2770. Taken together, these findings can be interpreted as a possible effect of the sGC activator BAY 60-2770 on muscarinic, nitrergic, and purinergic receptor sensitivity or affinity in the bladder.

5.3 Methodological considerations

Although various animal models have been suggested to imitate CP/CPPS, none of them seem to be perfect (Bjorling et al., 2011; Dos Santos Gomes et al., 2018; Schwartz et al., 2016; Vykhovanets et al., 2007; Wang et al., 2018). Zymosan was chosen as the drug to induce experimental chronic prostatitis in paper I, III, and IV because it has previously been proven to be a valid method to create an animal model of chronic prostatitis (Schwartz et al., 2016). In paper II, it was hypothesised that an infectious focus in the prostate could be the cause for bladder overactivity and the unsatisfactory management of men with CP/CPPS. An intraprostatic

injection with LPS was therefore chosen to imitate CP/CPPS induced by an infectious focus in the prostate.
There is a risk that the surgical procedure and the intraprostatic injections used to create a functional animal model for chronic prostatitis may damage adjacent structures and the pelvic floor. In addition, it is important to ensure that the substance used to induce chronic prostatitis does not diffuse into the bladder or urethra. Presently, the drugs used to induce chronic prostatitis were shown to not diffuse into the bladder (Figure 3). In addition, in paper II and III, no significant inflammatory changes were identified in the bladder after the induction of chronic prostatitis, and the surgical procedure was the same in all animals.
In the current thesis, we aimed to create a functional model for CP/CPPS for focusing on functional and histological changes. However, the degree of pelvic pain, which is often considered the main and most devastating symptom in men with CP/CPPS (Polackwich & Shoskes, 2016), was not evaluated. All intraprostatic injections and cystometry experiments were performed under deep anaesthesia using isoflurane, which could possibly affect nerve transmission. However, the same anaesthesia methods and substances were used in all animal groups, including controls, allowing valid statistical comparisons. The same argument, *i.e.,* that the same substance was used in all animals, can be made for DMSO used as the vehicle for s.c. treatments. Although drug doses were chosen according to previous similar studies, no dose-response studies have been conducted to ensure that the most effective and safe dose was used in the current thesis project (Fan et al., 2013; Fullhase et al., 2015; Lasker et al., 2013; Pankey et al., 2011). In addition, some factors like plasma testosterone levels, changes in blood flow and vascular relaxation, that could be important when evaluating the potential effects of NO/cGMP activation on LUT function, were not taken into consideration. However, all of these were beyond the scope of the current thesis project.

5.4 Translational potential and future perspectives

The findings in this thesis indicate that induction of chronic prostatitis leads to bladder dysfunction, mainly overactivity, without causing local histopathological changes in the bladder. Bladder overactivity was observed regardless of if the prostate inflammation was chemically induced (with zymosan) or induced by LPS, mimicking an infectious focus in the prostate. Cystometry revealed that the bladder changes were caused by sensitization of afferent signalling. Our findings thus indicated that induction of chronic prostatitis could lead to bladder dysfunction via cross-organ sensitization. Further, our findings showed that a sGC activator, BAY 60-2770, had a dramatic ameliorative effect on functional bladder alterations caused by induction of chronic prostatitis.

The underlying mechanisms of how induction of prostate inflammation leads to bladder overactivity and potential mechanisms for prostate-to-bladder cross-organ sensitization need to be investigated in future studies. Afferent and efferent signalling could be studied by utilizing a unique *in vivo* method known as the split bladder method (Aronsson, Carlsson, et al., 2014). In this method, the bladder is exposed, but instead of measuring contraction of the intact organ the bladder is carefully split into two halves. The blood supply as well as afferent and efferent nerve innervation is left intact on both sides. This allows manipulation (i.e., stretch, administration of drugs or direct electrical nerve stimulation) on one side, leading to activation of afferent nerve signalling, to be measured on the opposite side as an efferent response. By comparing responses (i.e., stretch, drug administration and electrical nerve stimulation) in healthy animals to those in animals in which CP/CPPS is induced, the afferent nerve sensitization hypothesis can be investigated. Further, by studying animals treated with a sGC activator, it can be elucidated how bladder afferent signalling in an animal model of CP/CPPS is affected by drug treatment.
Induction of prostatitis led to bladder overactivity *in vivo*, but the current *in vitro* findings showed that only purinergic responses were affected in the isolated bladder. This further highlights the importance of unaltered afferent signalling for normal bladder function. However, bladder function can also be affected by potential changes in the innate contractility of the prostate and urethra. Therefore, further studies are required to evaluate how the innate contractility and receptor expression of the prostate and urethra are affected by CP/CPPS. This could be investigated in an *in vitro* organ bath study.

From a clinical standpoint, the overall purpose of this thesis was to assess the potential correlation between CP/CPPS and bladder overactivity and examine how this can be alleviated by pharmacological treatment with a new class of drugs, the sGC activators. The current findings can help guide future clinical studies aimed at improving the management of male patients with CP/CPPS. A potential clinical study could include patients diagnosed with BPH, which have been scheduled for transurethral resection of the prostate (TUR-P). The reason for studying BPH patients would be mainly ethical, since it is easy to argue the appropriateness of performing an explorative study on patients that are already scheduled for invasive surgery. Upon informed consent, each patient could be examined preoperatively, in the same manner as all TUR-P patients, with blood- and urine sampling, urine culture, prostate specific antigen (PSA), c-reactive protein (CRP), uroflowmetry, residual urine, ultrasound to evaluate prostate volume and possible bladder stone. Prior to the surgery, each patient could be asked to fill out clinically relevant forms including NIH-CPSI, International prostate symptom score (IPSS), overactive

bladder questionnaire version-8 (OAB-V8) and a three-day micturition diary. These forms would allow the researchers to quantify perceived QOL as well as identify patients with concomitant chronic prostatitis and/or overactive bladder. Each patient could also fill out a genitourinary pain index (GUPI) to quantify the perceived degree of pelvic pain. After the surgery, resected prostate tissue and biopsies from the urinary bladder could be examined histopathologically. The patients could subsequently be divided into groups, depending on their degree and type (mononuclear, oedema, bleeding, mast cell activity) of prostate inflammation. Postoperatively, all patients could be asked to fill out the same forms as they did prior to the surgery and a new routine evaluation with blood- and urine sampling, urine culture, PSA, CRP, uroflowmetry, residual urine, ultrasound and x-ray could be performed. When all data is collected, comparative statistical analysis could be performed.

If such a study could clinically demonstrate a link between CP/CPPS and LUT dysfunction in men, the following clinical study could be an interventional drug study to further study the effects of sGC activators and to evaluate their potential usefulness for treating LUT comorbidities in men with CP/CPPS. If successful, these studies could lead to the proposal of a new pharmacological treatment option for a group of patients in great need of better care.

6. Conclusion

The current thesis showed that induction of chronic prostatitis could trigger cross-organ sensitization between the prostate and bladder. Induction of chronic prostatitis had negative effects on both afferent and efferent signaling in the bladder and led to bladder overactivity. Prostate-to-bladder cross-organ sensitization could also be initiated by an infectious focus in the prostate. These findings could be helpful to understand and improve some of the unsatisfactory treatment attempts of patients with CP/CPPS.

The sGC activator BAY 60-2770, alone or in combination with celecoxib, improved functional alterations in the bladder caused by induction of chronic prostatitis. The negative effects of chronic prostatitis on both afferent and efferent bladder signaling were normalized by BAY 60-2770 treatment. Targeting the NO/cGMP pathway with sGC activators could be a promising pharmacological treatment option for men with CP/CPPS.

Further preclinical and clinical studies are required to understand the mechanisms behind cross-organ sensitization in the pelvic region and the therapeutic effects of NO/cGMP pathway activation on LUTS caused by CP/CPPS. Figure 10 is a summary of all studies included in the current thesis, including potential future translational research.

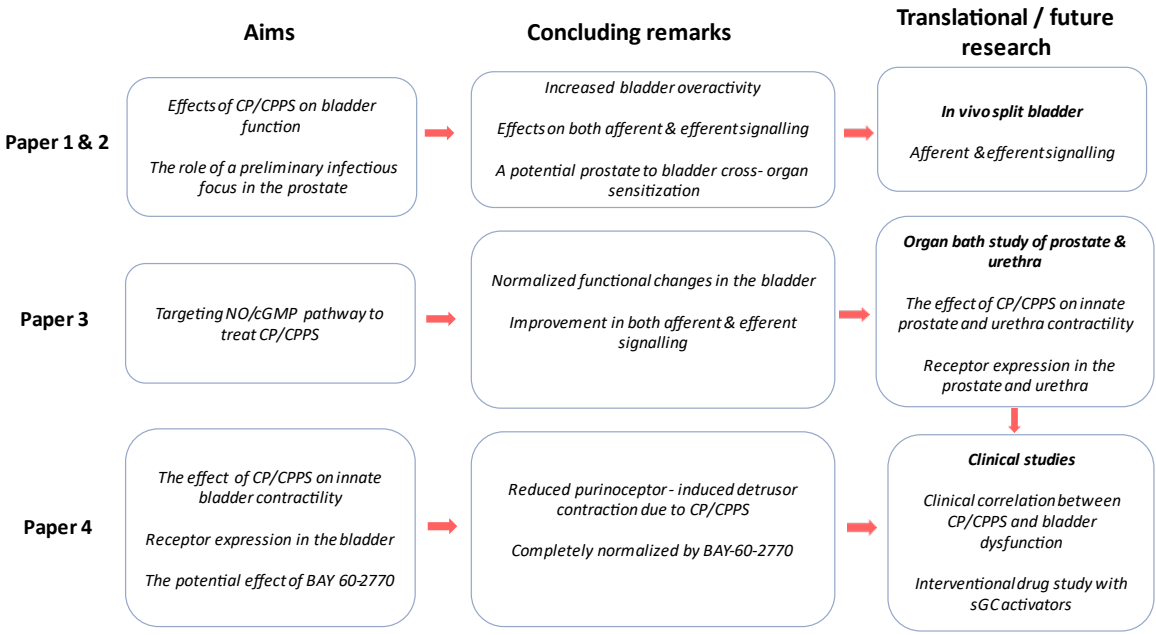

Figure 10. A summary of the studies included in the current thesis and potential future translational research.
CP/CPPS: Chronic prostatitis/chronic pelvic pain syndrome, NO/cGMP: Nitric oxide/cyclic guanosine monophosphate, sGC: soluble guanylate cyclase

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