Highlighting the relevance of proBDNF/mBDNF ratio and matrix metalloproteinase-9 activity in an aging female cohort with Overactive Bladder Syndrome

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TABLE OF CONTENTS

Abstract

Brain derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the human brain and is widely expressed in both the developing and adult mammalian brain. It is involved in various neural processes and is present also in peripheral tissues, such as bladder. Changes in neurotrophin levels, such as BDNF, and their precursor molecules have been associated with several physiological and pathological conditions including voiding dysfunction. By analyzing small metabolites found in urine along with the levels of neurotrophins, it may be possible to identify specific metabolic signatures and profiles associated with different types of voiding dysfunction such as overactive bladder syndrome (OAB). Nerve growth factor (NGF) and BDNF have been previously associated to OAB, with hypothesized mechanisms of their pathological involvement, specifically in the aging population. Herein, we study urinary BDNF in both its precursor and mature form and associated molecules as reliable biomarkers for OAB. Additionally, we use an in vitro model to further understand the mechanism of these molecular imbalance in the urine of OAB patients when compared to non-OAB controls.

Résumé

Le facteur neurotrophique dérivé du cerveau (BDNF) est la neurotrophine la plus abondante dans le cerveau humain et est abondamment secrété dans le cerveau des mammifères lors de leur croissance ainsi qu'à l'âge adulte. Il est impliqué dans divers processus neuronaux et est également produit dans les tissus périphériques, en particulier la vessie. Les changements des taux de neurotrophines, telles que le BDNF, et de leurs molécules précurseures (proBDNF) ont été associés à plusieurs conditions physiologiques et pathologiques, notamment le dysfonctionnement mictionnel. En analysant les petits métabolites présents dans l'urine ainsi que les niveaux de neurotrophines, il pourrait être possible d'identifier des signatures et des profils métaboliques spécifiques associés à différents types de dysfonctionnements mictionnels tels que le syndrome de la vessie hyperactive (OAB). Le facteur de croissance nerveuse (NGF) et le BDNF ont déjà été associés à l'hyperactivité vésicale, des hypothèses décrivant les mécanismes de leur implication pathologique, en particulier dans la population vieillissante. Ici, nous étudions le BDNF urinaire sous sa forme précurseure et mature ainsi que les molécules associées en tant que biomarqueurs fiables de l'hyperactivité vésicale. D'autre part, nous utilisons un modèle in vitro pour cerner le mécanisme de ces déséquilibres moléculaires dans l'urine des patients atteints d'OAB par rapport aux patients contrôles sans OAB.

Acknowledgements

I would like to begin by acknowledging my supervisor, Dr. Lysanne Campeau, who is an inspiring female urologist, scientist, mother, and mentor. Thank you for opening the door to your laboratory and for allowing me to partake on your research. Secondly, I would like to thank Dr. Philippe Cammisotto, for teaching me along the way, keeping my cell cultures alive, correcting my French, and for his patience in training me. Also, to Dr. Samer Shamout for transmitting his knowledge and training for the completion of this academic endeavour. I also have great appreciation for every integrant of the Urology Department of the Jewish General Hospital for helping me throughout my study recruitment process, especially Dr. Corcos, Dr. Anidjar, Dr. Alimi, Dr. Singh, and Dr. Perrin. My work would have been impossible without the help of the nursing team and administrative staff- singularly Nathalie, Mary, and Cherine. I also appreciate the kindness of Dr. Alameldin for allowing us to collaborate with the Pathology Department. I am particularly grateful to my lab colleagues and fellow graduate students, Aya Hajj and Stephanie Sirmekeysan, for being phenomenal coworkers and who will remain forever friends. Thank you for your sisterhood and for making our work environment so lively. My gratitude also goes out to the several funding agencies that supported my degree: CONACyT, LDI travel awards, RQRV, Experimental Surgery Department Excellence Awards, and the Claude Gagnon Urology studentships. Most importantly, to my friends and family, far and near, who have always supported my endeavors and have been my greatest cheerleaders. To my Québécois family; Laurence, Paul, Lyne, and Leo. Finally, to my sister and my mother, who are my greatest role models; thank you for your patience and unwavering support.

Preface

This work is presented in a manuscript-based format. Chapter 4 has been published. Chapter 5 is an in vitro study on rat bladder cells and is to be submitted for publishing.

Contribution of Authors

All author contributions are recorded at the start of their respective chapters. The entirety of the thesis document is written and prepared by me and revised by my supervisor Dr. Lysanne Campeau. The French translation of the abstract was revised by Philippe Cammisotto. The thesis consists of 6 chapters. The first chapter is an introduction to the main topics and chapter 2 is a more specific literature review, both written entirely by me. Chapter 3 focuses on the experimental designs, hypotheses, and objectives, where conceptualization was done by Dr. Lysanne Campeau and Philippe Cammisotto. Chapter 4 presents data in manuscript form, on the *clinical* study regarding the role of the neurotrophin BDNF in overactive bladder syndrome. Patient recruitment and sample collection was done by Samer Shamout and Abubakr Mossa. Urine creatinine, measurement of urinary metabolites (such as BDNF and natriuretic peptides), data entry, and statistical analysis was done by Philippe Cammisotto and I. Chapter 5 presents data regarding the *in vitro* study on rat bladder cells (urothelial and smooth muscle cells) and the potential involvement of natriuretic peptides in the pathology of overactive bladder syndrome. All experiments data analysis, and tables were done by Philipe Cammisotto and I. Text writing was done by me, Philippe Cammisotto, and revised by Dr. Lysanne Campeau. Chapter 6 was written entirely by me and revised by Philippe Cammisotto and Dr. Lysanne Campeau.

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 1

Chapter 4

List of Abbreviations

ACE: angiotensin-converting enzyme

ACh: acetylcholine

Akt: Ak strain transforming

ANCOVA: analysis of covariance

ANOVA: analysis of variance

ANP: atrial natriuretic peptide/ natriuretic peptide A

ANS: autonomous nervous system

ATP: adenosine triphosphate

AUA: American Urological Association

AUC: area under the curve

BDNF: brain-derived neurotrophic factor

BMI: body mass index

BNP: Brain natriuretic peptide/ natriuretic peptide B

BoNT-A: botulinum toxin A injections

BPH: benign prostatic hyperplasia

cAMP: cyclic adenosine triphosphate

cDNA: complimentary DNA

cGMP: cyclic guanosine monophosphate

CNP: C-type natriuretic peptide

CNS: central nervous system

COBaLT: Colombian overactive bladder and lower urinary tract symptoms

COVID-19: coronavirus disease 2019

CREB: cAMP-response element binding protein

CTR/CTL: control

CVD: cardiovascular disease

DB-cAMP: analog dibutyryl cyclic-AMP

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

DO: detrusor overactivity

DRG: dorsal root ganglia

ECD: extracellular domain (of p75NTR)

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

NOS: nitric oxide synthase

EPIC: Epidemiology of Incontinence Study

EpiLUTS: Epidemiology of Lower Urinary Tract Symptoms

FBS: fetal bovine serum

GAG: glycosaminoglycan layer

GC-A, B, C: guanylyl cyclase receptor type A, B, C

GFR: glomerular filtration rate

HDL: high density lipoprotein

HF: heart failure

HGN: hypogastric nerve

HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

ICIQ-SF: international consultation on incontinence questionnaire- short form

ICS: International Continence Society

IC/BPS: interstitial cystitis/ bladder pain syndrome

IIQ7: incontinence impact questionnaire 7

IMP: inferior mesenteric plexus

JNK: c-jun N-terminal kinases

LDI: Lady Davis Institute for Medical Research

L-NAME: nitro-L-arginine methyl ester

LUTS: lower urinary tract symptoms

M 2, 3: muscarinic receptor 2, 3

mBDNF: mature BDNF

miRNA: micro ribonucleic acid

MMP 1,2,7,9: matrix metalloproteinases 1, 2, 7, 9

fMRI: functional magnetic resonance imaging

mRNA: messenger ribonucleic acid

MUI: mixed urinary incontinence

NDO: neurogenic detrusor overactivity

NE: norepinephrine

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: nerve growth factor

NO: nitric oxide

NOBLE: National Overactive Bladder Evaluation

NPs: natriuretic peptides

NPR-A, B, C: natriuretic peptide receptor A, B, C

NTs: neurotrophins

NT-3: neurotrophin 3

NT-4/5: neurotrophin 4/5

OAB: overactive bladder syndrome

OAB-POLL: OAB on Physical and Occupational Limitations

OABSS: overactive bladder syndrome symptom score

P2X2/3: purinergic receptor

P75NTR: p75 neurotrophin receptor

PBS: phosphate-buffered saline

PC1/3: proconvertase enzyme

PET: photon emission computed tomography

PFMT: pelvic floor muscle training

PGE-2: prostaglandin 2

PI3K: phosphoinositide 3-kinase

proBDNF: proform (precursor) of BDNF

proNT: proform (precursor) of neurotrophin

PPARγ: peroxisome proliferator-activated receptor gamma

PVR: post void residual

QoL: quality of life

qPCR: quantitative polymerase chain reaction

RCTs: randomized controlled trials

REC: Research Ethics Committee

ROC: receiver operating characteristic

SEM: standard error of the mean

SMC: smooth muscle cell

SNP: sodium nitroprusside

SNS: sacral nerve stimulation

SOB: super optimal broth

SUFU: Society of Urodynamics, Female Pelvic Medicine & Urogenital Reconstruction

SUI: stress urinary incontinence

T4PNK: T4 polynucleotide kinase

TIMP: tissue inhibitor of metalloproteinases

tPA: tissue plasminogen activator

TrkA, B, C: tropomyosin kinase receptor A, B, C

UDS: urodynamic study

UI: urinary incontinence

UUI: urge urinary incontinence

URO: urothelial cell

US: ultrasound

UTI: urinary tract infection

UTR: untranslated regions

Chapter 1: Introduction to the human bladder and Overactive Bladder Syndrome

1.1 The urinary bladder: anatomy and physiology

1.1.1 Anatomical structure and function

The human urinary bladder forms an integral part of the genitourinary system. It is a hollow and viscous organ located extraperitoneal within the pelvic cavity that functions to store and void urine (Miftahof & Akhmadeev, 2019; Pouw, 2019). Urine, generated by the kidneys, is drained via the ureters into the bladder and is, once the bladder is full, expelled out of the body via the urethra. A physiologically normal adult bladder can accommodate up to 400 mL of urine, which translates into a sensation of fullness without causing significant rise of intravesical pressure (El-Zaatari & Ro, 2021). The bladder has an inverted pyramid shape and is divided into three major anatomical portions: the dome, the midportion, and the fundus or base (Miftahof & Akhmadeev, 2019; El-Zaatari & Ro, 2021). (Figure 1.1)

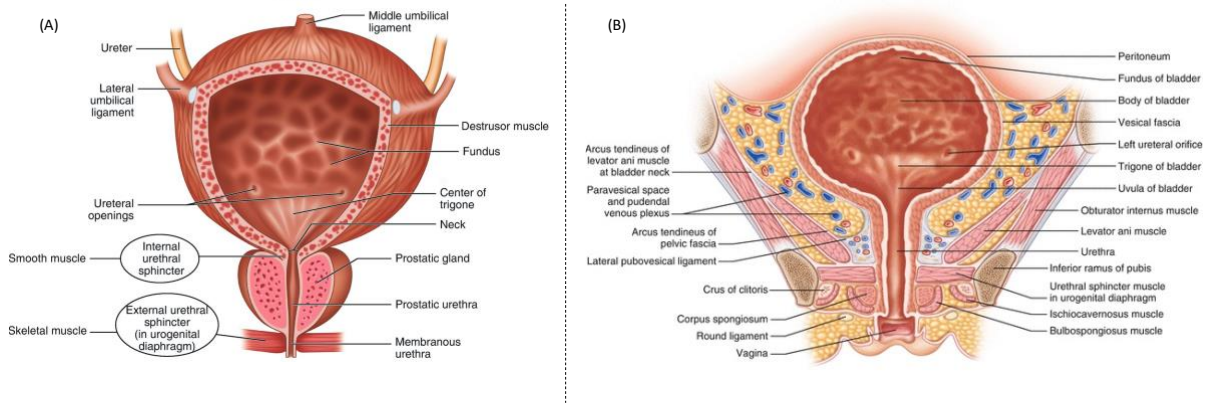

Figure 1.1 Anatomy of the urinary bladder in males and females (A) Urinary bladder in the male. The anterior wall of the urinary bladder and the anterior portion of the prostate gland have been sectioned to demonstrate the trigone of the urinary bladder and the prostatic urethra. Figure

from: (Smith et al., 2021) (B) Urinary bladder in the female highlighting adjacent structures. Figure from: (Larish & Kavaler, 2015).

The bladder's apex, located anterior-superiorly, sits at the tip of the dome and is the attachment site of the median umbilical ligament, also known as the urachus (El-Zaatari & Ro, 2021). Inferiorly at the base of the bladder is the trigone; a triangular anatomical landmark between the right and left ureteral orifices (uterovesical junctions) that connect to the bladder posterolaterally, as well as the urethral opening below. Also at the base of the bladder is the bladder neck, the region opening into the urethra that rests anterolaterally on the *internal obturator* and *levator ani* muscles (El-Zaatari & Ro, 2021). In males, the bladder lies superior to the prostate at the level of the bladder neck and anterior to the rectum, whereas in females it is anterior to the vagina and anteroinferior to the uterus (Pouw, 2019).

Blood Supply and lymphatic drainage

The superior and inferior vesical arteries are branches from the anterior trunk of the internal iliac artery, also known as hypogastric artery, that supply the bladder (Larish & Kavaler, 2015)**.** The anterosuperior bladder (midportion) is supplied by the superior vesical artery and the base (fundus) is supplied by the middle vesical artery that arises from the superior vesical artery. The remaining portions of the bladder, including adjacent structures, receive blood from the inferior vesical artery (Smith et al., 2021)**.** (Figure 1.2) Additional gender-specific blood supply comes from the obturator and inferior gluteal arteries (males), and the uterine and vaginal arteries (females), respectively, supplying small visceral branches to the bladder. The venous drainage is through a complicated plexus that empties in the hypogastric veins (Miftahof & Akhmadeev, 2019)**.** The lymphatics are

drained via numerous lymph nodes associated with the venous supply, but predominantly via the external iliac lymph nodes (El-Zaatari & Ro, 2021).

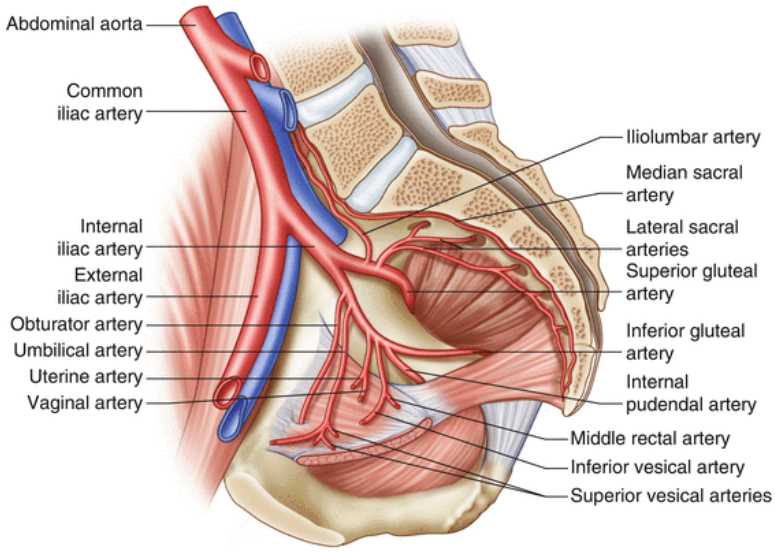

Figure 1.2 Branches of the internal iliac artery. Sagittal view of female blood supply to major pelvic structures. Figure from: (Larish & Kavaler, 2015).

Normal Histology

The bladder wall is comprised of two layers: the thin mucosa layer and the more prominent detrusor muscle that accounts for up to 70% of bladder wall thickness (El-Zaatari & Ro, 2021).Wall thickness is approximately uniform across the different parts of the bladder, apart from the trigone, which differs with respect to thickness and innervation, muscular alignment, and embryological background (El-Zaatari & Ro, 2021). The histomorphology of the bladder wall is divided into four layers, with the urothelium (mucosa) layer adjacent to the bladder lumen followed by the submucosal, muscular, and serous layers (Miftahof & Akhmadeev, 2019) (Figure 1.3). The superficial umbrella cell layer is covered by a glycosaminoglycan layer (GAG) and interacts with urine providing an active blood–urine permeability barrier (Jafari & Rohn, 2022). The mucosal

surface of the bladder is highly wrinkled so that it can undergo cycles of filling and voiding while maintaining its barrier function during all unfolding procedures (El-Zaatari & Ro, 2021).

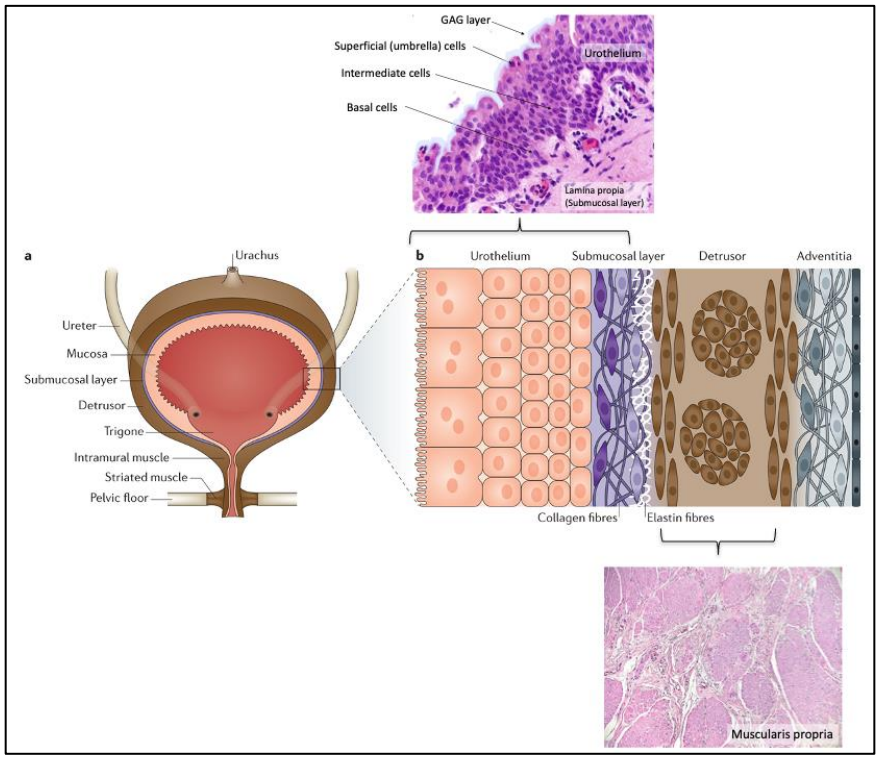

Figure 1.3 Bladder anatomy and bladder wall layers (a) The four layers of the urinary bladder wall: the mucosa (urothelium), which is the innermost layer facing the bladder lumen; the submucosal connective tissue layer (lamina propria); the muscular layer; and the serosal layer / adventitia covering the external surface. **(b)** The urothelium, covered by a glycosaminoglycan layer (GAG), is multilayered and includes basal cells, intermediate cells, and umbrella cells. The basement membrane sits beneath the urothelium and provides structural support. It separates the urothelial cells from the underlying connective tissue. The submucosal layer is composed of fibril-shaped or bundle-shaped collagens (types I and III) as well as an elastin fibrous network. Image adapted from:(Ajalloueian et al., 2018). Modified urothelium and detrusor muscle histology images taken from: (El-Zaatari & Ro, 2021).

There is substantial evidence that indicates that the urothelium functions as a transducer of several mechanoceptive and nociceptive stimuli in the bladder (Jafari & Rohn, 2022). Multiple receptors for acetylcholine (ACh), norepinephrine (NE), ATP, neurotrophins, prostacyclin, bradykinin, purines, cytokines, nitric oxide (NO), ion mechanosensitive Na^+ channels, myofibroblasts, and primary afferent and efferent neurons have been identified near to and within the suburothelium (*lamina propria*) and urothelial layers that can respond to various sensory inputs (Miftahof & Akhmadeev, 2019). Immediately beneath the urothelium layers lies a layer of loose connective tissue referenced as submucosa (*lamina propria*) followed by the muscularis mucosa and lastly, the *muscularis propria* (detrusor muscle) (El-Zaatari & Ro, 2021).

Detrusor Smooth Muscle

The muscular component of the bladder is arranged in widely spaced bundles, consisting of inner, middle, and external layers (El-Zaatari & Ro, 2021; Ajalloueian et al., 2018). The inner and outer layers are oriented longitudinally, whereas the medial layer is oriented circularly, allowing the bladder to maintain structural integrity and elasticity during the filling phase (Merrill et al., 2016; Ajalloueian et al., 2018).

Lastly, the serosa layer covers the lateral and superior external surfaces of the bladder wall, whereas the retroperitoneal bladder wall is surrounded by loose connective tissue (adventitia) (Merrill et al., 2016; El-Zaatari & Ro, 2021).

1.1.2 Innervation

The central and peripheral nervous systems work synergistically in the regulation of the urinary bladder involving cognitive, sensorimotor, and autonomic signals (H. A. Roy & Green, 2019) (Figure 1.4). Micturition, or urination, requires unique coordination of the urethral sphincter and

bladder functions that are dependent on their smooth and striated muscles (Miftahof & Akhmadeev, 2019). While the forebrain is responsible for deciding when urination is appropriate, the pons hosts micturition and continence centers and lastly, both voluntary and involuntary sphincter resistance is effected by spinal reflexes (Fletcher & Clarkson, 2011). Reflex voiding responses vary by age, due to neurodevelopmental stages, as well as presence or absence of neurogenic diseases. In a healthy adult, there is conscious awareness of bladder filling and voluntary control over the emptying process that account for major psychosocial factors of urination and not just expulsion of waste products from the body (H. A. Roy & Green, 2019).

Micturition Cycle: Storage and Voiding Phases

The determining factor that dictates whether the bladder relaxes (storage phase) or contracts (voiding phase) is a coordinated interplay between the autonomic nervous system (ANS) and somatic innervation (Miftahof & Akhmadeev, 2019). The bladder and the internal urethral sphincter receive autonomic sympathetic and parasympathetic stimulations, where parasympathetic innervation propagates micturition while its counterpart prevents it (Umans & Liberles, 2018) (Table 1.1). In the storage phase, the sympathetic division that arises from thoracolumbar spine (T10-L2) segments pass via the inferior mesenteric plexus (IMP) and travel in the hypogastric nerve (HGN) to contract the internal urethral sphincter and relax the detrusor smooth muscle. As volume expands the bladder, there is an increase in afferent nerve activity generated by tonic mechanoreceptors sensitive to bladder wall tension, which leads to micturition (Umans & Liberles, 2018; Fowler et al., 2008). Afferent activity increases, sending projection neurons to relay wall tension status to the forebrain. Perceptions of cognitive interpretation and considerations of emotional status are factored into a decision to either inhibit or initiate

micturition (Fletcher & Clarkson, 2011). Once the midbrain and pons have received the signal to switch from continence to micturition, the descending tracts from the pontine micturition center inhibit neurons to smooth (internal urethral) and striated (external urethral) sphincters and excite the parasympathetic preganglionic neurons to the detrusor muscle (Fowler et al., 2008). The parasympathetic fibers arise from sacral (S2-4) nerve roots and travel in the pelvic nerve, pass via the pelvic plexus, and innervate the detrusor muscle of the bladder, contracting it and thus propagating the voiding phase (Fletcher & Clarkson, 2011).

Table 1.1 Summary of Major Functions of the Somatic and Visceral Motor Systems: Bladder and Urethra

System	Division	Location of pre-ganglionic neurons	Location of ganglionic neurons	Actions	Neurotransmitter & targeted receptor	Bladder Function
Autonomic (involuntary)	Sympathetic Division (activated)	T10-L2	Hypogastric plexus	Relaxation of detrusor muscle	NE→ β3	Storage (filling) phase
			Pelvic (Sacral) plexus	Contraction of internal urethral sphincter	NE→ α-1	
	Parasympathetic Division (activated)	S2-S4	Pelvic (Sacral) plexus	Contraction of detrusor muscle	ACh → M2, M3 †	Voiding (micturition) phase
				Inhibition of internal urethral sphincter		
Somatic (voluntary)	-- (deactivated)	Onuf's nucleus (S2-S4)	Pudendal nerve (Sacral plexus)	Relaxation of external urethral sphincter	ACh→ +N ^	

Abbreviations: Ach: acetylcholine; NE: norepinephrine; β3: beta-3 adrenergic receptor; α-1: alpha-1 adrenergic receptor; M2/M3: muscarinic-2 and muscarinic-3 receptor; +N: nicotinic receptor. ^ Contraction of external urethral sphincter is mediated by activation of +N receptors through Ach and is essential during storage phase and urinary continence. † In the bladder, quantitatively M2 type receptors outnumber the M3 type at a ratio of 3:1, although functional affinity is shown to be greater for M3 than it is for M2 receptors.

Somatic innervation of adjacent bladder structures also plays a role in micturition by allowing volitional control to the pelvic floor and external urethral sphincter (Fowler et al., 2008;

Neuroscience, 3rd Ed., 2004). This is facilitated by α-motor neurons arising from Onuf's nucleus that is located in the ventral horn of the sacral spinal cord segments (S2-S4), as these fibers merge to become the pudendal nerve (*Neuroscience, 3rd Ed.*, 2004). The somatic innervation utilizes acetylcholine (ACh) to contract the external urethral sphincter through stimulation of nicotinic receptors, which leads to urinary continence (Fowler et al., 2008).

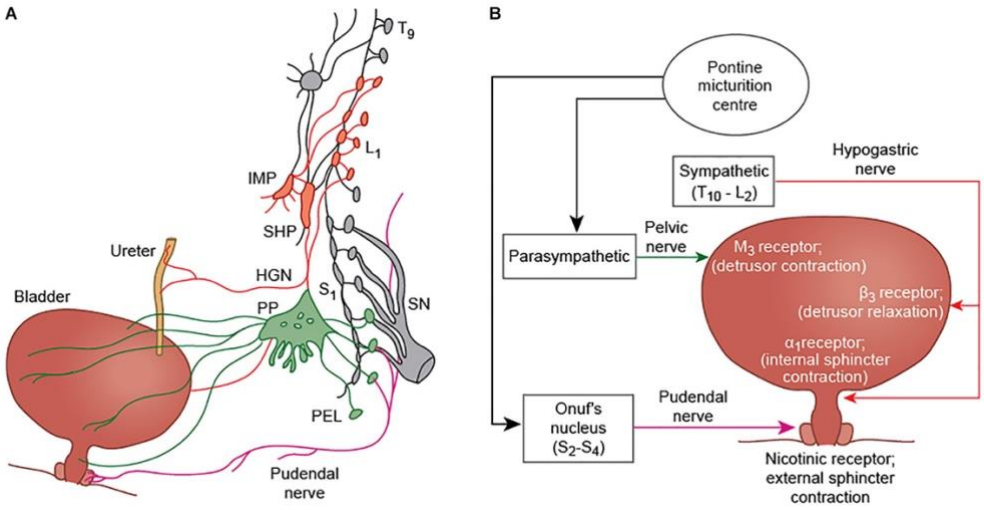

Figure 1.4 Efferent innervation of the bladder, ureter, and external urethral sphincter.

Image from: (H. A. Roy & Green, 2019) (A) Parasympathetic fibers (green) arising from S2-4 nerve roots and Sympathetic fibers (red) arising from T11-L2 segments. Somatic innervation of the external urethral sphincter is via the pudendal nerve and arises from Onuf's nucleus at S2-S4. (B) A simplified schematic showing the major nerves and neurotransmitters involved in bladder control. The pudendal nerve causes contraction of the external urethral sphincter via nicotinic receptors (+N); parasympathetic fibers produce contraction of the detrusor muscle by acting at M3 receptors; and sympathetic innervation inhibits detrusor contraction through β3 receptors, and contraction of the internal urethral sphincter by α1 receptor activation.

1.2 Overactive Bladder Syndrome

In September 2001, the Standardization Subcommittee from the International Continence Society (ICS) defined overactive bladder syndrome (OAB) as: urinary urgency, usually with urinary frequency and nocturia, with or without urgency urinary incontinence (Abrams et al., 2002; Wein & Rovner, 2002) (Table 1.2). Urinary urgency is defined as the complaint of a sudden, compelling desire to void which is difficult to defer, and is a particularly bothersome symptom, even in the absence of incontinence (Shaw & Gibson, 2023; Haylen et al., 2010). Over the past 22 years, the term OAB is still, by definition, an umbrella term that describes an idiopathic symptom complex characterized clinically once other organ diseases, from infectious to neoplastic, have been ruled out (Wein & Rovner, 2002). The definition has been subject to controversy as much of the terminology relating to OAB has been terminated or replaced over the years. Thus, as research expands to provide a better understanding of the pathophysiological mechanisms of OAB, so does the continuum of multifaceted etiological paradigms.

Table 1.2 Symptoms of OAB as defined by AUA/SUFU 2019 Guideline ((Lightner et al., 2019)

Symptom	***Definition***	***Clinical Assessment***
Urinary urgency*	complaint of a sudden, compelling desire to pass urine which is difficult to defer	Subjective
Urgency urinary incontinence	involuntary leakage of urine, associated with a sudden compelling desire to void	Voiding diary / Pad tests
Urinary frequency	8 or more micturition episodes per 24 hours	Voiding diary
Nocturia	complaint of interruption of sleep one or more times because of the need to void per night	Voiding diary

*Urgency is the hallmark symptom of OAB.

1.2.1. Epidemiology and impact on Quality of Life (QoL)

There have been several population studies reporting variability in the prevalence of OAB across the globe, with heterogeneity on OAB criteria and age of population studied (Tahra et al., 2022). The Epidemiology of Incontinence (EPIC) study, which was conducted in five countries including Canada, was one of the largest population-based surveys that studied the prevalence of lower urinary tract symptoms (LUTS) and OAB (Irwin et al., 2008). It demonstrated an overall prevalence of OAB of 11.8% (10.8% in men and 12.8% in women), with overall prevalence of OAB in adults aged 35 years or older in Canada estimated at 18.1% (Irwin et al., 2008; Irwin et al., 2006). Moreover, a more recent study on a Canadian representative adult population found that OAB was experienced by 12.3% of the 1,000 respondents included in the study (Shaw et al., 2020). Moreover, this syndrome has been correlated to aging and in European countries alone, it is estimated that 17% of people over the age of 40 suffer from OAB.(Mansfield et al., 2022; Milsom et al., 2001). Table 1.3 highlights several important population-based studies on LUTS and OAB that have been conducted around the world. The NOBLE study (Stewart et al., 2003), Korean EPIC (Y.-S. Lee et al., 2011), Milsom study (Milsom et al., 2001), the multi-ethnic OAB-POLL study (Coyne et al., 2013), EpiLUTS study (Coyne et al., 2011) , Colombian COBaLT study (Plata et al., 2019), and the Turkish study are all included, demonstrating an overall OAB prevalence ranging from 11.8% to 35.6%. Overall, females are shown to be affected at a higher rate with varying degree of difference from their male counterpart.

Table 1.3 Population-based studies on LUTS and OAB. Adapted from: (Eapen & Radomski, 2016)

	EPIC	NOBLE	EpiLUTS	MILSOM	OAB-POLL	COBaLT	Korean EPIC	Turkish Study
Year published	2006	2003	2011	2001	2013	2019	2011	2014
Location	Canada, Germany, Italy, Sweden, UK	USA	USA	France, Germany, Italy, Spain, Sweden, UK	USA	Colombia	Korea	Turkey
Number of participants	19,165	5,204	20,000	16,776	10,000	1,060	2,000	1,555
Age	≥ 18	≥ 18	≥ 40	≥ 40	18-70	18-89	≥ 18	≥ 18
Survey technique	Telephone	Telephone	Internet	Telephone/direct	Internet	Direct	Telephone	Direct
Overall OAB prevalence (%)	11.8	16.5	35.6	16.6	23.2	31.8	12.2	29.3
Male	10.8	16.0	27.2	15.6	16.4	2.4	10.0	6.1
Female	12.8	16.9	43.1	17.4	30.0	3.9	14.3	23.8
Overall prevalence of UUI (%)	1.5	6.1	24.4	2.0-4.0	-	15.3	3.6	6.5
Male	5.4	2.6	-	-	4.7	8.4	2.9	3.9
Female	13.1	9.3	-	-	14.0	21.5	28.4	8.2

Abbreviations: EPIC: Epidemiology of Incontinence; NOBLE: National Overactive Bladder Evaluation; EpiLUTS: Epidemiology of Lower Urinary Tract Symptoms; OAB-POLL: OAB on Physical and Occupational Limitations; COBaLT: Colombian overactive bladder and lower urinary tract symptoms; UUI: urge urinary incontinence.

Quality of life (QoL)

OAB is a chronic condition that can have detrimental and debilitating effects on the overall quality of life (QoL), severely impacting an individual's physical and social activities as well as leading to significant economic burden (An et al., 2016)**.** Survey-based studies utilizing validated QoL tools (e.g., International Consultation on Incontinence Questionnaire-QoL- ICIQ LUTS-QoL) have documented that individuals with OAB can experience a range of psychological stressors such as shame, anxiety, frustration, and embarrassment (An et al., 2016)**.** Population studies have shown that OAB-wet patients, those with UI, report lower QoL, decreased work productivity, increased sleep deprivation and overall burnout when compared to OAB-dry patients (Coyne et al., 2008; Sexton et al., 2009)**.** Nonetheless, overall severity of symptoms is the single most important factor associated with impaired QoL and motive to seek medical help (Barentsen et al., 2012; Shaw et al., 2020)**.** Lastly, caregivers of patients that experience LUTS symptoms have also identified with overall physical and mental burden as often times it requires significant time and energy expenditure out of their own personal needs (Shaw & Gibson, 2023)**.**

1.2.2. Pathogenesis

The pathophysiology of overactive bladder is yet to be fully understood and is currently supported by various hypotheses that include neurogenic, myogenic, integrative, and afferent theories (Van Rensburg & Cassim, 2018; Gulur & Drake, 2010). Originally, overactive bladder symptoms were presumably caused by ACh-induced involuntary contractions of the detrusor muscle during the filling phase of the micturition cycle (Robinson et al., 2023; Hutchinson et al., 2020). Albeit, only a moderate 64% of individuals who suffer OAB will present with detrusor overactivity (DO) on urodynamic evaluation, and symptoms suggestive of OAB have been reported by approximately

83% of patients with DO (Hashim & Abrams, 2006; Shaw & Gibson, 2023; Robinson et al., 2023). DO can be classified as neurogenic (NDO) or idiopathic DO, which on its own can account for important differential diagnoses for OAB upon clinical assessment.

Neurogenic OAB

The neurogenic hypothesis suggests that damage to the central inhibitory pathways or sensitization of afferent nerves can lead to unmasking primitive voiding reflexes, propagating desynchronized detrusor contractions (Gulur & Drake, 2010). Research utilizing functional brain imaging, such as functional MRI (fMRI) and photon emission computed tomography (PET), has advanced our understanding of complex neural-bladder control mechanisms during functional voiding and filling phases of the bladder. It is well understood that damage in any of these control centers can impair the functional abilities of the bladder, propagating neurogenic voiding dysfunction (Shy et al., 2014; Shaw & Gibson, 2023)**.**

Phenotype-specific etiologic risk factors for OAB have been identified with clear age and gender differences (Gulur & Drake, 2010). Currently, overactive bladder can be classified into OAB-wet and OAB-dry, based on the presence or absence of urinary incontinence (UI) or, like DO, based on etiology such as neurogenic-OAB versus idiopathic OAB (Haylen, De Ridder, et al., 2010). As an individual ages, the list of possible contributing factors grows and therefore creates a multifaceted phenotype of the syndrome, with an overlap between risk factors and differential diagnosis for OAB (Peyronnet et al., 2019) (Table 1.4).

Table 1.4 Risk Factors and Differential Diagnoses for OAB. Adapted from: (K.-S. Lee & Lee, 2007) and (Peyronnet et al., 2019)

Risk factors		**Differential Diagnosis**	
Non-modifiable	Modifiable	Neurological (NDO)	Non-neurological
Age Gender (female) Menopause	- Alcohol - Smoking - Obesity - Caffeine intake - Carbonated beverages - Spicy foods - Bladder stones - Medication side effects (diuretics, anticholinergics, narcotics) - Metabolic syndrome - Pregnancy - Vaginal Delivery	<u>Supraspinal neurologic lesions:</u> - Stroke - Parkinson's disease - Hydrocephalus - Brain tumor - Traumatic brain injury - Multiple sclerosis <u>Suprasacral spinal lesions:</u> - Spinal cord injury - Spinal cord tumor - Multiple sclerosis - Myelodysplasia - Transverse myelitis Diabetic neuropathy	- UTI - Pelvic mass or malignancy - SUI/MUI - Atrophic vaginitis - Bladder or ureteric calculus - Bladder outlet obstruction - Foreign body - Obstructive sleep apnea - Congestive heart failure - IC/BPS - BPH - Pelvic organ prolapse
Phenotypes			
Metabolic Syndrome	Affective disorders	Sex hormone deficiency	Urinary microbiota
Functional gastrointestinal disorders		Autonomic nervous system dysfunction	

Abbreviations: NDO: neurogenic detrusor overactivity; BPH: benign prostatic hyperplasia; UTI: urinary tract infection; IC/BPS: interstitial cystitis/ bladder pain syndrome; SUI: stress urinary incontinence; MUI: mixed urinary incontinence

1.2.3 Diagnosis and Management

Idiopathic OAB is a diagnosis of exclusion. Although the hallmark urodynamic feature is detrusor overactivity (DO), the diagnosis of OAB is solely based on clinical symptoms (Corcos et al., 2017). Given that there is no clear underlying cause of OAB, treatment of this condition remains purely symptomatic, and does not address the problem at the root. Nevertheless, a thorough medical history and physical exam, validated patient questionnaires (ICIQ-LUTS, ICIQ-SF, OAB-SF) and voiding diaries remain the cornerstone of OAB diagnosis (Corcos et al., 2017; Lightner et al., 2019). Additional exams such as urinalysis, ultrasound (US) with postvoid residual volume (PVR), cystoscopy, and urodynamics (UDS) assist in ruling out potential causes of OAB-related symptoms as well as identifying objective voiding dysfunction (Nambiar et al., 2022).

Management

Treatment options follow a stepwise approach from conservative to pharmacological and ultimately surgical management strategies (Corcos et al., 2017) (Table 1.5). Multiple guidelines and consensuses highlight the importance of conservative management prior to second- or third-line treatments. First line conservative management ranges from: bladder training, caffeine restriction, pelvic floor muscle training (PFMT), fluid intake normalization, and management of comorbidities and polypharmacy (Robinson et al., 2023). Other experimental alternatives, such as acupuncture, are currently being evaluated through randomized controlled trials (RCTs) (Hargreaves et al., 2023; Hargreaves et al., 2022).

Oral medications can be given in monotherapy or combination therapy and include selective and non-selective antimuscarinic agents and β_3 agonists. The choice of medication is driven by the side effect profile, patient tolerance, and relative and absolute contraindications to use (Lightner et al., 2019). For example, antimuscarinics can cause a range of side effects including dry mouth, dry

eyes, constipation, blurred vision, dyspepsia, urinary retention, and impaired cognitive function (Robinson et al., 2023). β_3 agonists are reserved for patients who cannot tolerate antimuscarinics, but also carry their own set of side effects (increased blood pressure, nasopharyngitis, UTIs, and urinary retention) (Lightner et al., 2019).

Individuals whose symptoms have failed to improve with lifestyle modification and pharmacotherapy are, in clinical terms, considered to have refractory OAB (Corcos et al., 2017). With careful patient selection and counseling, management can be escalated to interventional treatments such as intravesical botulinum toxin A injections (BoNT-A) or sacral nerve stimulation (SNS) (Lightner et al., 2019). In rare cases, urinary diversion surgeries, such as bladder augmentation cystoplasty, are reserved as a last resort in carefully selected patients (Robinson et al., 2023; Nambiar et al., 2022; Lightner et al., 2019).

Table 1.5 Contemporary treatments for non-neurogenic OAB symptoms in adult women

Conservative		Interventional (Refractory OAB)	
First Line: Lifestyle modifications	**Second Line: Pharmacotherapy**	**Third line: Procedural**	**Surgical Intervention**
- Medication adjustment - Urine containment (e.g., pads) - Caffeine reduction - Fluid management - Weight loss - Smoking cessation - Behavioral therapies - PFMT - Treat constipation/ bowel disorders - Acupuncture *	***Antimuscarinic drugs***	Intravesical botulinum toxin A injection	Augmentation/clam cystoplasty
	- Darifenacin - Fesoterodine - Oxybutynin (oral or transdermal) - Propiverine - Solifenacin succinate - Tolterodine - Trospium chloride	Sacral nerve stimulation (SNS)	Urinary diversion (+/- cystectomy)
		PTNS	
	β_3-adrenoreceptor agonists		
	- Mirabegron - Solabegron ^ - Vibegron ^ - Ritobegron ^		

Abbreviations: PFMT: Pelvic floor muscle training; PTNS: Percutaneous posterior tibial nerve stimulation. * The use of acupuncture in OAB-related symptoms is currently being evaluated in RCTs for validation of use in the clinical setting. ^ Solabegron Ritobefron, and Vibegron are currently under clinical development.

1.3 Exploratory biomarkers in OAB

Current diagnostic work-up relies on imaging and functional biomarkers. As phenotyping patients with OAB purely based on symptoms is insufficient, the search of chemical biomarkers to reveal the underlying pathology and determine the presence of OAB has detected that urinary neurotrophins, cytokines, prostaglandins, and microbiota appear dysregulated in patients with OAB (Fry et al., 2014). For example, normalized level of urinary ATP detected by luciferin-luciferase were significantly increased in women with OAB in the presence of DO (Silva-Ramos et al., 2013). Additionally, stretch-mediated release of ATP and other metabolites from the

urothelium in OAB patients, such as intermediates of the Kreb's cycle, may represent changes in energy utilization and hypoxia (Mossa et al., 2020). Other lines of research study OAB-specific gene expression and microRNA profiles hopes to identify unique genetic and molecular markers which could potentially lead to future development of gene therapies for OAB (Cammisotto et al., 2022; Fırat et al., 2019; Fırat et al., 2020).

1.3.1 Urinary metabolomics and neurotrophins in the context of voiding dysfunction

The use of biomarkers in a context of phenotyping and theranostics can be applied to individualized management according to OAB phenotype by means of urinary metabolomics. The non-invasive nature of urine collection, as well as its stable homeostatic condition, makes urinary metabolomics advantageous over other forms of body-fluid analysis. Urinary metabolomic and proteomic assays can provide a qualitative and quantitative overview of peptides and low molecular weight (less than 2000 Da) metabolites present in urine, that may be rapidly eliminated from the bloodstream (Bouatra, Aziat, Mandal, Guo, Wilson, Knox, Bjorndahl, Krishnamurthy, Saleem, Liu, et al., 2013). Several urinary analyses of metabolomics in patients with OAB have been published in recent years, with success in identifying target metabolites, highlighting pathogenic cellular pathways (Kira et al., 2022; Mossa et al., 2020; Tellechea et al., 2022). Nonetheless, there is yet to be a systematic review published on the combined findings.

Urinary Neurotrophins

Neurotrophins (NTs), or neurotrophic factors, are essential for neural plasticity, development, and maintenance of the nervous system. Nerve growth factor (NGF) is the most studied in voiding dysfunction, followed by brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (Shimizu et al., 2023; Tsiapakidou et al., 2021).

Alterations to the afferent limb of the micturition reflex, including bladder afferent neurons in the dorsal root ganglia (DRG) and urothelial cells in the bladder wall mucosa have been associated with OAB (Gonzalez et al., 2014). The functional bladder urothelium hosts nerves and inflammatory cells, capable of releasing neurotrophins as soluble factors that are activated via specific neurotrophin receptors: three isoforms of tropomyosin receptor kinase (Trk) family (TrkA, TrkB, TrkC), and neurotrophin receptor ($p75^{NTR}$) (Keefe et al., 2017). Each receptor presents with specific binding affinities depending on the neurotrophic factor present and activate both regenerative and apoptotic signalling pathways. All NTs are synthesized in premature forms or pro-neurotrophins (proNTs) (Keefe et al., 2017). Specifically, $p75^{NTR}$ receptors bind with low affinity to all pro-neurotrophins through sortilin (Gabryelska et al., 2023). Once this complex is activated, it operates in an antagonistic manner to cell survival and growth, triggering several pro-apoptotic cascades such as Jun N-terminal (JNK) kinases, Bak, and Fas-L via the activation of p53 (Keefe et al., 2017). On the other hand, the activation of mature neurotrophin/Trk complexes in the neuronal cell bodies activate a signal cascade propagating: target organ dysfunction, neurotransmitter phenotype, synaptic reorganization, and increased synaptic efficacy (MacInnis & Campenot, 2002) (Figure 1.5).

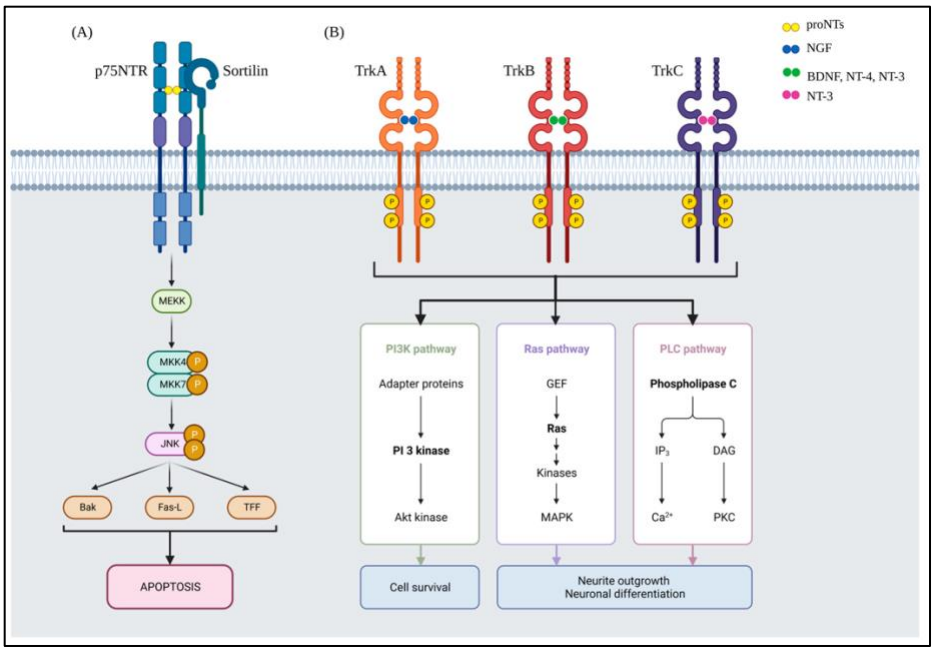

Figure 1.5 Neurotrophins, their receptors, and associated signaling cascades. The neurotrophins forgo specific interactions with three isoforms of tropomyosin receptor kinase (Trk) family. Mature neurotrophins bind with their respective receptors to activate transcription, pro-survival, and pro-differentiation of genes. NGF binds TrkA, BDNF and NT-4 bind TrkB, and NT-3 binds TrkC. Through sortilin, all pro-neurotrophins bind to p75 neurotrophin receptor ($p75^{NTR}$), activating pro-apoptotic cascades. Image from (Gabryelska et al., 2023).

Elevated levels of urinary neurotrophins have been found in women with LUTS, such as IC/BPS and OAB, which may underlie or contribute to pathological bladder activity and afferent cell hyperexcitability (Gonzalez et al., 2014). A systematic review and meta-analysis that included twelve studies evaluated the levels of urinary neurotrophins where it found that NGF, BDNF to creatinine ratio and NGF to creatinine ratio were all increased in female OAB patients when compared to healthy controls (Tsiapakidou et al., 2021a). Additionally, it has been shown that male patients with moderate to severe LUTS related to benign prostatic hyperplasia (BPH) present with elevated urinary BDNF levels when compared to controls, proving to be a promising biomarker

for the diagnosis of DO in male patients with BPH (L. Wang et al., 2017). Other studies utilizing neurotrophins as biomarkers have focused on OAB related LUTS and DO in the pediatric population, with conflicting results (Stemberger Maric et al., 2022; Deng et al., 2019)**.** Several limitations regarding the differences between results across the literature predominantly focus on study design, incomplete raw data, variable reliability of commercially available ELISA kits, and lack of extensive clinical studies (Deng et al., 2019; Tsiapakidou et al., 2021a)**.**

Current data proposing NTs as biomarkers for voiding dysfunction lacks proper validation, showing conflicting and contradicting results and heterogeneity across the methodology with limited clarification in the biomarker effect. Although the mechanistic effects of NTs in bladder function are being clarified through basic and translational research, more studies are warranted to adapt these biomarkers in the clinical setting.

Chapter 2: Literature Review

In the following sections, I will elucidate the intricate association between neurotrophins, their signaling cascades, and the manifestation of voiding dysfunction. Specifically, my emphasis will be on the domain of overactive bladder syndrome (OAB) and the pivotal role that urinary biomarkers play therein. This comprehensive literature review aims to furnish contemporary evidence pertaining to the involvement of brain-derived neurotrophic factor (BDNF) and matrix metalloproteinase-9 (MMP-9), highlighting the underlying cellular mechanisms that could potentially be targeted for precision diagnosis, tailored therapeutics, and objective quantification of treatment efficacy.

2.1 Urinary Metabolomics and Neurotrophins

Urinary metabolomics is a rapidly emerging field that focuses on the comprehensive analysis of small molecules in urine to gain insights into various physiological and pathological conditions both outside and within the domain of urology. In the context of voiding dysfunction, urinary metabolomics has shown promise in identifying potential biomarkers and unraveling the underlying mechanisms involved in bladder dysfunction. For example, a recent study demonstrated that OAB female participants display increased urinary levels of metabolic stress intermediates and inflammatory markers that correlate significantly and specifically with the severity of OAB symptoms (Mossa et al., 2020). Additionally, neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), have been implicated in the regulation of bladder function in both physiological and pathological states, linked to conditions such as overactive bladder (OAB), bladder outlet obstruction, diabetic voiding dysfunction, and

interstitial cystitis/bladder pain syndrome (IC/BPS) (Frias et al., 2011; (Antunes-Lopes et al., 2013; Mossa et al., 2021; Steers & Tuttle, 2006).

2.1.1 Brain-derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that plays a crucial role in the growth, development, and plasticity of neurons as well as synaptic remodeling during cognitive processes (Carlino et al., 2013; Colucci-D'Amato et al., 2020). It is the most abundant neurotrophin in the developing and adult human brain and the central nervous system (CNS) but can also be found in peripheral tissue, where it has been shown to control many aspects of survival, development, and function of peripheral neurons (Reichardt, 2006; Sánchez-García et al., 2023; Satoh et al., 2001; Cunha, 2010). In circulation, BDNF can be found in lymphocytes and monocytes but is primarily secreted by platelets as a response to coagulation processes, with concentrations reaching up to 1,000 fold than those found in nerve tissue (Boukhatem et al., 2021; Savic et al., 2022).

BDNF has been implicated in various pathological conditions, where differences in concentration levels have been observed in individuals with substance use disorders, major depressive disorder, and traumatic brain injury (Valério et al., 2022; Paoli et al., 2022; Do et al., 2022). Other neuropathologies linked to decreased BDNF levels and signaling are neurodegenerative disorders, such as: Alzheimer's disease, Parkinson's disease, Huntington's disease, and neuropsychiatric diseases such as schizophrenia (Cunha, 2010; Sánchez-García et al., 2023; Bianchi and Siena, 2016; Stern et al., 2008). In the preclinical stages of AD, both the precursor BDNF molecule (proBDNF) and mature BDNF (mBDNF) levels are decreased in the parietal cortex, suggesting that the decrease in BDNF occurs early in the progression of cognitive decline (Peng et al., 2005). Animal model studies have highlighted that different forms of learning and memory require either

proBDNF or mBDNF (Carlino et al., 2013). The balance between proBDNF and mBDNF is crucial for physiological as well as pathological conditions. The diverse effects of BDNF are mediated through the p75 neurotrophin receptor ($p75^{NTR}$) receptor, which binds to the proBDNF form (Angoa-Perez et al., 2017).

2.1.2 BDNF synthesis and signalling pathways: the proBDNF and mBDNF isoforms

BDNF is initially synthesized as a precursor called proBDNF. It is then converted to mBDNF through post-translational processing and cleaved by several proteases, plasmin, furin, MMP-9, MMP 3/7 and proconvertase enzyme (PC1/3), depending on the host cell type (Angoa-Pérez et al., 2017; Mowla et al., 2001; Thomas et al. 2016). The human BDNF gene is located on chromosome 11p13-14 and is composed of various noncoding exons and a single coding exon (Notaras & Van Den Buuse, 2019).

Mature BDNF and signaling pathways involve the activation of tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC) and $p75^{NTR}$ (Reichardt, 2006). Each of the four mammalian neurotrophins, including BDNF, has been shown to activate one or more of these receptors as previously highlighted in Chapter 1. Through Trk receptors, BDNF activates Ras, phosphatidylinositol-3 (PI3)-kinase, phospholipase C-gamma1, and signaling pathways controlled by these proteins, such as the MAP kinases (Reichardt, 2006). Activation of $p75^{NTR}$, through the complex formation of the proBDNF peptide driven by sortilin, results in the activation of the nuclear factor-kappaB (NF-kappaB) and Jun kinase, as well as other signaling pathways that lead to apoptosis (Reichardt, 2006; Notaras & Van Den Buuse, 2019). Ultimately, homeostasis can only be sustained by maintaining the appropriate balance between the two isoforms as these elicit opposing biological effects.

2.1.3 Genetic Polymorphisms

Over one hundred polymorphisms have been linked to the BDNF gene, and the understanding of how that translates to the functional pathways is still being evaluated. Val66Met is the single nucleotide polymorphism of main interest, linked to abnormal synaptic connections, and is currently paving way to BDNF-targeted gene and cellular therapies (Notaras & Van Den Buuse, 2019).

2.1.4 The balance of mBDNF and proBDNF in bladder pathology

The balance between mBDNF and proBDNF is important for healthy bladder physiology (Carlino et al. 2012). BDNF has been shown to play a role in the regulation of bladder function, including bladder contraction and relaxation, as well as the maintenance of bladder epithelial integrity (Ochodnicky et al., 2012). Studies have demonstrated that BDNF is expressed in the bladder urothelium and detrusor muscle, making it a myokine as it can be released from the muscle and act on nerve terminals as a retrograde messenger (Bogacheva et al., 2022). BDNF levels are regulated by various factors, including nerve growth factor (NGF) and inflammation. Alterations in the balance between mBDNF and proBDNF are associated with bladder dysfunction, such as OAB and interstitial cystitis/bladder pain syndrome (IC/BPS) (Kim, 2018; Covarrubias et al., 2023)**.**

Animal studies have also demonstrated that modifications on BDNF expression are involved in the control of bladder contraction and relaxation, as well as the sensation of bladder fullness (Reynolds, Dmochowski, et al., 2016). The changes in mBDNF/proBDNF levels have been specifically associated with alterations in bladder sensory signaling, increased bladder sensitivity, and pain (D. S. Kim, 2018). There are several intracellular and extracellular proteases that regulate

the expression of BDNF isoforms, including furin, prohormone proconvertases PC1, PC2, and PC7, plasmin, and matrix metalloproteinases (MMPs) (Arévalo and Deogracias 2023) (Figure 2.1). Of specific interest, MMP-9 cleavage of pro-BDNF into mature BDNF may play a key role in the development of several diseases that involve dysregulation of physiologically active BDNF, including voiding dysfunction (Reinhard et al., 2015). Understanding these changes can provide insights into the underlying mechanisms of bladder dysfunction and may lead to the development of novel therapeutic strategies for these conditions.

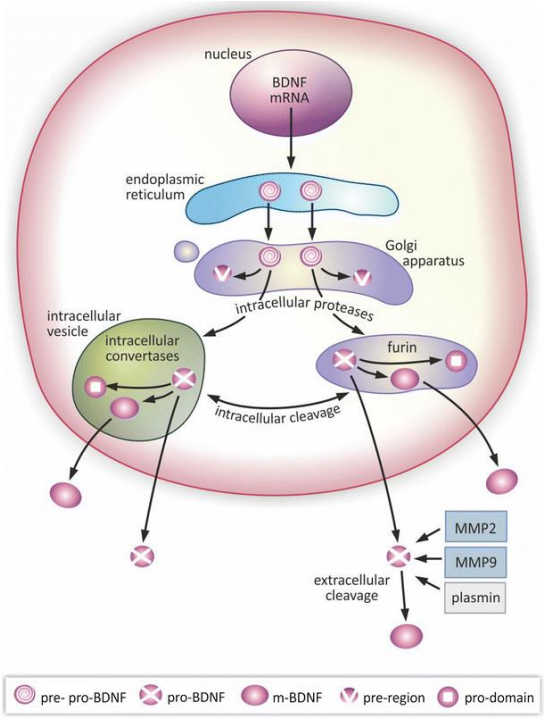

Figure 2.1 Schematic representation of BDNF synthesis and maturation. Several intra and extracellular stages account for BDNF synthesis. In the intracellular pathway, the pre-pro-BDNF precursor sequence is produced in the endoplasmic reticulum and transported to the Golgi apparatus. During intracellular cleavage, the pre-region sequence is removed, resulting in formation of immature pro-BDNF. After the removal of the pro-domain sequence, the mature isoform of BDNF (m-BDNF) is produced. Intracellular cleavage leading to formation of m-BDNF

also occurs in intracellular vesicles, allowing transport of this neurotrophin to axonal terminals and subsequent release into the extracellular space, via presynaptic membrane. Processing of BDNF is accomplished by intracellular proteases, regulated convertases, and furin. As a result, both pro-BDNF and m-BDNF isoforms are released into the extracellular space. In the extracellular pathway, pro-BDNF released into the extracellular space is processed by metalloproteinases 2 and 9 (MMP-2 and MMP-9), plasmin, and extracellular proteases. Consequently, functionally effective isoforms of m-BDNF and pro-BDNF can be found in the extracellular space. (Figure and description from: Kowiański et al. 2018)

2.2 Matrix Metalloproteinases: MMP-9 Enzymatic Activity

Matrix metalloproteinases (MMPs) are a group of zinc-containing endopeptidases that degrade the components of the extracellular matrix (ECM), such as collagen laminin and fibronectin (Shahzad et al., 2023). These enzymes are essential for proper tissue remodeling during would healing, playing a crucial role in the remodeling and degradation of the ECM, the complex network of proteins that provides structural support to tissues and organs. MMP-9, also known as matrix metalloproteinase-9 (collagenase type IV; gelatinase B) is one of the most widely investigated MMPs due to its established role in carcinogenesis, inflammation, tissue remodeling, tumor metastasis, Alzheimer's disease, diabetic retinopathy, epilepsy, and most recently COVID-19 (Charzewski et al., 2021; Shahzad et al., 2023). MMP-9 specifically can break down and degrade various components of the ECM, including collagen types IV, V, and VII, gelatin, and elastin. It is produced and released by various cell types, including neutrophils, monocytes, macrophages, fibroblasts, and epithelial cells , such as bladder urothelium (Charzewski et al., 2021).

Due to its involvement in the various pathological conditions previously described, MMP-9 has gained attention as a potential biomarker and therapeutic target. Its levels and activity can be

measured in biological samples, such as blood, tissue, or bodily fluids, to assess disease progression or response to treatment. Inhibition of MMP-9 activity has been explored as a therapeutic strategy in certain diseases characterized by excessive ECM degradation and tissue damage (L. Kumar et al., 2022).

2.2.1 MMP-9 synthesis: proMMP-9, transcription, and translation

MMP-9 is initially secreted as an inactive pro-form called proMMP-9, particularly by neutrophils. Subsequently, the proenzyme undergoes activation mediated by proteases, stromelysin-1/MMP-3, cathepsins, and plasmin, cleaving off the inhibitory pro-peptide and producing the active form of MMP-9 (Y. Wang et al., 2022). To avoid widespread tissue damage and hampered homeostasis, MMP-9 enzymatic activity is carefully controlled and regulated by tissue inhibitors of matrix metalloproteinases (TIMPs 1-3)(Brew & Nagase, 2010; Y. Wang et al., 2022)**.** MMP synthesis is dependent on zinc as it regulates gene expression through epigenetic modifications. Zinc deficiency has been shown to contribute to changes in the MMP-9 chromatin state and therefore the epigenetic transcriptional regulation of the MMP-9 gene (Nosrati et al., 2019; Shahzad et al., 2023).

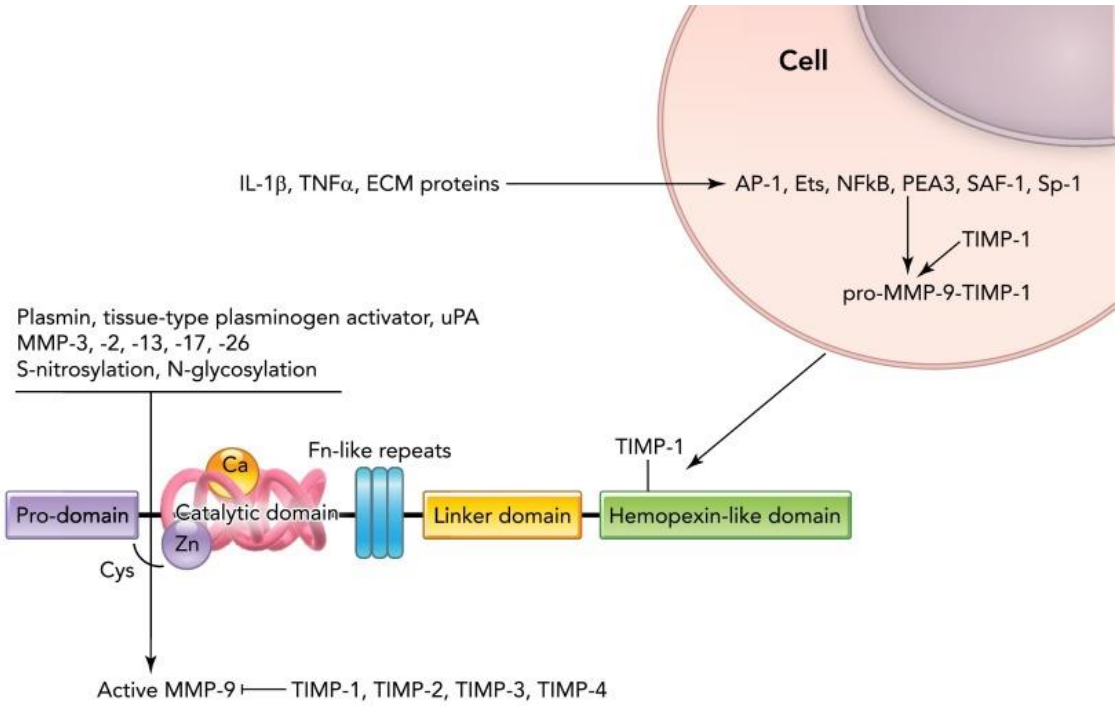

Figure 2.2 MMP-9 structure and factors regulating MMP-9 transcription and translation

At the transcriptional level, MMP-9 is positively regulated by multiple factors, including Ets, NF-κB, PEA3, AP-1, Sp-1, and SAF-1. Abbreviations: Ets, E-26 transcription factor; NF-κB, nuclear factor κB; PEA-3, polyomavirus enhancer A-binding protein-3; AP-1, activator protein 1; SAF-1, serum amyloid A-activating factor 1; HSP60, heat shock protein 60; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase plasminogen activator. Figure and description from: (Yabluchanskiy et al., 2013)**.**

2.2.2 Factors controlling MMP-9 activity

The activity of MMP-9 is controlled by various factors. According to Loffek et al., the catalytic function the MMP enzymes is rigorously regulated across four levels: (1) control of gene expression involving both transcriptional and post-transcriptional mechanisms (Figure 2.1), (2) management of extracellular localization and the particular tissue or cell types where MMPs are released (known as compartmentalization), (3) activation of pro-enzymes through the removal of

their pro-domains (4) inhibition via specific inhibitors, such as TIMPs, and non-specific proteinase inhibitors, like α2-macroglobulin. Once MMPs become active, they can influence the overall proteolytic potential in the extracellular environment through processes such as zymogen activation (conversion from the MMP pro-form) and the degradation or inactivation of other proteases (Loffek et al., 2011).

2.2.3 MMP-9 in neurotrophin synthesis and voiding dysfunction

The MMP-9 and neurotrophin interaction is complex due to their pleiotropic nature and has been shown to be dynamic throughout the course of disease (Li et al., 2022). In the context of voiding dysfunction, standalone MMP-9 has been implicated in conditions such as neurogenic bladder dysfunction (A. Kumar et al., 2018) and dysfunctional voiding (Pohl et al., 2002). The study by Kumar et al. (2018) investigated voiding dysfunction in patients with traumatic brain injury and found that it can occur secondary to impaired cognitive or behavioral functioning, brain damage, associated spinal cord injury, and direct bladder trauma. Dysfunction voiding, on the other hand, is characterized by impaired bladder emptying and a lack of coordination between the sphincter and detrusor during emptying (Everaert et al., 2000; Pohl et al., 2002). Additionally, a study by (C. Zhang et al., 2020) demonstrated that an enlarged prostate in old rats may lead to complications associated with voiding dysfunction involving ionotropic P2X2/3-type purinergic receptors, which are known to upregulate the release of MMP-9. This suggests a potential role of MMP-9 in the regulation of neurotrophin signaling in the context of male voiding dysfunction.

In summary, MMP-9 enzymatic activity is involved in the pathogenesis of voiding dysfunction. Its synthesis is regulated at the translational level, and factors such as inflammatory cytokines can control its activity. Increased MMP-9 activity has been associated with various pathologies,

including neurogenic bladder dysfunction and dysfunctional voiding (Mossa et al., 2020). Furthermore, MMP-9 may play a role in neurotrophin synthesis, which is important for the development and function of the nervous system. MMP-9/BDNF ratio was recently assessed in a patient population with COVID-19, linking these mediators with stages of disease development where they found that the MMP-9/BDNF ratio was significantly depleted in severely as well as in ill patients, compared to the mild group (Savic et al., 2022)**.** Understanding the mechanisms underlying MMP-9 activity and neurotrophin regulation in voiding dysfunction can provide insights into potential therapeutic targets for the management of OAB.

Chapter 3: Hypothesis and Objectives

3.1. Rationale: BDNF in Overactive Bladder Syndrome (OAB)

A focused evaluation of individuals with OAB could help classify them according to different aetiologies and potentially predict response to certain treatments. In reference to our recently published pilot study, we performed urinary metabolomic analysis of 40 female patients (20 controls, 20 OAB) aiming to identify biomarkers of OAB, and consequently improving treatment and prognosis. We found that increased levels of urine metabolites (malate, fumarate and α-hydroxyisobutyrate) as well as nerve growth factor (NGF) proteolysis imbalance were significantly predictive of OAB severity (Mossa et al., 2021). These findings have prominent repercussions by providing potential new diagnostic tools, biomarkers and pharmacological molecules targeting specific mitochondrial enzymes. Having said that, we aim to expand this study to include other exploratory biomarkers, such as brain-derived neurotrophic factor (BDNF) and interacting molecules that share its cellular pathways. Current reports debate the utilization of BDNF as a biomarker for OAB due to contradicting conclusions, so we challenged this concept by focusing the light on the significance of proBDNF/BDNF imbalance rather than BDNF levels alone.

The data reproduced will be coupled with an expanded analysis of urinary metabolite markers which will help overcome the effect of variability in the data and improve statistical inferences, therefore increasing reliability of our overall findings.

3.2. Hypotheses

3.2.1 Hypothesis 1: BDNF in OAB

We hypothesize that altered levels of urinary and /or plasma inflammatory/oxidative markers, such as neurotrophins and anaerobic metabolites, could be successful biomarkers of OAB severity and diagnosis.

3.2.2 Hypothesis 2: The role of natriuretic peptides in neurotrophin synthesis

We hypothesize that natriuretic peptides play a role in cellular biosynthetic pathways unique to neurotrophins, thus indirectly affecting voiding function within the bladder itself.

3.3 Objectives: BDNF in OAB

3.3.1 Primary Objective

Our primary objective is to determine changes in BDNF and proBDNF levels in the urine samples of female patients with OAB, and to relate these data to transcriptional and translational systems.

3.3.2 Secondary Objectives

1. Establish correlation between plasma markers and urine profiles of both OAB and control groups.

2. Assess the feasibility of utilizing levels of neurotrophins as a biomarker for OAB in an aging population.

Experimental Design 1: BDNF in OAB

After obtaining approval by the local ethics committee (Project 2016-328, 15-022 approved on June 2017 by the CIUSSS West-Central Montreal Research Ethics Board) and informed consent from all participants, a total of 40 female participants between 50 and 80 years-old were recruited to the study. 20 patients as controls (without OAB) and 20 patients diagnosed with OAB (with or without treatment). Study recruitment was active from 2017-2021, done at the Urology Department of the Jewish General Hospital, Montreal, Canada. Participants were required to withhold all OAB treatments (anticholinergic and beta-3 agonists) for at least 3 weeks before the sample collection. A routine negative screening urine culture to exclude urinary tract infection (UTI) was also performed. (Figure 3.1) The control subjects (20) group were normal volunteers or patients attending the same clinic who had no urinary symptoms, no current or prior use of OAB medications, and a negative urine test for any infection. Exclusion criteria were as follows: established diabetes mellitus, history of malignancies or pelvic radiotherapy, pelvic organ prolapse, urinary tract infection, neurogenic lower urinary tract dysfunction, and hepatic or renal impairment (creatinine clearance <70 mL/min). All patients were interviewed in person. Urine sample handling was done at the Lady Davis Institute for Medical Research (LDI). Blood samples were collected and handled by the hospital. Data processing and statistical analysis was done using IBM SPSS version 23.0 software.

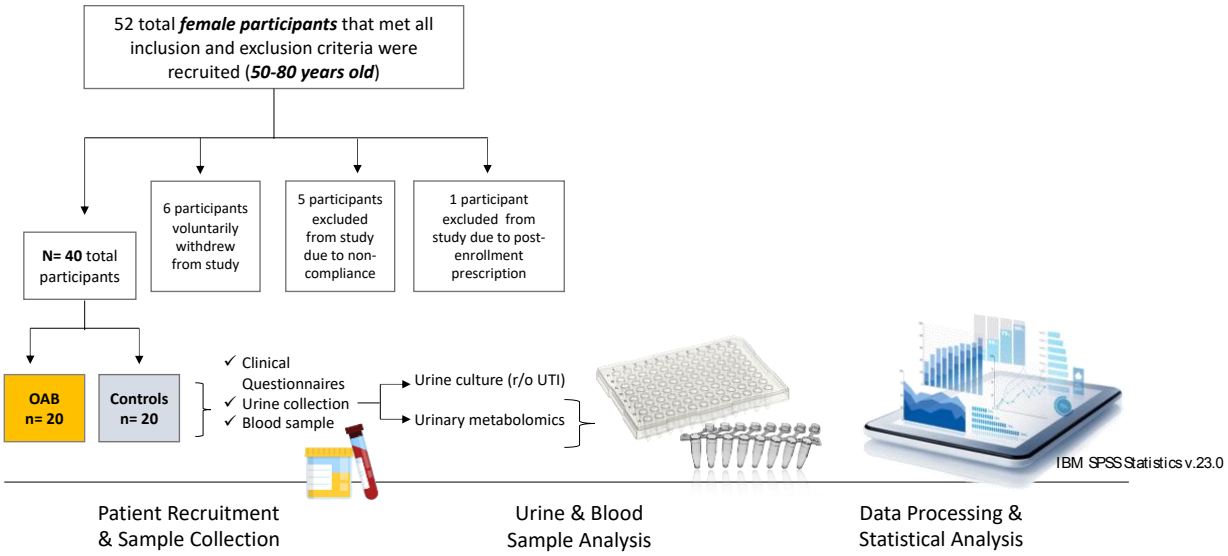

Figure 3.1 Timeline of experimental design: BDNF in OAB. A prospective cohort study was carried out from 2017-2021. Urine samples were frozen at -80°C until processing initiated, limiting freeze-thaw cycles. Sample analysis, experiments, and data processing was done consequently.

3.3.2 Objective 2: Natriuretic peptides in neurotrophin synthesis

Our primary objective was to examine how natriuretic peptides A, or atrial natriuretic peptide, (ANP) and brain natriuretic peptide (BNP), known to control neurotrophin secretion in other tissues, may be involved in neurotrophin synthesis in rat bladder cells in vitro. (In the context of the clinical data observed in our OAB cohort).

Experimental Design 2

Urinary ANP levels were measure in our clinical cohort and found to be significantly increased in the OAB group when compared to controls. Primary culture of female Sprague Dawley rat urothelial (UROs) and smooth muscle cells (SMCs) were incubated for 24 hours with either ANP or BNP. Gene expression was measured by RTqPCR. MMP-9 levels were assessed by immunoblotting and enzymatic kits and neurotrophin ones by ELISA kits. CrisCas-9 was used to target MMP-9 genomic sequence. Figure 3.2 highlights the experimental design timeline of the *in vitro* study.

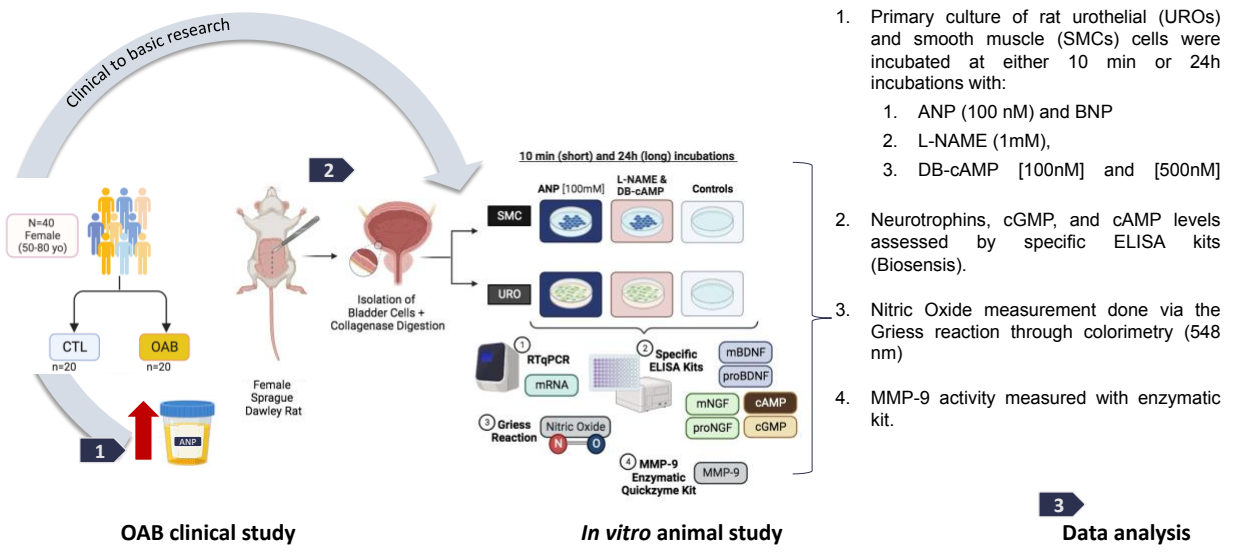

Figure 3.2 Timeline of experimental design for *in vitro* study. Urinary Atrial Natriuretic Peptide (ANP) levels were significantly elevated in the OAB cohort, which led to an in vitro study arm to assess the role of ANP in bladder cells.

Chapter 4: Decrease in the Ratio proBDNF/BDNF in the Urine of Aging Female Patients with OAB

This chapter includes work recently published in *Metabolites. (June 2023)*

Here we investigated the urinary levels of BDNF in an aging female population with OAB.

Contribution of authors: Conceptualization, Philippe Cammisotto, Samer Shamout and Lysanne Campeau; methodology, Claudia Covarrubias and Philippe Cammisotto; validation, Claudia Covarrubias, Philippe Cammisotto, Samer Shamout and Lysanne Campeau.; formal analysis, Claudia Covarrubias, Philippe Cammisotto, and Lysanne Campeau.; investigation, Claudia Covarrubias, Philippe Cammisotto, Samer Shamout and Lysanne Campeau; resources, Lysanne Campeau; data curation, Claudia Covarrubias, Philippe Cammisotto, Samer Shamout and Lysanne Campeau; writing—original draft preparation Claudia Covarrubias and Philippe Cammisotto; writing—review and editing, Claudia Covarrubias, Philippe Cammisotto, Samer Shamout and Lysanne Campeau; supervision, Philippe Cammisotto and Lysanne Campeau ; project administration, Lysanne Campeau ; funding acquisition, Lysanne Campeau. All authors have read and agreed to the published version of the manuscript.

Decrease in the Ratio proBDNF/BDNF in the Urine of Aging Female Patients with OAB

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Abstract

Imbalance in the levels of neurotrophins, growth factors crucial in the development, function, and survival of neurons is commonly observed in many pathological states. Concentrations of brain-derived neurotrophic factor (BDNF) and its precursor (proBDNF) were measured in the urine of a cohort of aging female patients with overactive bladder disease (OAB). When reported to creatinine, levels were similar between OAB patients and healthy controls. However, the ratio proBDNF/BDNF was significantly decreased in the OAB group. Receiver operating characteristic (ROC) curve analysis of the ratio proBDNF/BDNF displayed a good diagnostic value for OAB (AUC = 0.729). Clinical questionnaires of symptom severity (OABSS and IIQ-7) were negatively correlated with this ratio. On the other hand, microRNAs (miRNA) involved in proBDNF gene translation were expressed at comparable levels between groups. However, urinary enzymatic activity of matrix metalloproteinase-9 (MMP-9), the enzyme that cleaves proBDNF into BDNF, was increased in OAB compared to controls. Levels of miR-491-5p, the main miRNA that downregulates MMP-9 synthesis, were greatly decreased in urine from OAB patients. These results suggest that the ratio proBDNF/BDNF could be useful in the phenotyping of OAB in an aging population, and the difference could originate from enhanced MMP-9 enzymatic activity rather than translational control.

Keywords: BDNF; proBDNF; urine; overactive bladder; proteases; MMP-9; microRNAs

Introduction

Overactive bladder (OAB) is defined by the International Continence Society (ICS) as a syndrome of urinary urgency, frequency and nocturia, in the absence of urinary tract infection (UTI) or other obvious pathology. Urgency, the hallmark symptom of OAB, is described as the "complaint of a sudden, compelling desire to pass urine which is difficult to defer" (Haylen et al. 2010; Lightner et al. 2019) and can accompany or precede incontinence (urgency urinary incontinence), characterized as involuntary leakage of urine. OAB can be sub-categorized as dry (OAB-dry), meaning without incontinence, or wet, with incontinence (OAB-wet) (Haylen, Ridder, et al., 2010).

The overall prevalence of OAB is estimated at 10–12% within the adult population, with increasing rates with aging (Irwin et al., 2011; Irwin, Milsom, Hunskaar, Reilly, Kopp, Herschorn, Coyne, Kelleher, Hampel, & Artibani, 2006; Reynolds, Fowke, et al., 2016). Additionally, the current literature describes women having a higher prevalence of OAB-wet when compared to their male counterpart (Al Edwan et al., 2020; Irwin et al., 2011; Reynolds, Fowke, et al., 2016). Through population-based studies, the increase in the prevalence of OAB in women has been linked to several factors including advanced age, menopause, marital status, increased body mass index (BMI), and high parity rates (Al Edwan et al., 2020; Irwin, Milsom, Hunskaar, Reilly, Kopp, Herschorn, Coyne, Kelleher, Hampel, & Artibani, 2006; Y. Wang et al., 2011). Altogether, the symptoms of OAB have detrimental effects on psychosocial functioning and overall quality of life (QoL) (Reynolds, Fowke, et al., 2016). There is no clear etiology of non-neurogenic OAB; rather, it is likely a manifestation of several contributing factors. Treatment modalities primarily provide symptomatic relief as there is currently a lack of curative treatment (Lightner et al., 2019).

Behavioral therapy is recommended as first-line treatment, followed by second-line treatment with oral pharmacotherapy, which includes antimuscarinics and β3-adrenoceptor agonists (Lo et al., 2020). Refractory OAB or intolerable medication side effects can warrant third-line therapeutics such as intravesical OnabotulinumtoxinA, sacral neuromodulation, or peripheral tibial nerve stimulation (Lightner et al., 2019; Lo et al., 2020).

Over the past several years, various hypotheses have been proposed to explain the pathophysiological mechanisms and identify clinical subtypes of OAB (Peyronnet, Mironska, Chapple, Cardozo, Oelke, Dmochowski, Amarenco, Gamé, Kirby, & Aa, 2019). It is well documented that bladder function, such as urine storage and voiding, is controlled by the peripheral and central nervous systems, relying on the interconnection between the autonomic and somatic nervous systems (N. Yoshimura et al., 2014). Neurotrophins are a class of growth factors that were originally found in the nervous system where they promote growth and survival of neurons, directly regulating nerve ending activity (Gibon & Barker, 2017). In particular, brain-derived neurotrophic factor (BDNF) is synthesized by many cell types outside of the nervous system (e.g., megakaryocytes, neurons, endothelial cells) including bladder smooth muscle cells and constitutes the most abundant neurotrophin in the human body (Lommatzsch et al., 1999). Mature BDNF originates from a precursor (proBDNF) after proteolytic cleavage (R. Lee et al., 2001). Mature BDNF binds preferentially to high-affinity receptor tropomyosin receptor kinase B (TrkB), to promote neuro-regeneration and axonal growth. On the other hand, proBDNF binds the proinflammatory/apoptotic p75 neurotrophin receptor ($p75^{NTR}$), triggering inflammation and apoptosis (Allen & Dawbarn, 2006; Coelho et al., 2019). The ratio between mature BDNF and proBDNF determine the balance between survival and apoptotic cellular pathways and depends upon the expression of several intracellular and extracellular metalloproteinases (MMPs) and

convertases (R. Lee et al., 2001). In particular, the metalloproteinase MMP-9 promotes extracellular proBDNF conversion into mature BDNF (mBDNF) (Kuzniewska et al., 2013). Both neurotrophins have been proposed to be markers of OAB (Mossa et al. 2021).

OAB is a clinical diagnosis where severity is quantified with patient reported outcomes (Lightner et al., 2019). Identifying the different phenotypes of OAB according to their underlying causative factors could possibly highlight therapeutic targets. Given the aforementioned, it is necessary to develop a non-invasive, objective, valid and reproducible test for the diagnosis, phenotyping and therapeutic targeting of OAB. Herein, we propose the use of urinary neurotrophins and associated metalloproteinases as biomarkers for OAB in an aging female population. The objectives of our study were to: (1) measure the ratio proBDNF/BDNF to improve the phenotyping of OAB and (2) determine changes in the concentrations of microRNAs and proteins associated with these neurotrophins to understand the molecular mechanisms of the underlying pathophysiology.

Materials and Methods

Patient Profiles

Participants in the OAB group were women aged between 50 and 80 years-old (n = 20) who were diagnosed with OAB (with or without treatment) and were recruited at the urology department of the Jewish General Hospital, Montreal, Canada. Their symptoms include urinary frequency and urgency, with or without urge incontinence, for at least 3 months. They also were required to withhold all OAB treatments (anticholinergic and beta-3 agonist) for at least 3 weeks before the sample collection. A routine negative screening urine culture to exclude urinary tract infection (UTI) was also performed. The control subjects (20) group were normal volunteers or patients attending the same clinic within the same age group (50–80 years old) who had no urinary symptoms, no current or prior use of OAB medications, and a negative urine test for any infection.

Exclusion criteria were as follows: established diabetes mellitus, history of malignancies or pelvic radiotherapy, pelvic organ prolapse, urinary tract infection, neurogenic lower urinary tract dysfunction, and hepatic or renal impairment (creatinine clearance <70 mL/min). All patients were interviewed in person. Informed written consent was provided by all patients. This study was approved by the Medical-Biomedical Research Ethics Committee (REC) of the Integrated Health and Social Services University Network for West-Central Montreal (IRB: 2016-328, 15-022, approved on 20 June 2017).

A sample size calculation was previously carried out using the original project estimation based on the Human Metabolome Data Base. Urine succinate level was used as a reference based on previous metabolomic studies performed (Bouatra, Aziat, Mandal, Guo, Wilson, Knox, Bjorndahl, Krishnamurthy, Saleem, & Liu, 2013; Mossa et al., 2020; Wishart et al., 2009). Differences between control and disease conditions were 3.4 μmol/mmol creatinine (with normal urine succinate at 5.6 and abnormal of 9.0 μmol/mmol creatinine), with a standard deviation (sd) of 3.8, a study power at 80% and significance at 0.05.

Demographic and Clinical Differences

Complete medical history, physical examination, screening urinalysis, 1-day voiding diary and validated symptom questionnaires were carried out on every participant. Voiding dairies allowed us to estimate: 24 h, daytime and nighttime frequencies, total 24 h voided volume, nocturnal voided volume, mean voided volume per micturition, and maximum voided volume. All participants completed the Overactive Bladder Symptom Score (OABSS), the International Consultation on Incontinence Questionnaire-Short Form (ICIQ-SF), and the Incontinence Impact Questionnaire (IIQ-7) (Blaivas et al., 2007; Uebersax et al., 1995). Few patients only recorded their urgency and leakage episode in the voiding diary and hence they were not statistically tested due to insufficient

data. Fasting glucose and insulin levels were measured to calculate Homeostatic Model Assessment of Insulin resistance (HOMA-IR) as an index for insulin resistance. Significant insulin resistance was determined by values above 2.9.

Collection and Preparation of Urine Samples

Midstream early morning urine samples were gathered by patients in two sterile plastic containers. One was kept at 4 °C for bacterial culture. The other container was kept at −20 °C. Dietary restrictions were not requested during urine collection. Upon reception at the hospital, samples were thawed, aliquoted and stored at −80 °C. Laboratory staff was blinded to which samples were OAB or controls.

MiRNAs Isolation from Urine Samples

Urine aliquots were kept on ice then centrifuged at 10,000 rpm for 20 min to remove particles (cells and cellular debris). Subsequently, a urine microRNA (miRNA) purification kit (Norgen Biotek protocol Corp, Thorold, ON, Canada) was used to isolate miRNAs from supernatants, according to manufacturer's protocol. These columns isolate total miRNAs (cell-free and vesicular/exosomal ones), as well as small nuclear RNAs (Braicu et al., 2019). No DNAse or RNAse treatments were required. Assessment of contamination by DNA was carried out by using the Nanodrop system for single strand DNA and double strand DNA: no significant amount of DNA was found. An RNA contamination test was not carried out as the Norgen isolation kit specifically isolates miRNA and small RNAs, the latter being used as reference to standardize miRNA measurements. Quantification of nucleic acid was carried out on a nanodrop system. The purity of the nucleic acid (RNA) (A260/A280) was close to 2 for all samples. RNA integrity was also assessed by the Nanodrop system.

MicroRNA Poly-Adenylation and Synthesis of cDNA

A polymerase tailing kit from Lucigen (Middleton, WI, USA) was used to add poly(A)(adenine) tails to mature miRNAs. In short, purified RNAs were incubated with adenosine triphosphate (ATP, 1 mM) and *Escherichia coli* (*E. coli*) poly(A) polymerase (200 U/mL) for 30 min at 37 °C. The reaction was ended by incubating samples for 5 min at 95 °C. Subsequently, complementary DNA (cDNA) synthesis was performed using a custom-made stem loop primer containing a poly-T(thymidine) tail (Integrated DNA Technologies (IDT, Coralville, IA, USA)). Reverse transcriptase kit (OneScript cDNA synthesis kit) from abm (Richmond, BC, Canada) was used according to the manufacture's protocols, with the following incubation settings: Reverse transcription (RT) for 30 min, 50 °C for 50 min and 85 °C for 5 min. Samples were held at 4 °C, transferred to −20 °C for long term storage. Reference gene small nuclear RNA U6 (snU6) was amplified with a specific primer (Braicu et al., 2019). Total cDNA obtained was quantified using a Nanodrop system.

Quantitative PCR (qPCR)

Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Universal primer complementary to the stem loop primer was used together with forward primers specific for each miRNA of interest. The reference gene snU6 was detected with its own set of forward and reverse primers. Quantitative PCRs were carried out on a Sensifast Probe Low-ROX (low carboxyrhodamine) kit containing Synergy Brands SYBR-green, on an Applied Bioscience 7500 Fast Real-Time PCR, under the following conditions: 95 °C 10 min, 45 cycles of 95 °C 15 s and 60 °C 35 s, always followed by melt curve analysis. Samples were analyzed in triplicates. Primers were tested for specificity and efficiency (90–110%). Relative miRNA expressions were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Enzyme-Linked Immunosorbent Assay (ELISA) and Enzymatic Kits

ELISA kits for BDNF, proBDNF and $p75^{NTR}$ extracellular domain ($p75^{ECD}$) were purchased from Biosensis (Thebarton, Australia), those for sortilin and cortisol were from Abcam (Cambridge, MA, USA). Enzymatic kits were from the following providers: matrix metalloproteinase-3 (MMP-3) (AAT Bioquest, CA, USA), matrix metalloproteinase-7 (MMP-7), matrix metalloproteinase-9 (MMP-9) (Quickzyme, Leidan, The Netherlands), and plasmin (Sigma-Aldrich, Oakville, ON, Canada).

Statistics

Comparisons between groups were achieved by Student *t*-test (demographics, voiding diary, serum data and questionnaires) or Mann–Whitney test (not normally distributed). Significance was set at $p < 0.05$. One-way analysis of covariance (ANCOVAs) for confounders (age, homeostatic model assessment for insulin resistance (HOMA-IR) and estimated glomerular filtration rate (eGFR)) were performed to compare differences between control and OAB cohorts. Spearman's correlation was used to analyze urinary parameters, questionnaires' scores, and voiding diary parameters. Receiver operating characteristic (ROC) was computed to determine sensitivity and specificity. IBM SPSS Statistics ver. 23.0 (IBM Co., Armonk, NY, USA) was used for all statistics.

Results

Subject Characteristics

Of the 52 total female participants enrolled in this study, only 40 participants successfully completed the study protocol and are included in the analysis. The mean age for the OAB group was higher than the control group (68.9 ± 11.38 vs. 56.25 ± 5.22 years in controls, $p < 0.001$) (Table 4.1). There was no significant difference in the body mass index (BMI), demographics, or vital signs between both groups. The OAB group was found to have higher HOMA-IR level (3.11±

1.18 vs. 2.13± 1.03 in controls, $p = 0.020$), prevalence of metabolic syndrome, and hypertension when compared to the control group. Additionally, OAB symptom severity as reflected on the voiding diary and questionnaires' scores, were significantly higher in the OAB group (Table 4.1). Patients from the OAB group presented higher HOMA-IR level and higher prevalence of metabolic syndrome (40%) and hypertension (65%) compared to patients from the control group (20% of metabolic syndrome and hypertension, each). On the other hand, voiding diary, and total questionnaires' scores, which reflect OAB symptom severity and its impact on quality of life, were as well significantly higher in the OAB group (Table 4.1).

Table 4.1. Characteristics of control and OAB groups: Demographic, serum, symptom questionnaires, and urine analysis data.

	CTR	**OAB Group**	***p* Value**
Demographic and serum analysis:			
Age (years)	56.25 (5.22)	68.9 (11.38)	**<0.001**
BMI (kg/m^2)	29.75 (7.65)	28.82 (5.45)	ns
eGFR (mL/min/1.73 m^2)	98.5 (14.52)	76 (19.78)	**<0.001**
HOMA-IR	2.13 (1.03)	3.11 (1.18)	**0.020**
Total Cholesterol/HDL	3.50 (1.18)	3.23 (0.81)	ns
Questionnaires' scores:			
OABSS (0–28)	7.3 (3.56)	17.45 (4.45)	**<0.001**
ICIQ--SF (0–22)	3.26 (3.98)	8.05 (3.83)	**<0.001**
IIQ-7 (0–100)	2.4 (5.2)	28.9 (23.2)	**<0.001**
Voiding diary parameters:			
24 h frequency	9.15 (2.28)	11.4 (3.03)	**0.012**
Daytime frequency	8.5 (2.04)	9.5 (2.09)	ns
Night frequency	0.65 (0.81)	1.9 (1.71)	**0.005**
24 h voiding volume (mL)	2705 (2346.02)	1859.6 (865.37)	ns
Night voiding volume (mL)	495.25 (253.88)	449.75 (270.77)	ns
Mean voided volume (mL)	322.25 (311.1)	167.36 (75.2)	**0.037**
Maximum voided volume (mL)	480.75 (193.44)	327.25 (126.7)	**0.005**

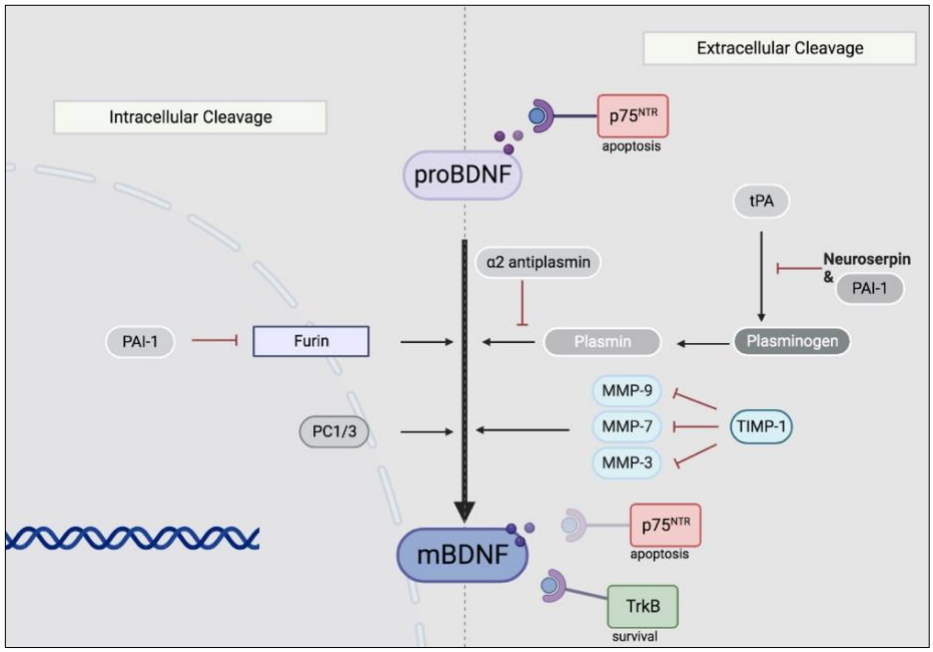
Data are presented as mean (Standard Deviation) for variables compared with independent *t*-test. Statistically significant differences are reported with *p* value. (ns) non-significant. Abbreviations:

Body Mass Index (BMI), estimated Glomerular Filtration Rate (eGFR), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), high density lipoproteins (HDL), Overactive Bladder Symptom Score (OABSS), International Consultation on Incontinence Questionnaire-Short Form (ICIQ-SF), and Incontinence Impact Questionnaire (IIQ-7).

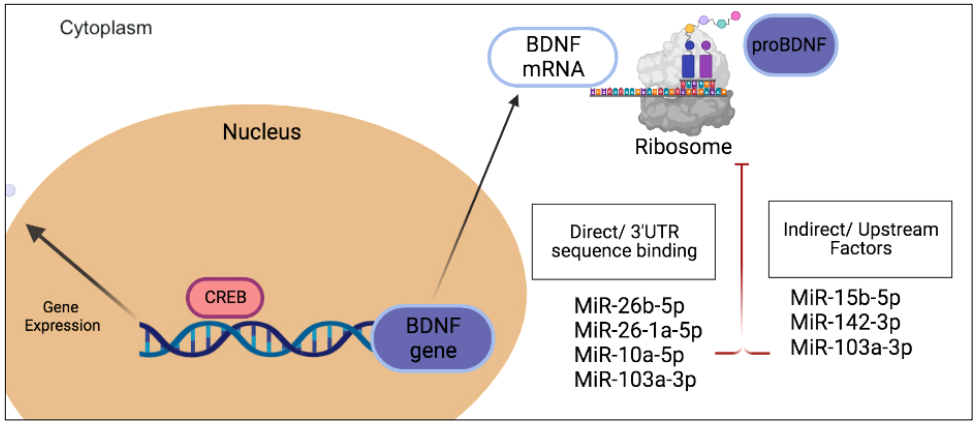
Biochemical Urinalysis

All variables tested summarized in Figure 4.1 were corrected to creatinine levels.

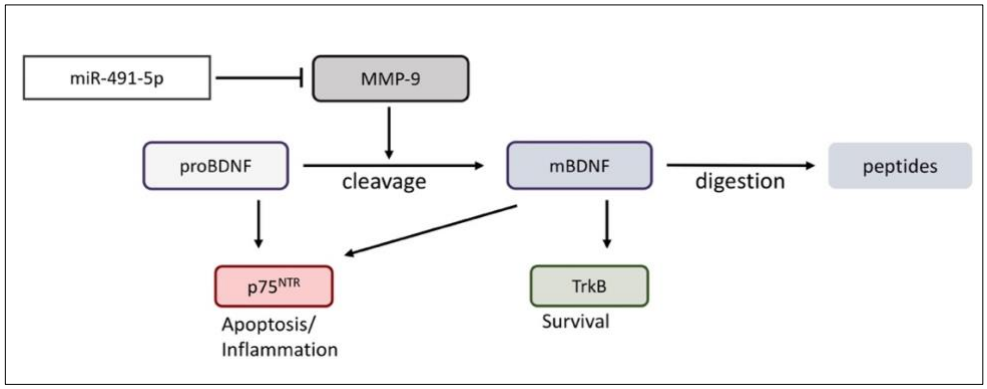
There were no differences in single standing BDNF/creatinine levels in the urine of controls versus OAB patients (Table 4.2 and Figure 4.2), while proBDNF/creatinine measures were lower in the OAB population, yet not statistically significant. The ratio of proBDNF/BDNF was significantly lower in the OAB group (p = 0.023) (Table 4.2 and Figure 4.2).



(a)



(**b**)



(**c**)

Figure 4.1. (**a**) Schematic representation of proteolytic pathways converting precursor proBDNF to its mature form mBDNF. (**b**) General illustration of BDNF ribosomal synthesis. Several miRNAs elicit direct and indirect inhibition of BDNF mRNA expression. Binding of BDNF and proBDNF to TrkB rand $p75^{NTR}$ receptors, respectively. (**c**) MiR-491-5p inhibition of MMP-9 implicated in the metabolism of BDNF. MiR, microRNA; TrkB, tyrosine kinase receptor B; PAI-1, plasminogen activator inhibitor-1; PC1/3, proprotein convertase 1/3; tPA, tissue plasminogen activator; TIMP-1, tissue inhibitor of metalloproteinase-1; mBDNF, mature brain-derived neurotrophic factor; MMP, matrix metalloproteinases; $p75^{NTR}$, p75 neurotrophin receptor; UTR, untranslated regions; CREB, cAMP-response element binding protein, cAMP response element-binding protein. Figure (**a**, **b**) were created using BioRender.

Table 4.2. Urinary BDNF, proBDNF, MMP-9, and proMMP-9 levels compared between control and OAB groups without considering confounders.

	Ctl Group	**OAB Group**	***p* Value**
BDNF (pg/mg creat)	19.67 ± 4.25	30.92 ± 6.84	0.265
proBDNF (pg/mg creat)	1543.5 ± 381.7	733.1 ± 131.8	0.231
proBDNF/BDNF	27.24 ± 3.09	16.89 ± 3.02	**0.023**
MMP-9 (ng/mg creat)	0.325 ± 0.124	1.802 ± 0.481	**0.035**
proMMP-9 (ng/mg creat)	0.391 ± 0.162	0.646 ± 0.216	0.301
MMP-9/pro-MMP-9	2.401 ± 0.60	3.446 ± 0.61	0.251

Data are presented as mean ± SEM for variables compared with the non-parametric Mann–Whitney test. Significant differences are reported ($p < 0.05$).

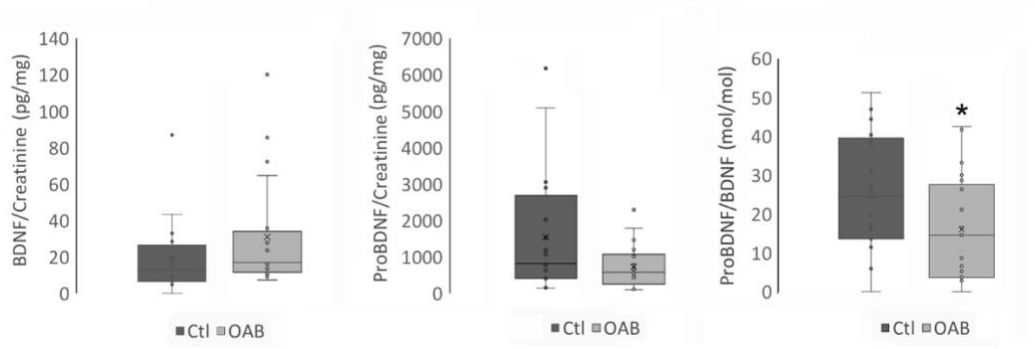


Figure 4.2. BDNF and proBDNF levels in urine samples from control (Ctl) and OAB patients (OAB). Levels of BDNF and proBDNF were measured in parallel and normalized to creatinine. The ratio proBDNF/BDNF is also represented. (Ctl $n = 20$, OAB $n = 20$). Non-parametric Whitney test was carried out. * $p < 0.05$.

Receiver operating characteristic (ROC) for proBDNF/BDNF demonstrated high sensitivity for OAB diagnosis (AUC = 0.729) compared to each neurotrophin taken separately (Figure 4.3).

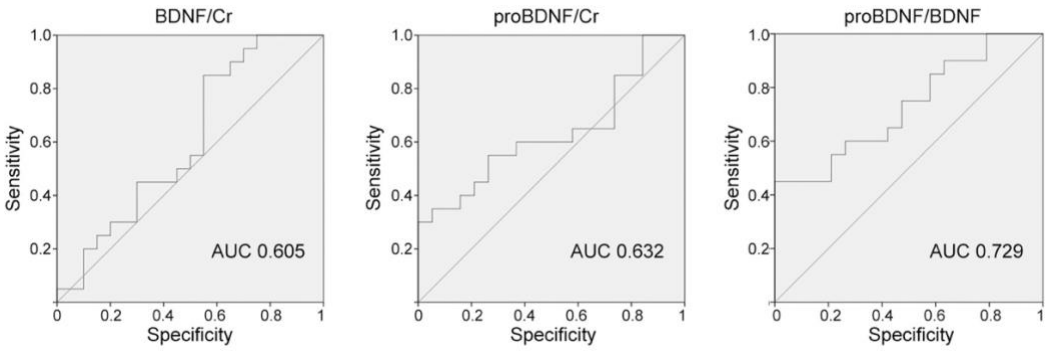


Figure 4.3. Receiver Operating Characteristics (ROC) curve for BDNF/creatinine and proBDNF/creatinine levels in urine samples. Area under curve (AUC) was computed for BDNF/creatinine (BDNF/Cr) (pg/mg creatinine), proBDNF/creatinine (proBDNF/Cr) (pg/mg creatinine), and the ratio proBDNF/BDNF (mol/mol). The highest AUC was found for the ratio proBDNF/BDNF.

Additionally, enzymatic activity of MMP-9, one of the main enzymes converting proBDNF to BDNF, was higher in the OAB group: 0.325 ± 0.124 vs. 1.802 ± 0.481 ng/mg creatinine in control and OAB group, respectively, p = 0.035 (Table 4.2). MMP-9 and its precursor proMMP-9 enzymatic activities were also plotted to further assess this trend (Figure 4.4). The ratio imbalance suggested an increased conversion of proBDNF to BDNF due to an enhanced activity of MMP-9.

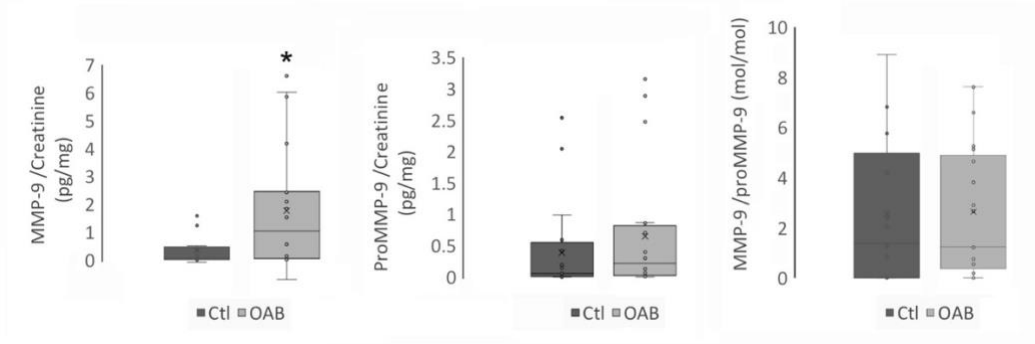


Figure 4.4. Matrix metalloproteinase-9 (MMP-9) and its precursor proMMP-9 enzymatic activity in urine samples from control (Ctl) and OAB patients (OAB). Activity of MMP-9 and total amount of proMMP-9 were measured and normalized to creatinine. The ratio MMP-9/proMMP-9 were also plotted. (Ctl $n = 20$, OAB $n = 20$). Non-parametric Whitney test was carried out. *$p < 0.05$.

We further adjusted the urinary levels of BDNF, MMP-9, and their precursor molecules with metabolic confounders of age, HOMA-IR index, and estimated kidney function level (eGFR). The urinary levels of proBDNF/BDNF ratio in the OAB group remained lower in a statistically significant fashion after adjusting for HOMA-IR and eGFR separately ($p < 0.05$) (Table 4.3).

Table 4.3. Urinary BDNF, MMP-9, and their precursors compared between control (CTR) and OAB groups considering confounders.

	Confounders	**CTR**	**OAB Group**	***p* Value**
BDNF (pg/mg creat)	Age	27.51 (20.63–34.39)	26.87 (19.99–33.76)	0.952
	HOMA-IR	20.78 (13.95–27.6)	27.66 (20.59–34.74)	0.509
	eGFR	22.97 (16.1–29.9)	31.41 (13.99–48.84)	0.431
proBDNF (pg/mg creat)	Age	1528 (1202–1853)	749 (424–1074)	0.133
	HOMA-IR	1753 (1390–2117)	729 (352–1105)	0.072
	eGFR	1488 (1169–1807)	789 (470–1108)	0.162
proBDNF/BDNF (mol/mol)	Age	24.81 (21.44–28.18)	19.31 (15.94–22.68)	0.302
	HOMA-IR	29.45 (26.08–32.82)	18.89 (15.4–22.38)	**0.047**
	eGFR	27.59 (24.18–31.01)	16.53 (13.11–19.95)	**0.042**
MMP-9 (ng/mg creat)	Age	0.530 (0.082–0.978)	1.645 (1.225–2.065)	0.111
	HOMA-IR	0.581 (0.108–1.054)	1.677 (1.204–2.150)	0.128
	eGFR	0.445 (0.014–0.876)	1.722 (1.316–2.128)	0.054
proMMP-9 (ng/mg creat)	Age	0.583 (0.374–0.792)	0.455 (0.246–0.664)	0.697
	HOMA-IR	0.387 (0.170–0.604)	0.539 (0.314–0.764)	0.645
	eGFR	0.331 (0.117–0.545)	0.707 (0.376–1.038)	0.262
MMP-9/proMMP-9	Age	2.419 (1.682–3.156)	3.431 (2.740–4.122)	0.367
	HOMA-IR	2.727 (1.889–3.565)	3.764 (2.957–4.571)	0.405
	eGFR	3.010 (2.342–3.678)	2.900 (2.271–3.529)	0.913

Data are presented as estimated marginal mean (95% CI) for variables compared with ANCOVA. Statistically significant differences are considered at $p < 0.05$. Abbreviations: Body Mass Index (BMI), estimated Glomerular Filtration Rate (eGFR), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR).

Subsequently, correlation between BDNF, proBDNF and clinical questionnaires in the total cohort showed that the OABSS score had a significant negative correlation with standalone proBDNF levels (p <0.05). The proBDNF/BDNF levels also negatively correlated with OABSS and IIQ-7 (p <0.05) but did not show this trend with the ICIQ-SF questionnaire (Table 4.4).

Table 4.4. Correlation between BDNF, proBDNF, and symptom questionnaires in the total cohort.

		Correlation	***p* Value**
BDNF (pg/mg creat)	OABSS	0.035	0.828
	ICIQ-SF	0.165	0.314
	IIQ-7	0.144	0.377
proBDNF (pg/mg creat)	OABSS	−0.336	**0.034**
	ICIQ-SF	−0.176	0.285
	IIQ-7	−0.267	0.096
proBDNF/BDNF (mol/mol)	OABSS	−0.392	**0.012**
	ICIQ-SF	−0.290	0.073
	IIQ-7	−0.391	**0.013**
MMP-9 (ng/mg creat)	OABSS	0.259	0.117
	ICIQ-SF	0.307	0.065
	IIQ-7	0.207	0.212

BDNF, proBDNF and the ratio proBDNF/BDNF were correlated to three questionnaires, OABSS, ICIQ-SF and IIQ-7. The p value for Spearman correlation was considered statistically significant for $p < 0.05$. Abbreviations: Overactive Bladder Symptom Score (OABSS), International

Consultation on Incontinence Questionnaire-Short Form (ICIQ-SF), and Incontinence Impact Questionnaire (IIQ-7).

To provide insights in the regulation of proBDNF synthesis, we measured diverse urinary factors and microRNAs known to control the translation of proBDNF mRNA (Table 4.5). MiR-26b-5p, miR-26a-5p, miR-10a-5p, and miR-103a-3p that bind the 3′UTR sequence of proBDNF mRNA were not expressed differently between control and OAB patients (Caputo et al., 2011; Q. Zhang et al., 2021). Levels of other miRNAs (miR-15b-5p, miR-142-3p and miR-103a-3p) that control proBDNF expression through downstream or upstream pathways were also not different (Boone et al., 2017; Gupta et al., 2020; Q. Zhang et al., 2021). On the other hand, concentration of miR-491-5p, which negatively controls MMP-9 expression, was significantly decreased in the OAB group: 0.122 (Q1, Q3: 0.0221, 0.392) vs. 0.533 (Q1,Q3: 0.302, 1.643) in the control group, $p < 0.05$ (Figure 4.1c) (Guo et al., 2021). Another factor associated with BDNF synthesis, cortisol, was not significantly different (Table 4.5) (Jeanneteau & Chao, 2013). Finally, level of soluble extracellular domain of receptor p75 ($p75^{ECD}$) resulting from the cleavage of membrane-bound receptor $p75^{NTR}$ was increased in the OAB group while concentrations of the $p75^{NTR}$ co-receptor sortilin were similar (Luu et al., 2022; Meeker & Crooks, 2022). Adjustments of miR-491-5p and $p75^{ECD}$ data to clinical questionnaires were still highly correlated.

Table 4.5. Urinary factors involved in proBDNF synthesis and proteolysis in the control and OAB groups.

	Ctl	OAB	*p* Value
miR-26b-5p	0.891 (0.423, 1.864)	1.865 (0.0830, 2.842)	0.99
miR-26a-5p	0.808 (0.495, 1.911)	1.634 (0.0852, 2.911)	0.989
miR-10a-5p	0.884 (0.420, 3.489)	0.344 (0.101, 1.083)	0.108
miR-103a-3p	1.200 (0.500, 2.156)	0.592 (0.0570, 1.105)	0.102
miR-15b-5p	0.825 (0.494, 3.076)	1.504 (0.307, 4.070)	0.841
miR-142-3p	0.438 (0.0728, 1.511)	0.449 (0.267, 1.930)	0.369
miR-202-3p	1.300 (0.318, 2.750)	0.257 (0.146, 2.531)	0.211
miR-124-5p	0.939 (0.382, 4.121)	1.356 (0.259, 2.234)	0.813
miR-152-5p	1.362 (1.166, 3.902)	0.721 (0.298, 5.568)	0.792
miR-491-5p	0.533 (0.302, 1.643)	0.122 (0.0221, 0.392)	**0.008**
MMP-7 (ng/mg creat)	0.232 (0.140, 0.455)	0.443 (0.228, 1.02)	0.079
MMP-3 (mU/mg creat)	0.0147 (0.00584, 0.0262)	0.0104 (0.00038, 0.235)	0.583
Plasmin (ng/mg creat)	18.1 (14.57, 30.90)	27.65 (15.65, 38.40)	0.512
Cortisol (ng/mg creat)	38.18 (29.72, 53.74)	35.29 (17.89, 56.70)	0.529
p75^{ECD} (ng/mg creat)	2.471 (2.149, 2.855)	2.851 (2.623, 3.593)	**0.035**
Sortilin (pg/mg creat)	1710 (737, 2984)	1752 (1286, 3266)	0.398

MicroRNAs binding the 3′UTR part of the BDNF gene (miR-26-50, miR-26-1a-5p, miR-10a-5p, miR-103a-3p) or involved in its indirect upstream control (miR-202-3p, miR-10a-5p, miR-15b-5p, miR-142-3p) were measured by RTqPCR and normalized to snU6. Enzymatic activities other than MMP-9 involved in the conversion of proBDNF to BDNF (MMP-3, MMP-7, and plasmin) were measured. Another factor associated with BDNF synthesis, cortisol, and membrane-bound proteins involved in proBDNF signaling (sortilin and p75^{ECD}) were assessed as well. Data are presented as median (interquartile range, Q1, Q3) for variables compared with the non-parametric Mann–Whitney test. Significant differences are reported in bold ($p < 0.05$).

Discussion

The present study examined the diagnostic and phenotyping value of the urinary ratio proBDNF over mature BDNF for OAB. Levels of microRNAs and proteins related to proBDNF synthesis and proteolysis of mature BDNF were determined along with the concentration of receptors sortilin and $p75^{ECD}$, which are involved in proinflammatory processes after binding proBDNF.

A recent metanalysis gathered evidence on urinary mature BDNF/creatinine as a potential biomarker for OAB (Tsiapakidou et al., 2021b). The concentrations of BDNF measured in the present study are in the range of those reported in previous publications (between 4.7 to 859 pg/mg creatinine for controls and between 4.0 to 1627 pg/mg creatinine for OAB patients). Differences in proBDNF/BDNF ratios between OAB and control group were still found after correcting for HOMA-IR and eGFR confounders, suggesting that they do not contribute to the proBDNF/BDNF ratio imbalance found in OAB. Unlike previously published studies on OAB patient cohorts (Rada et al., 2020; Utomo et al., 2021), we did not observe single standing difference of BDNF/Cr levels between both groups (Antunes-Lopes et al., 2013; L.-W. Wang et al., 2014). An explanation for this can be that BDNF is the most abundant of the neurotrophins in the human body (Farach et al., 2012). Likewise, studies have shown that the aging process has no significant impact on BDNF concentrations, which is a characteristic that is not shared by nerve growth factor (NGF) (Lanni et al., 2010). The activation of MMP-9 proteolysis of proBDNF should lead to a subsequent increase in BDNF, which we did not observe. This could be explained by other pathways targeting the downregulation of BDNF activity. In vitro, we observed that nitric oxide (NO), which has been shown to be increased during OAB, decreases BDNF release by bladder smooth muscle cells (SMCs) in culture (unpublished observations). The interaction between NO and BDNF could be

explained by impaired release of BDNF by cells caused by NO induced decreased in calcium influx in smooth muscle (Berridge, 2008).

We have reported a higher proNGF/NGF ratio in this OAB population compared to controls (Jeanneteau & Chao, 2013). Compensatory mechanism between inter-neurotrophin level variations has been described in Sprague Dawley rats (Liao et al., 2020). Therefore, the inverse finding of a lower proBDNF/BDNF ratio here described could be explained by a compensatory MMP-9 proteolysis to increase BDNF levels.

According to recent reports, little is known regarding the role of BDNF in bladder function, due to the limited number of studies, the low number of patients involved and the diversity of detection kits used (Tsiapakidou et al., 2021b). In particular, the ELISA kits used were not tested for their specificity to distinguish between BDNF and proBDNF, given the similarity in sequence present in both forms. We confirmed the specificity of our kits (unpublished materials), as previously conducted for NGF and proNGF (Mossa et al. 2021). Nevertheless, we can extrapolate the results on BDNF and proBDNF thanks to their specific signaling pathways. It is well known that neurotrophins and their precursors trigger different pathways by binding specific membrane-bound receptors associated with cell survival and growth or to inflammation and apoptosis (Ioannou et al., 2017). The relative amount of pro- and mature neurotrophins could have more clinical value than each taken separately (Ioannou et al., 2017).

On the other hand, the ratio proBDNF/BDNF presents a similar sensitivity than the proNGF/NGF in detecting patients with OAB (ROC analysis 0.729 vs. 0.735, respectively) (Jeanneteau & Chao, 2013). Our findings are in accordance with previous studies reporting a diagnostic value of BDNF/creatinine between 0.67 and 0.95, which is considered as a ‘fair’ performance (Meeker & Crooks, 2022).

Levels of miRNAs involved in the control of proBDNF translation were unchanged, confirming our previous report that the balance of proNGF/NGF appears to be controlled at the level of protease activity rather than at a transcriptional step (Mossa et al. 2021). In accordance, enzymatic activity of MMP-9 converting proBDNF to BDNF was enhanced in OAB. In vitro experiment on bladder primary cell cultures using the gene editing enzyme Crispr-Cas9 to partially delete MMP-9 gene led to massive accumulation of proBDNF in the culture medium (unpublished observation), highlighting the crucial role of MMP-9 in proBDNF proteolysis. Indeed, the upregulated MMP-9 activity observed in the OAB group can be caused by the associated lower levels of miR-491-5p, which negatively controls MMP-9 expression by direct binding of a 3′-UTR sequence present on MMP-9 mRNA. Within the field of oncology, the tumor suppressor miR-491-5p expression has been reported to inhibit important metastatic pathways (Guo et al., 2021). Exploring the role of microRNAs in OAB pathophysiology could uncover potential therapeutic targets.

Finally, we found a statistically significant increase in the extracellular portion of $p75^{NTR}$ receptor in urine of OAB patients. The $p75^{ECD}$ is downregulated in neurological disease and has been suggested to possess neuroprotective properties (Meeker & Crooks, 2022). A recent report showed an increase in plasma $p75^{ECD}$ after 4 weeks treatment of diabetic type 1 mice with an antagonist of $p75^{NTR}$ (Luu et al., 2022). In the present study, a relatively lower level of proBDNF binding to $p75^{NTR}$ would decrease its activation and lead to an increase in $p75^{ECD}$. The physiologic implications of this phenomenon remain to be elucidated.

The outcome of this pilot study provides empirical evidence to further support the utility of neurotrophins as biomarkers for OAB and simultaneously presents the underlying pathophysiological mechanisms of OAB symptomology. ProBDNF/BDNF ratio was found to have a significantly negative correlation with the scores of OAB symptom questionnaires (OABSS, IIQ-

7). This suggests that the dysregulated levels of the balance of the biological isoforms of BDNF, proinflammatory/apoptotic proBDNF, and neuroprotective BDNF contribute more to the pathogenesis of OAB than either one alone. For instance, research within the domain of neuropsychology has suggested that proBDNF/BDNF ratio may be more an indicator of cognitive change than proBDNF and BDNF levels alone (Cechova et al., 2020). Our data demonstrate that the ratio of proBDNF/BDNF and MMP-9 activity complement each other in the presence of OAB, and that this can be directly linked to microRNA activity and common metabolic comorbidities highly prevalent in the aging population (e.g., systemic arterial hypertension, dyslipidemia, and impaired glucose tolerance). Additionally, our previous study found an increased urinary level of NO and prostaglandin E2 (PGE2), two co-activators of MMP-9, which further highlights the importance of MMP-9 activity in the pathophysiology of OAB through the dysregulation of BDNF isoforms (Khan et al., 2012; Mossa et al., 2021; Ridnour et al., 2007).

This pilot study is subject to several limitations, including a small sample size. As we did not consider patients with non-idiopathic OAB or male patients, our results cannot be generalized to other patient groups outside of our inclusion criteria. Other clinical limitations associated with this study are further discussed in our previous publications (Mossa et al. 2021; Mossa et al. 2020). Future larger clinical studies should assess a larger sample of subjects with varying disease evolution and severity, with follow-up and response to treatments. This would allow a better understanding of the clinical value of proBDNF and associated metabolites as prognostic, diagnostic, and early markers of OAB development, and particularly help phenotype patients according to different etiologies. This in turn could allow us to further validate results that use proBDNF metabolism as a tool for tailored treatment protocols.

Conclusions

In conclusion, the present report suggests that the ratio proBDNF/BDNF could help improve diagnosis and phenotyping of OAB in female patients in a context of metabolic syndrome. More research is required to comprehend the role of these neurotrophins in the development of the pathology. It also confirms our previous report on NGF suggesting that neurotrophins are regulated at the levels of proteolytic enzymes converting pro-form to mature form rather than the translational levels. Finally, it strengthens the fact that the ratio between precursor and mature neurotrophins has more diagnostic value than isolated values of mature neurotrophins alone.

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Institutional Review Board Statement: This study was approved by the Medical-Biomedical Research Ethics Committee (REC) of the Integrated Health and Social Services University Network for West-Central Montreal (IRB: 2016-328, 15-022, approved on June 20, 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are unavailable due to privacy or ethical restrictions.

Conflicts of Interest: The authors declare no conflict of interest.

Chapter 5: BDNF secretion controlled by natriuretic peptides in bladder cells in vitro: a matched study on urine proteomics from female patients with overactive bladder syndrome

This chapter helps bridge the gap between clinical findings found in chapter 4 and *in vitro* findings on rat bladder cells. It illustrates relevant translational findings in OAB in the aging population.

Here we investigated the urinary levels of natriuretic peptides in an aging female population with OAB and compared these findings using rat bladder cells *in vitro*.

Contribution of authors: Conceptualization, Philippe Cammisotto and Lysanne Campeau; methodology, Claudia Covarrubias and Philippe Cammisotto; validation, Claudia Covarrubias, Philippe Cammisotto and Lysanne Campeau.; formal analysis, Claudia Covarrubias, Philippe Cammisotto, and Lysanne Campeau.; investigation, Claudia Covarrubias, Philippe Cammisotto and Lysanne Campeau; resources, Lysanne Campeau; data curation, Claudia Covarrubias, Philippe Cammisotto and Lysanne Campeau; writing—original draft preparation Claudia Covarrubias and Philippe Cammisotto; writing—review and editing, Claudia Covarrubias, Philippe Cammisotto and Lysanne Campeau; supervision, Philippe Cammisotto and Lysanne Campeau ; project administration, Lysanne Campeau ; funding acquisition, Lysanne Campeau. All authors have read and agreed to this version of the manuscript.

BDNF secretion controlled by natriuretic peptides in bladder cells *in vitro*: a matched study on urine proteomics from female patients with overactive bladder syndrome

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Abstract

In patients with overactive bladder syndrome (OAB), neurotrophin ratios of nerve growth factor brain-derived neurotrophic factor (BDNF) to its precursor proBNDF is significantly associated with symptom severity. Nitric oxide (NO), through stimulation of soluble guanylate cyclase, controls neurotrophin secretion. Atrial (ANP) and brain (BNP) natriuretic peptides also stimulate cGMP synthesis through the particulate guanylyl synthetase. We examined the relationship between NPs and neurotrophins in clinical samples and in rat bladder cells *in vitro*. ANP, but not BNP, displayed higher levels in urine samples from OAB patients compared to controls. Smooth muscle cells (SMCs) appeared as the main source of BDNF and proBDNF, urothelial cells (UROs) secreting very low amounts. Sodium nitroprusside (SNP), a NO donor, decreased the secretion of BDNF. On the other hand, ANP, but not BNP, increased BDNF secretion in SMCs. It also stimulates MMP-9 secretion from SMCs and UROs. Knockdown of MMP-9 confirmed the essential role of this enzyme in the processing of proBDNF into BDNF. In SMCs, ANP decreased NO, and L-NAME increased BDNF secretion in a manner similar to ANP but without stimulating MMP-9. On the other hand, ANP increased intracellular cyclic AMP and the analog dibutyryl cyclic AMP mimicked the effect of ANP on both BDNF and MMP-9. These data suggest that ANP acts on bladder cells to increase BDNF by at least two pathways, involving decrease in nitric oxide and increase in cyclic AMP.

Keywords: Atrial natriuretic peptide; BDNF, Overactive bladder; bladder cells, second messengers

Introduction

Overactive bladder syndrome (OAB) is a common condition characterized by urgency, frequency, and nocturia. It can significantly impact the quality of life (QoL) for affected individuals (Abrams et al., 2002). The pathophysiology of OAB is multifactorial, including pathways associated with neurotrophin secretion and their precursors (Tsiapakidou et al., 2021a). Neurotrophins, a family of proteins that play a crucial role in the development and maintenance of the nervous system, are involved in the regulation of bladder function (Shimizu et al., 2023). In a matched study on urine metabolomics and proteomics from aging female patients with OAB, we found neurotrophin ratios of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (proNGF/NGF; proBDNF/BDNF) to be significantly associated with symptom severity while also highlighting the role of proteolytic enzymes in their regulation (Covarrubias et al. 2023; Mossa et al. 2021).

Additionally, our recent publication demonstrated that intracellular cyclic GMP (cGMP) stimulated by nitric oxide (NO) plays an important role in neurotrophin secretion (Sirmakesyan et al 2023). Natriuretic peptides, mostly the atrial (ANP) and brain (BNP) ones, also stimulate the synthesis of cGMP but in a different way. While NO activates the soluble guanylate synthase located in cell cytoplasm, natriuretic peptide receptors are linked to the particular guanylyl synthase close to the plasma membrane (Friebe et al., 2020). There are therefore two pools of cGMP, one cytoplasmic under control of the soluble guanylate synthase, and one in compartments close to the plasma membranes for the particulate guanylyl cyclase. The two pools do not mix and trigger different signaling pathways leading to distinct cellular effects.

Natriuretic peptides (NPs), ANP, B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) have been found to be secreted by tissues such as the brain, exocrine pancreas, salivary glands, lungs, kidney, and the gastrointestinal tract, but in significantly lower quantities when

compared to the myocardium (Najenson et al., 2019). They have natriuretic, diuretic, and vasorelaxant properties. NPs counteract fibrosis, cardiac hypertrophy, and remodeling in the heart (Pankow et al., 2007). In addition to their direct cardiovascular effects, natriuretic peptides have also been shown to inhibit nicotine-induced whole-cell currents and catecholamine secretion in bovine chromaffin cells, which directly inhibits catecholamine release and increases catecholamine uptake (Babinski et al., 2002). Furthermore, CNP and its receptor, guanylyl cyclase receptor type B (GC-B/NPR-B), are positioned to play a role in prenatal and postnatal neurogenesis in the olfactory system, where it has been identified as a factor that can act on neuronal precursors when primed by BDNF or NGF, mediating their differentiation and survival (Simpson et al., 2002). Natriuretic peptides have also been shown to carry anti-cancer effects by inhibiting the growth and invasion of human small cell lung cancer, breast cancer, bladder cancer, and other tumors (Kawanishi et al. 2008; Xu et al. 2022).

The principal receptor activated by ANP and BNP is the transmembrane guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPR-A) while CNP activates natriuretic peptide receptor-B (CG-B/NPR-B). Both receptors catalyze the synthesis of cGMP through activation of particulate guanylyl cyclase, which activates intracellular second messengers and mediates most known effects of natriuretic peptides through pathways independent from soluble guanylate cyclase. A third natriuretic peptide receptor, natriuretic peptide receptor-C (NPR-C), clears all three natriuretic peptides from circulation through receptor-mediated internalization and degradation without increasing levels of intracellular cGMP (Pandey, 2021; Potter et al., 2009).

Inhibition of natriuretic peptides, particularly BNP, has been proposed as a therapeutic strategy in the management of bladder cancer. Studies have shown that BNP inhibits bladder cancer

invasiveness and tumor growth (Ceci et al., 2016). Additionally, activation of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor involved in the regulation of cell proliferation and differentiation, has been found to suppress bladder cancer growth by inhibiting the PI3K-Akt signaling pathway (Lv et al., 2019).

Herein we analyze the role of NPs and their receptors in neurotrophin secretion in bladder cells *in vitro*. Given the knowledge gap and the shared molecular pathways between neurotrophins and NPs, the objective of this study was to examine the relationship between NPs and neurotrophins in clinical samples and in rat bladder cells *in vitro*. Due to the ability of NPs to impact micturition, we hypothesized that they likely play a role in the pathophysiology of OAB through unique molecular mechanisms enacted on neurotrophins.

Materials and Methods

Patient samples

Urine samples from 20 controls and 20 OAB patients (50-80 years) were obtained with validated medical questionnaires. Detailed patient characteristics are described in chapter 4.

Cell cultures

Female Sprague-Dawley rats (8-week-old) were used in accordance with the Canadian Council for Animal Care. The Animal Ethics Committee of McGill University (Quebec, Canada) approved all protocols. Animals were kept under standard Purina chow and had free access to water. Euthanasia by cardiac exsanguination was carried out under isoflurane anesthesia and bladders were sampled and placed in cold sterile phosphate-buffered saline (PBS) (pH 7.4). Urothelial cells were isolated after digestion of the urothelium in DMEM medium containing 100 U/mL of collagenase IV for 15-20 minutes at 37ºC with gentle shaking. Cells were washed twice in Dulbecco's Modified Eagle

Medium (DMEM) 10% fetal bovine serum (FBS) then grown in DMEM low glucose/Keratinocyte (50/50) media containing FBS (10%), Glutamax (X1), hormones mix (insulin 5 µg/mL, dihydrocortisone 0.5 µg/mL, adenine 15 µg/mL, ethanolamine 0.1 mM), Rho Inhibitor Y27632 (10 µM) and 1% penicillin/streptomycin (100 U/mL, 100 µg/mL) in a humidified incubator under 5% CO_2 atmosphere. Medium is replaced every 2-3 days until cell confluency. Cells were starved 24 hours prior to use in MCDB medium (replacing keratinocyte medium) without Y27632. Smooth muscle cells were isolated from minced bladder tissue incubated in DMEM containing 250 U/mL of collagenase IV for 45 minutes, with intense shaking. After two washings, SMCs were seeded in SK medium (Wisent, St-Bruno, Canada) and supplemented with FBS (10%), high glucose (27 mM) and penicillin/streptomycin (100 U/mL, 100 µg/mL). SMCs were used after a 72-hour period under normoglycemic conditions. Incubation with ANP (100 nM) or BNP (1 microM) was carried out for 24 hours.

ELISA kits

Specific kits were used as follows: ANP and BNP (Sigma-Aldrich, Oakville, Ontario, Canada) cGMP and cAMP (Cayman Chemical (Ann Arbor, MI), and BDNF and proBDNF (Biosensis, Australia). Activity of matrix metalloproteinase-9 (MMP-9) was measured using an enzymatic kit (Quickzyme Biosciences, Netherland).

Crispr-cas9 plasmids

Sg primers were designed on https://design.synthego.com. The sequences were generated from the genome of Mus musculus (NM+013599.4) and purchased from IDT (Coralville, IOWA, USA): forward 5'-CAC CGC GGC CCG GGT GTA ACC ATA G -3' and reverse 3'-AAA CCT ATG GTT ACA CCC GGG CCG C-5'. DNA sequence between rat and mouse MMP-9 gene presented high homology and this plasmid found suitable for knockout in rat cells. Bacteria containing

plasmid CRISPR-Cas9 were obtained from Addgene Cas9 (LentiCRISPRv2 #52961) (Watertown, MA, USA). After overnight growth on petri dish, isolated colonies were added to a liquid broth containing ampicillin (100 μM) and incubated at 37°C for overnight with shaking. The following day, plasmids were extracted (Sigma-Aldrich kit, Oakville, Ontario, Canada). A Nanodrop system was used to measure plasmid concentrations. Plasmids were then digested with BsmBI (New England Biolabs, MA, USA) and the products run on a 0.7-1% agarose gel. Bands of linear plasmids were purified using a gel DNA extraction kit (Sigma-Aldrich, Oakville, Ontario, Canada) and plasmid amounts were measured with the Nanodrop system. Ligation of Sg primers was carried out in the presence of ATP (1mM) and T4 polynucleotide kinase (T4PNK) (New England Biolabs, MA, USA) for 30 minutes at 37°C followed by 5 minutes at 95°C. Insertion of ligated primers into linear plasmid was done with T4 ligase enzyme, at room temperature for 1 hour. Termination was achieved by incubating at 65°C for 10 minutes. Next, competent stbl3 cells were transfected after heat shock (42°C for 45 seconds then 4°C for 30 minutes). Cells were cultured in super optimal broth (SOB) *medium* at 30°C for 2 hours. Bacteria were then seeded on petri dishes with ampicillin (100 μM), overnight at 37°C. Single colonies were homogenized for plasmid characterization. UROs and SMCs were transfected using plasmids containing the sg primers for MMP-9 or empty plasmids (sham control) for 24 hours. Selection was done using puromycin (5 μg/mL). Cells were grown until confluence in their respective growth medium and used as previously described.

RT-qPCR

RNA was isolated using Trizol reagent (Biomatik Corporation) and chloroform. Concentration and purity were assessed by a Nanodrop spectrophotometer ND-1000 (ThermoFisher Scientific,

Wilmington DE). Complementary DNA (cDNA) was synthesized using a reverse transcriptase kit (OneScript cDNA synthesis kit) according to the manufacture's protocol (ABM, Richmond BC, Canada). For quantitative polymerase chain reaction (qPCR), the following primers were purchased from Integrated DNA Technologies (IDT, Coralville, IOWA, USA): BDNF forward primer (5'-CTACGAGACCAAGTGCAATCC -3'), BDNF reverse primer (5'-AACCGCCAGCCAATTCTCTTT -3'), MMP-9 forward primer (5'-CCA TGC ACT GGG CTT AGA TCA T-3'), MMP-9 reverse primer (5'-CAG ATA CTG GAT GCC GTC TAT GTC-3'), 18S forward primer (5'-GCA ATT ATT CCC CAT GAA CG-3'), 18S reverse primer (5'-GGC CTC ACT AAA CCA TCC AA-3'). Samples were amplified using Applied Bioscience 7500 Fast Real-Time PCR with the Sensifast Probe Low-ROX kit containing SYBR-green. Conditions were as follow: 35 cycles of heating and cooling, 30s at 95°C for denaturation, 30s at 57°C for annealing and 30s at 72°C for extension followed by a final extension at 72°C for 10 min. Samples were done in duplicates and melt curves performed each time. Specificity of primers was performed to ensure specificity and efficiency (90-110%). Using purified RNA without reverse transcription, controls were also assessed. Final data analysis was done using the $2^{-\Delta\Delta CT}$ method.

Classic RT-PCR

RNA samples were submitted to cDNA synthesis as described previously. Primers were designed as follows: NPR-A forward 5-AAGAGCCTGATAATCCTGAGTACT-3 and reverse 5-TTGCAGGCTGGGTCCTCATTGTCA-3; NPR-B forward 5-TCAAACACATGAGAGATGTTC -3 and reverse 5-TATTGGCATACTGTTCCATGC-3; NPR-C forward 5-TGACACCATTCGGAGAATCA-3 and reverse 5-CATCTCCGTAAGAAGAACTGTTGA-3. Amplification was carried out using the 2X Taq

FroggaMix (FroggaBio, Toronto, ON, Canada). PCR products were resolved on a 1% agarose gel containing ethidium bromide and were photographed under UV light.

Results

Urine samples from OAB patients were characterized by higher levels of ANP/creatinine, compared to healthy controls (Figure 5.1a). BNP levels over creatinine were similar between groups. ROC curves displayed an AUC of 0.695 for ANP while it was close to 0.5 for BNP. Receptors of natriuretic peptides, NPR-A, NPR-B and NPR-C were found expressed in both UROs and SMCs from rat bladder using classic RT-PCR. (Figure 5.1b)

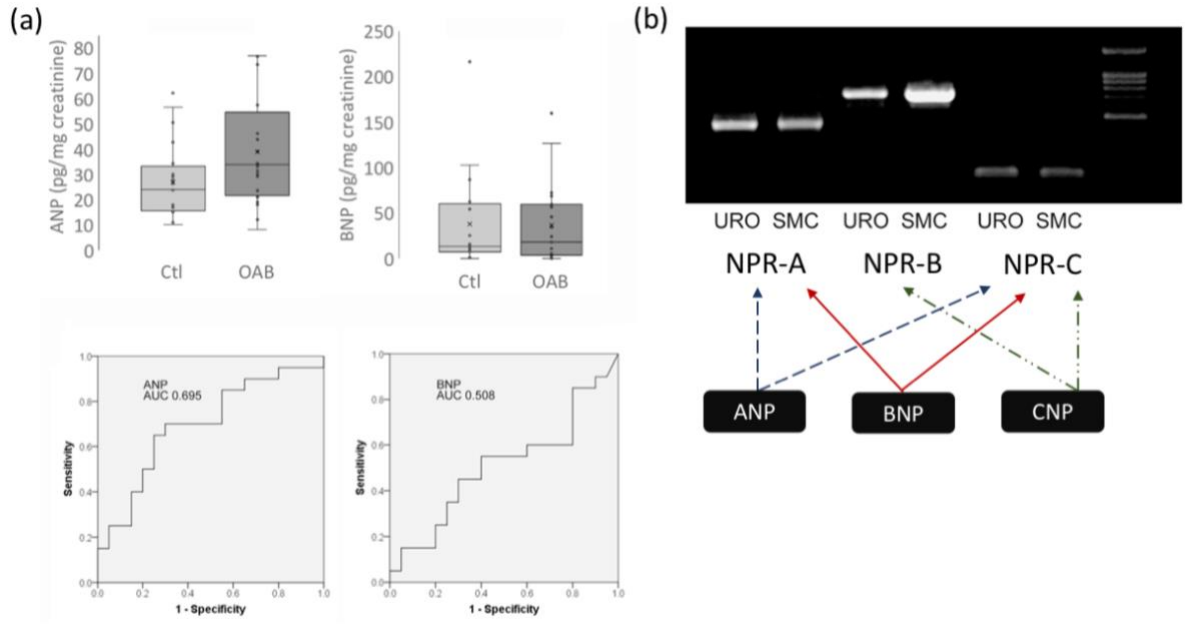

Figure 5.1. Increase levels of urinary ANP in OAB patients: (a) In the urine of OAB patients, levels of ANP, but not BNP, were increased. ROC curve for ANP was close to 0.70, similar to the ones reported for proBDNF/BDNF and proNGF/NGF in previous clinical publications. (b) Receptors of natriuretic peptides, NPR-A, NPR-B and NPR-C were found expressed in both UROs and SMCs. Known affinities to each natriuretic peptide for these receptors are denoted by corresponding arrows.

Subsequently, we studied the secretion of BDNF and proBDNF from bladder cells in culture. We observed that SMC are the main source of both proteins while urothelial cells secrete only a minor amount (Figure 5.2a). To determine if the secretion of these hormones is affected by nitric oxide in a way comparable to NGF and proNGF, cells were incubated with a nitric oxide donor, sodium nitroprusside (SNP) (300 microM) for 24 hours. We observed a decrease in BDNF by SNP only in SMCs, with a concomitant decrease in the ratio BDNF/proBDNF (figure 5.2b). This is the opposite of what we observed in vivo in human urine samples, which prompted us to assess the effect of natriuretic peptides on the same cultures.

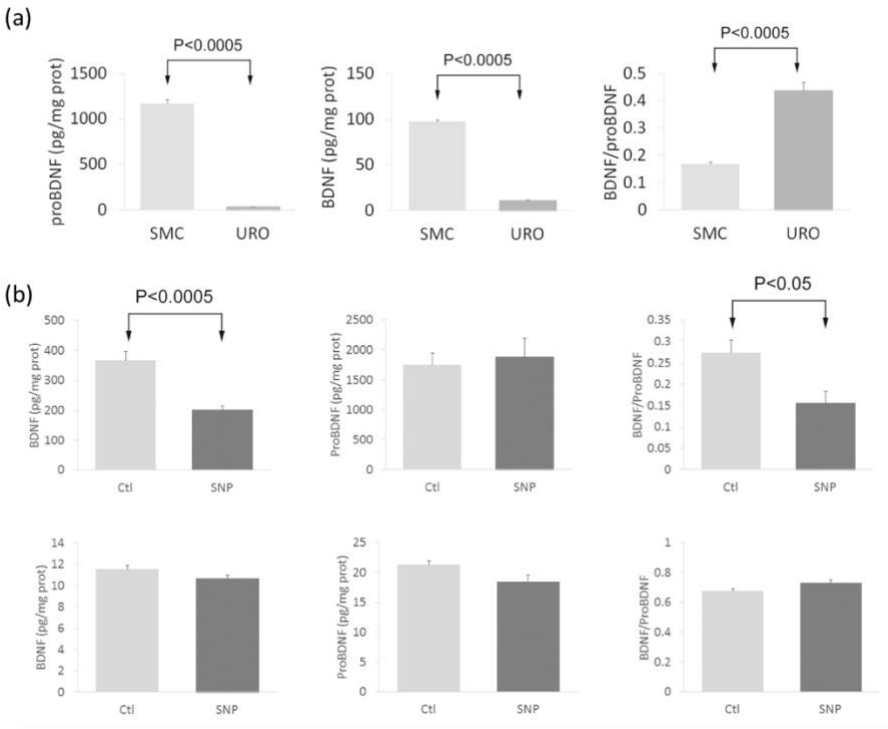

Figure 5.2. Levels of mBDNF and proBDNF in rat bladder SMCs and UROs: (a) Smooth muscle cells (SMCs) from rat bladder are the principal source of secreted BDNF and of its precursor (proBDNF), while urothelial cells (UROs) display a much smaller amount. (b) Addition of sodium nitroprusside (SNP) to the medium, a nitric oxide generator (300 microM), decreases secretion of BDNF in SMCs (upper lane) while the proBDNF levels were unaffected. No change

was observed in UROs (lower lane) probably given the small amount of neurotrophins released. This suggest that a mechanism other than nitric oxide is to account for the observation obtained in the urine of OAB patients.

Incubation of rat bladder cells with atrial natriuretic peptide (ANP, 100 nM) or brain natriuretic peptide (BNP, 1 microM) was carried out for 24 hours. While there was no effect on urothelial cells neither with ANP or BNP, in SMCs, there was a significant decrease in proBDNF and increase in BDNF with ANP, resulting in an increase ratio BDNF/proBDNF, as observed in our clinical samples. (Figure 5.3a). On the other hand, matrix metalloproteinase-9 (MMP-9), the main enzymes that digest proBDNF into BDNF, were expressed by both cell types where UROs appear to be the main source (Figure 5.3b). ANP, but not BNP, increases MMP-9 extracellular activity, which could explain the decrease in proBDNF being generated into mature BDNF. To confirm the role of MMP-9 in this process, knockdown of MMP-9 was performed, resulting in the accumulation of proBDNF in SMCs (Figure 5.3c). Urothelial cells did not show any changes in proBDNF, conceivably due to the small amounts of proBDNF present in these cells. Within the same samples we observed a surge of BDNF in the medium, thus confirming the essential role of MMP-9 in the conversion of proBDNF into BDNF.

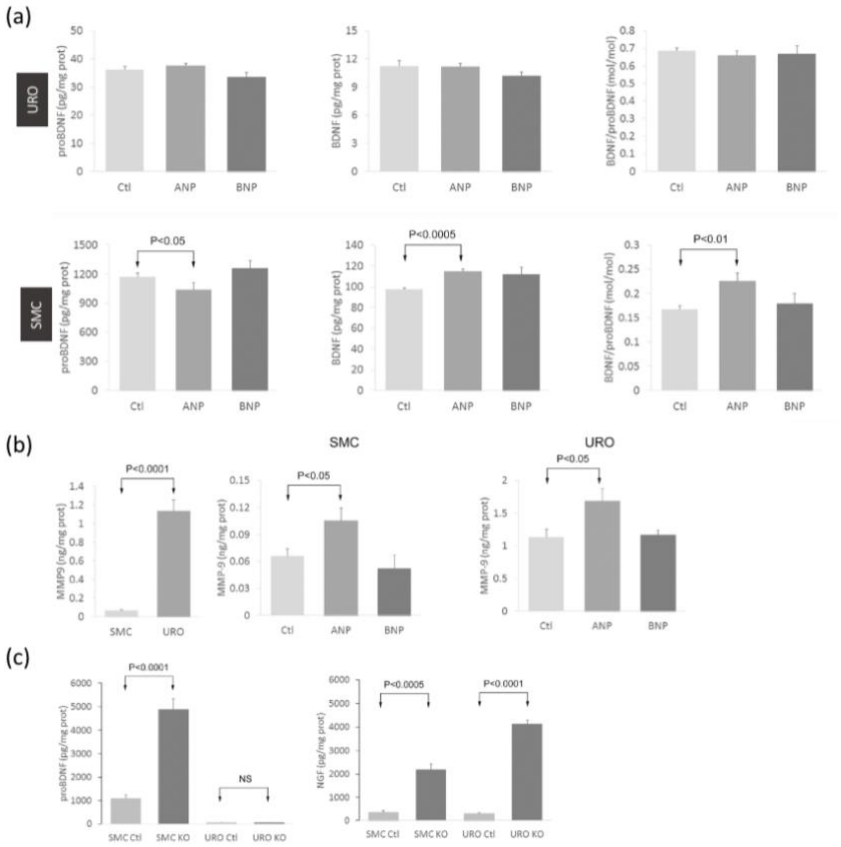

Figure 5.3. Natriuretic peptides, MMP-9, and CrisprCas9 in UROs and SMCs: (a) 24-hour incubation of ANP and BNP in UROs and SMCs. (b) MMP-9, the main enzyme cleaving proBDNF into BDNF, is mainly secreted by UROs and ANP increases its release in both URO and SMC media. (c) Knock-out of MMP-9 by CrisprCas9 confirmed the essential role of this protease in the cleavage of proBDNF. The knockout is confirmed as well by the surge in NGF secretion in the same cells.

Natriuretic receptors are associated to several intracellular pathways, including cyclic AMP and cyclic GMP, nitric oxide synthesis and phospholipase C (Münzel et al., 2003). To understand which second messengers might be involved in the control of the secretion of BDNF, proBDNF and MMP-9 by ANP, SMCs were incubated with ANP (100 nM) and several agents, in a short (10 min) and long-term fashion (24 hrs).

Nitric oxide is linked to natriuretic receptors through nitric oxide synthase (NOS) enzymes (Kone, 2001). Addition of ANP (100 nM) or nitro-L-arginine methyl ester (L-NAME), a non-selective NO inhibitor, (1 mM) for 10 min or 24 hours significantly decreased secretion of nitric oxide by SMCs (Figure 5.4a). L-NAME per se after 24 hours increased BDNF and decreased proBDNF secretions in the medium, resulting in an enhanced ratio of BDNF/proBDNF at levels similar to ANP (Figure 5.4b). On the other hand, we observed a decrease of MMP-9 activity in the presence of L-NAME after 24 hours which is in line with the ability of nitric oxide to stimulate proMMP-9 catalytic activity (O'Sullivan et al., 2014). As ANP increases MMP-9 activity, the decrease in nitric oxide must be stimulating BDNF secretion through a mechanism independent from MMP-9 (Figure 5.4c).

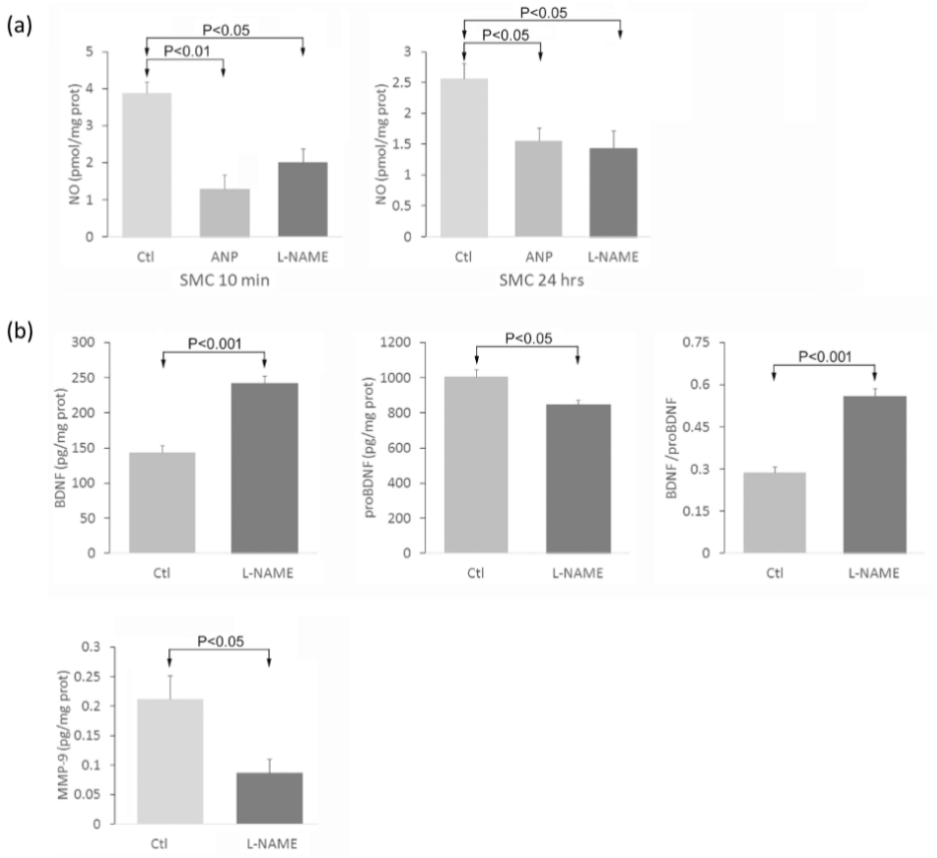

Figure 5.4. NO secretion in SMCs treated with ANP (100 nM) or L-NAME (1 mM) for 10 min or 24 hours: (a) SMCs treated with ANP (100 nM) or L-NAME (1 mM) for 10 min or 24 hours significantly decreases secretion of nitric oxide. (b) L-NAME mimicked the effect of ANP on BDNF, proBDNF, BDNF/proBDNF ratio, but not on MMP-9.

In consequence, we investigated cyclic AMP. In SMCs, its level was increased by ANP (100 nM) and decreased by L-NAME (1 mM) only in the short term (Figure 5.5a). When incubating SMCs with dibutyryl cyclic-AMP (500 µM), a membrane-permeable analog of cylic AMP, MMP-9 activity, BDNF secretion, and the ratio BDNF/proBDNF were increased (Figure 5.5b). Secretion of proBDNF was in parallel decreased, mimicking the effect of ANP alone. Dibutyryl cyclic-AMP did not change nitric oxide levels (results not shown). This suggests that ANP might control BNDF and proBDNF secretions by at least two independent mechanisms.

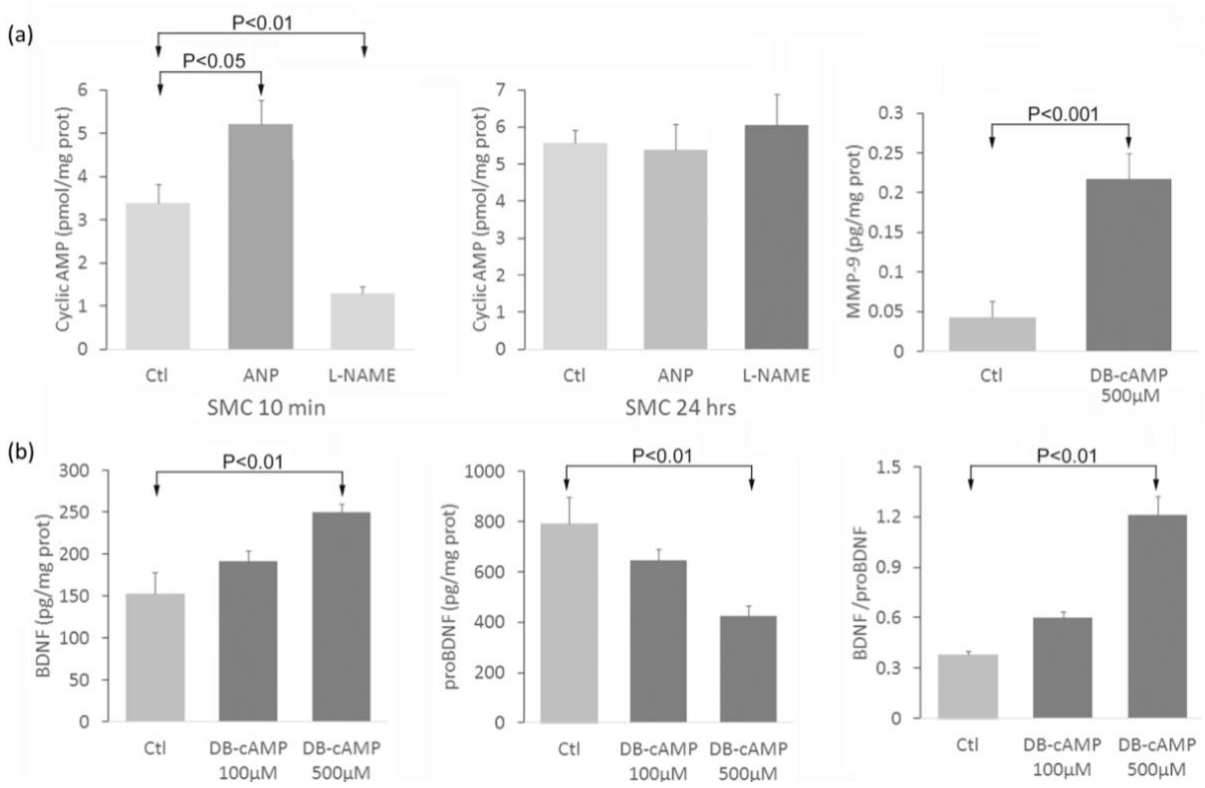

Figure 5.5. Cyclic AMP in the secretion of BNDF/proBDNF in smooth muscle cells (SMC). (a) Incubation of SMC with ANP (100 nM) for 10 minutes led to a significant increase in cyclic AMP while L-NAME (1 mM) decreased it. No changes were observed for longer incubations. Extracellular MMP-9 was increased by the cell-permeable analog dibutyryl cyclic-AMP (DB-cAMP) (500 μM) (a). This analog also dose-dependently increased the secretion of mature BDNF and decrease proBDNF (b).

Finally, ANP did not elicit changes in the levels of mRNA for BDNF nor MMP-9 (Figure 5.6). There was an increase in cyclic GMP after short and long incubations with ANP, but this pathway remained to be assessed as our kits cannot distinguish the origin of this nucleotide. These results suggest that ANP increases BDNF and decreases proBDNF secretion by at least two mechanisms, one involving an MMP-9-independent nitric oxide stimulated pathway (long term) and a separate pathway requiring an increase of MMP-9 through increase in cyclic AMP (short term).

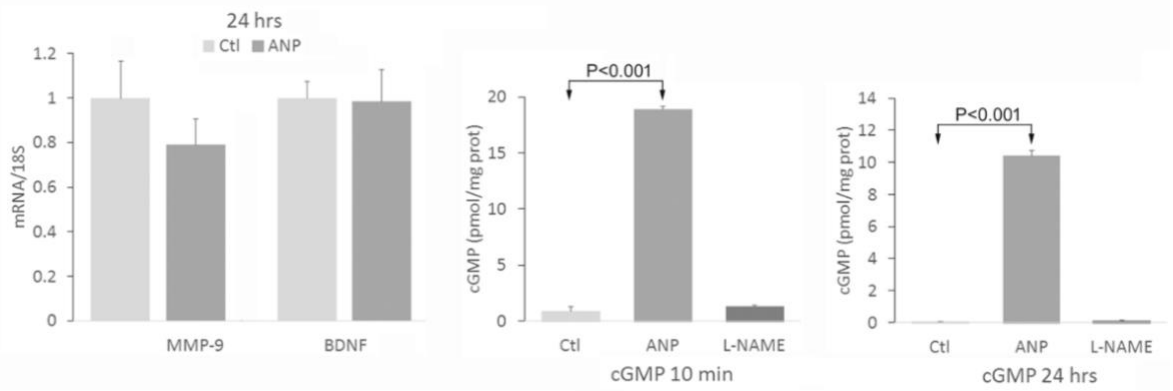

Figure 5.6. Levels of MMP-9 and BDNF mRNA were measured after 24 hours with ANP. Cyclic GMP was measured after 10 min or 24 hrs in the presence of ANP (100 nM) or L-NAME (1 mM). n=5-6, student t-test or one-way ANOVA using control (Ctl) as reference.

Discussion

In the present study, we sought to test the hypothesis that natriuretic peptides were related to neurotrophin regulation in bladder cells. We first observed that female OAB patients present with a significantly elevated urinary ANP level when compared to matched controls, suggesting that these changes could also be observed in bladder cells *in vitro*. We additionally found differential relationships of ANP and BNP levels dependent on cell subtype, urothelial and smooth muscle cells respectively, and mediating factors including MMP-9 enzymatic activity and NO. Looking into intracellular pathways regulating BDNF secretion in SMCs, ANP triggered a decrease in NO and an increase in cyclic AMP. L-NAME, a NOS enzyme inhibitor, decreased NO and proBDNF and increased BDNF mimicking ANP, but decreased MMP-9. On the other hand, Dibutyryl cAMP (500 µM) yielded the same results than ANP including increases in MMP-9 activity. These results demonstrate that ANP controls the secretion of BDNF and its precursor in bladder tissue by acting on both URO and SMC cells. Two distinct pathways might be stimulated by ANP in SMCs.

One of the limitations of the present study is our lack of knowledge regarding the prevalence of cardiovascular comorbidities in the 40 human urine samples as these could confound the levels of NPs present in the urine of these patients. Although the original study cohort was thoroughly screened to not include subjects taking medication or have diagnosis of disease that could confound results of voiding dysfunction, we did not specifically exclude those who had underlying cardiovascular disease such as heart failure (HF). There is a direct association between bladder dysfunction and HF pathophysiology due to compensatory secretion of natriuretic peptides (H.-N. Kim & Januzzi, 2010). Alternatively, medications used to treat HF (e.g., b-blockers, angiotensin-converting enzyme (ACE) inhibitors, and diuretics) and aging-related comorbidities such as

diabetes mellitus and renal failure, may indirectly prompt the precipitation of lower urinary tract symptoms. The therapeutic effects of diuretics in HF increase urine sodium excretion and volume of urine, which can lead to urinary frequency, urgency, and incontinence. Conversely, pharmacological agents that treat OAB appear to worsen or precipitate HF symptoms through pathways unique to their specific therapeutic effect (Tannenbaum & Johnell, 2014).

Previous reports have indicated that up to 50% of patients with heart failure (HF) suffer from urinary incontinence (UI) and OAB, where studies have measured the impact of aging and urinary NPs in nocturnal polyuria and nocturia, symptoms that overlap with OAB diagnosis (Yoshimura et al. 2012; Khosla et al. 2022). Additionally, OAB has been described as a distress symptom in HF pathogenesis (Son & Kwon, 2018). A study on over five thousand Japanese women correlated plasma BNP levels with prevalence and severity of nocturia (Yoshimura et al., 2012). However, these associations still lack sufficient data to fully understand the relationship between UI, OAB, HF and the role of NPs therein.

Another limitation to our study is the lack of presenting data on the male counterpart as all the *in vitro* assessments used female Sprague Dawley rat models. It is well established that there is inter-variability between genders in rat studies, including sex-hormone regulated expression and action of transporters in renal function (A. K. Roy & Neuhaus, 1967; Morel, 1990; Sabolić et al., 2007; Grötsch et al., 1985). At puberty, male rats excrete more protein than female rats, and this amount increases with aging (Stonard, 1990). Gautier et al. showed that there were differences between the sexes for the majority (14 of 16) of the urinary biomarkers that they studied (Gautier et al., 2014). In rat urodynamics, studies have shown that estrogen, and lack of testosterone, are attributed to the enhanced bladder capacity of female compared to male rats (Patra et al., 2008,

2009). Unfortunately, much of the available data on metabolic cage studies in rats has generated conflicting conclusions when describing the comparison between both genders.

Lastly, we did not include morphological changes of bladder wall architecture via microscopy that can further unveil the functional role and expression of these molecules. Nevertheless, the present results could help bridge gaps between these two conditions, OAB and HF, that are highly associated with aging and their interrelation can be considered in future OAB clinical studies as part of the inclusion/exclusion criteria.

Conclusions

Despite the implicit limitations of an *in vitro* study, to the best of our knowledge, we are the first to compare urinary natriuretic peptides in patients with OAB and to provide in vitro analysis on their impact of neurotrophin secretion in bladder cells. Urinary biomarkers yield non-invasive assessment over other biomarker studies, such as plasma or urodynamic studies (UDS). Overall, the study demonstrated that by using a matched study on urine metabolomics and proteomics from female patients with OAB, neurotrophin secretion in bladder cells is controlled, partly, by natriuretic peptides and that an enhanced secretion of active MMP-9 appears to be essential. Additionally, this could yield new insights on the implications of urinary metabolomics in theranostic and phenotypic signatures of OAB and related voiding dysfunction. It is yet to be fully understood how the mechanisms underlying the interaction between neurotrophins and natriuretic peptides can translate into applicability in better understanding the pathophysiology of bladder function, particularly in OAB. NPs, specifically ANP, possess unique cell signaling, paracrine, and autocrine effects at a systemic level. As we highlight in this study, their effect in bladder cells represent a promising starting point for future directions in OAB. Future studies should assess NPs

levels in human bladder tissue and translate these findings in preclinical studies to establish causality.

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Institutional Review Board Statement: This study was approved by the Medical-Biomedical Research Ethics Committee (REC) of the Integrated Health and Social Services University Network for West-Central Montreal (IRB: 2016-328, 15-022, approved on 20 June 2017). The animal study was approved by the Animal Ethics Committee of McGill University (Quebec, Canada).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the OAB study.

Data Availability Statement: Data are unavailable due to privacy or ethical restrictions. **Conflicts of Interest:** The authors declare no conflict of interest.

Chapter 6: Discussion and Conclusion

6.1 Summary of findings

With this project, we were able to combine two research approaches, clinical and basic research. The conglomeration of the findings highlights important concepts involving the role of urinary mBDNF and proBDNF in overactive bladder syndrome (OAB) as observed in an aging female population. Chapter 4 places emphasis on our clinical work, demonstrating the important role of the neurotrophin regulator MMP-9, with its increased enzymatic activity leading to an increase of proNGF/NGF ratio (Mossa et al., 2021) and a decrease of the proBDNF/BDNF ratio (Covarrubias et al., 2023). These ratio imbalances prompted by the enzymatic activity of MMP-9, could partly be caused by aging and local tissue factors, such as natriuretic peptides (NPs), nitric oxide and prostaglandin E2 (PGE2), all metabolites found to be elevated in our OAB cohort (Covarrubias et al., 2023; Mossa et al., 2020). After finding atrial natriuretic peptide (ANP) to be significantly increased in OAB patients, we sought out to study bladder cells *in vitro* focusing on shared intracellular pathways that could untangle mechanisms tethered to neurotrophins and NPs.

We illustrate our *in vitro* findings in chapter 5, where we isolated and cultured both urothelial and smooth muscle cells from 8-week-old female Sprague Dawley rat bladders. Consequently, we treated these cells with ANP and measured changes in mBDNF, proBDNF, and MMP-9. We found that particularly in smooth muscle cells (SMCs), ANP caused an increase of mBDNF and a decrease in proBDNF, which could, in part, explain the decrease of the proBDNF/BDNF ratio found in our OAB cohort. We further supported this using CrisprCas9 KO of MMP-9, confirming its essential role in the proteolysis of mNGF into peptides and the conversion of proBDNF to mBDNF. This *in vitro* study additionally revealed key second messengers that linked and further supported our *in vivo* findings. First, we found that ANP-treated cultures increased cAMP levels,

which could explain its control on mBDNF and proBDNF secretion. Secondly, the cGMP second messenger was also elevated in ANP-treated cultures, but given the limited specificity of our kits, we were unable to deduce the mechanism of action of this pathway.

6.2 Areas of Opportunity

Platelets are a major source of BDNF. Although we excluded patients who had underlying urological conditions that could confound the results, such as macrohematuria, we conducted a verification protocol in these samples using a human platelet membrane glycoprotein (CD41) ELISA kit (Novus Biological, CO, USA) in the circumstance that there was microhematuria not accounted for. We did not find a statistically significant difference on platelet count between controls and OAB patients (presented as CD41/Cr (pg/mg); CTL 0.23±0.05(SEM) vs. OAB 0.16± 0.02(SEM)) (Figure 6.1a). To further verify these insignificant urinary platelet traces, we cross-checked for immunoreactivity (LSBio, MA, USA)) for urinary α2-macroglobulin (A2M) levels (Figure 6.1b), showing absence of this plasma protein in the preparations (presented as A2M/Cr (pg/mg); CTL 4334.9±1163.7(SEM) vs OAB 12370.69± 5139.6(SEM)). Mature neurotrophins have been shown to bind reversibly to α2-macroglobulin, a plasma protease inhibitor and transporter, protecting them against proteolytic degradation and clearance pathways (Le Blanc et al., 2020). As a learning point, we determined that future urinary biomarker studies assessing BDNF should consider platelet cross-contamination in their analyses.

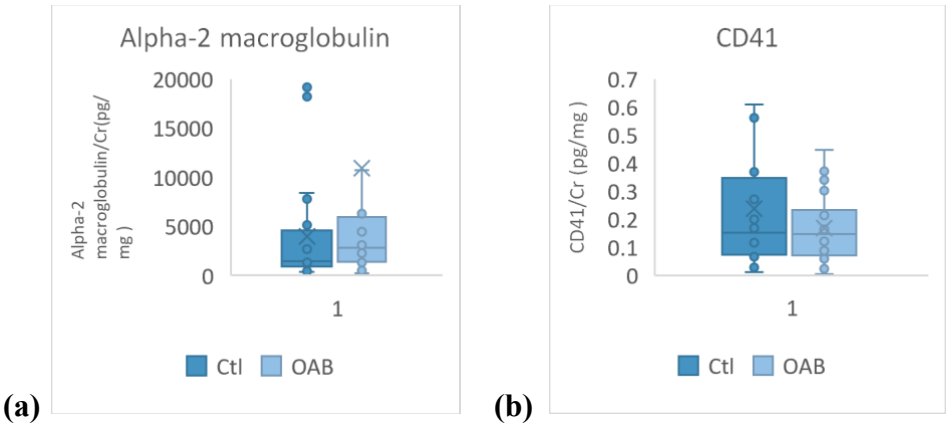

Figure 6.1. Urinary Platelet CD41 marker and α2-macroglobulin levels. Levels of (a) CD41/Cr (pg/mg) and (b) α2-macroglobulin (A2M) levels as shown in control (ctl) and OAB groups. There were no noted statistically significant changes.

Additionally, we speculate a noteworthy impact of long-term storage and freeze-thawing cycles of our urine samples as this could have led to a loss of exosomes, propagating a drop of microRNA levels to below detection limits. Meticulous research is warranted to assess the storage stability of purified exosomes and exosomes frozen in urine samples collected in the home environment, as used in this study. Another area of opportunity is to further explore the relationship between cardiovascular disease (CVD) and voiding dysfunction. We saw a clear correlation between ANP, an established biomarker for heart failure (HF) and important hormone that regulates water homeostasis. As we move towards phenotyping OAB contingent on the patient's comorbidities, we must place scrupulous attention to age-related factors that can exert influence on urinary symptoms. Such is the case for HF and related CVDs. To our knowledge, we are the first to assess the role of ANP in bladder cells *in vitro* and its interactivity with neurotrophins. Nonetheless, more attention should be noted in both the clinical and research domains.

Current strategies investigating viable urinary biomarkers for OAB fail to expand beyond single-standing neurotrophin levels, which could explain the vast heterogeneity amongst the consensus reached to date.

6.3 Conclusion

This work unburies mechanisms that further establish the role of neurotrophins in overactive bladder syndrome. The integration of urinary metabolomics and neurotrophin analysis could provide valuable insights into the underlying mechanisms, and together with neurotrophins and their related metabolites, make up a phenotype-centered diagnostic panel that can complement urodynamic studies and clinical diagnostic workup. Thus, further investigation is warranted to identify the molecular effect of neurotrophins, natriuretic peptides, and their corresponding receptors on the bladder and to find an association to their levels that correspond to disease progression and treatment response. These exploratory biomarkers have the potential to guide theranostic, personalized treatment strategies and monitor disease progression for individuals suffering from OAB.

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