Establishing a new rodent model of partial bladder outlet obstruction and the role of macrophages in bladder obstruction

by

Martin Sidler, MD

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

© Copyright by Martin Sidler 2017
Establishing a new rodent model of partial bladder outlet obstruction and the role of macrophages in bladder obstruction

Martin Sidler
Master of Science
Institute of Medical Science
University of Toronto
2017

Abstract

Partial bladder outlet obstruction has a high prevalence, develops insidiously, and can severely affect quality of life and health of patients. Obstruction leads to bladder hypertrophy and ultimately to fibrosis. While macrophages are known as central regulators of fibrosis in many organs, their role in bladder remodeling is poorly understood. We hypothesized that macrophages modulate bladder remodeling in the context of partial outlet obstruction. We developed a partial bladder outlet obstruction rodent model that avoids dissection around the bladder neck, thus avoiding a wound healing response of that area affecting the bladder. Importantly, our model also preserved bladder innervation, in contrast to the traditionally used model. Bladder outlet obstruction increased the number of macrophages and depletion of macrophages not only affected cytokine levels in bladder tissue but also helped preserve bladder function. Macrophages seem to contribute to obstruction-related bladder dysfunction.
Acknowledgments

First, I want to express my gratitude to my parents, who invested so much in me, offering me all the opportunities in life that they themselves never had! Mom, Dad, you are amazing examples, you never gave up in life, and words cannot express how thankful I am for all you instilled in me. I also thank my dearest wife, Claudia – you are such a lovely and supportive woman, I would not be who I am without you! Most of all, I am deeply grateful to God, who sent His son Jesus Christ to lay down His life to pay for my failure, so I can live free from guilt and shame, and have the hope of eternal life. No words can express what You did for me!

I would also thank my supervisor, Dr. Darius Bagli, who taught me principles of scientific thinking and mentored my journey through the course of my program. I greatly benefitted from his extensive experience and creative ideas on how to address the relevant questions. My deep gratitude also goes out to Dr. Karen Aitken, you are an amazing friend, mentor, and teacher to me. I am also grateful to my program advisory committee members, Drs. Benjamin Alman and John Coles, both dedicated hard working surgeons and brilliant scientists.
Contributions

Several people have contributed to this thesis: Janet Jiang with her practical support in tissue sectioning and staining, Dominika Bijos with her expertise in measurement of tissue strip contractility, and the whole team at the Sick Kids animal facility providing excellent and dedicated care to our study animals.
# Table of Contents

Acknowledgments .......................................................................................................................... iii

Contributions ................................................................................................................................. iv

Table of Contents ............................................................................................................................ v

List of Tables ........................................................................................................................................ ix

List of Abbreviations ........................................................................................................................ x

List of Figures ..................................................................................................................................... xiii

1 Literature Review .......................................................................................................................... 1

1.1 The Urinary Bladder and its Function ......................................................................................... 1

1.2 Partial Bladder Outlet Obstruction .............................................................................................. 2

1.2.1 Clinical Consequences of partial Bladder Outlet Obstruction ........................................... 2

1.2.2 Anatomical Bladder Outlet Obstruction .............................................................................. 4

1.2.3 Neurogenic and Functional Bladder Outlet Obstruction .................................................... 4

1.2.4 Pathophysiology of the Obstructed Bladder ....................................................................... 5

1.2.5 Tissue and Cellular Response to Obstructive Stimuli ......................................................... 6

1.3 Treatment of partial Bladder Outlet Obstruction ...................................................................... 7

1.3.1 Pharmacologic and Surgical Options .................................................................................. 7

1.3.2 Treatment of LUTS ........................................................................................................... 8

1.4 Macrophages and their Subtypes ............................................................................................... 9

1.5 Tissue Fibrosis and the role of Macrophages in Remodeling .................................................. 9

1.6 Immune cells in Bladder Remodeling ....................................................................................... 11

1.6.1 Circulating Immune Cells in Bladder Outlet Obstruction ................................................. 11

1.6.2 Macrophages in Bladder Outlet Obstruction ..................................................................... 11

1.7 Limitations in Modelling pBOO in Animals ............................................................................ 12

2 Hypothesis and Aims .................................................................................................................... 16

2.1 Hypothesis .................................................................................................................................. 16

2.2 Aims ........................................................................................................................................... 17
3 Novel Model for Partial Bladder Outlet Obstruction in Mice

3.1 Introduction

3.2 Material and Methods
3.2.1 Experimental animal groups
3.2.2 Surgical Procedures
3.2.3 Bladder functional parameters
3.2.4 Contractility assay
3.2.5 Histology
3.2.6 Statistics

3.3 Results
3.3.1 Obstructed Animals
3.3.2 Sham Animals
3.3.3 Structural Responses to Dissection
3.3.4 Mid-term characteristics of NeMO

3.4 Discussion
3.4.1 NeMO safely models functional and structural changes
3.4.2 NeMO shams lack the confounding features of PU shams
3.4.3 Detrusor strip contractility
3.4.4 Denervation
3.4.5 Time course
3.4.6 Relating NeMO to other pBOO models

3.5 Conclusion

4 Application of Nerve-Sparing mid-urethral Obstruction (NeMO) in Female Mice

4.1 Introduction

4.2 Material and Methods
4.2.1 NeMO in female rats
4.2.2 NeMO in female mice

4.3 Results
4.3.1 Nerve-sparing Mid-urethral Obstruction (NeMO) in female rats
4.3.2 Nerve-sparing Mid-urethral Obstruction (NeMO) in female mice

4.4 Discussion
5 Assessment of Murine Bladder Function

5.1 Introduction

5.2 Material and Methods
5.2.1 Cages and Recording Equipment
5.2.2 Data Analysis Algorithm
5.2.3 Surgical Procedure and Drug Treatment
5.2.4 Statistical Analysis

5.3 Results
5.3.1 Micturition recording and analysis method
5.3.2 Functional Changes associated with pBOO
5.3.3 Literature review

5.4 Discussion
5.4.1 Equipment and data analysis for CUWR
5.4.2 Overview of options to assess murine bladder function

5.5 Conclusion

6 Role of Macrophages in Obstruction-Induced Bladder Remodeling

6.1 Introduction

6.2 Material and Methods
6.2.1 Preliminary assessment of macrophage density in rat bladder after pBOO
6.2.2 Depletion of bladder macrophages using Clodronate Liposomes®
6.2.3 Cytokine assay
6.2.4 Partial bladder outlet obstruction and macrophage depletion trial I
6.2.5 Partial bladder outlet obstruction and macrophage depletion trial II
6.2.6 Recording and analysis of bladder function
6.2.7 Histologic evaluation
6.2.8 Statistics

6.3 Results
6.3.1 Macrophage density correlates with residual urine and bladder mass
6.3.2 Bladder macrophage depletion and quantification in mouse
6.3.3 Dose Finding and Cytokine Screen
6.3.4 Macrophage Depletion during pBOO Trial I
6.3.5 Macrophage Depletion during pBOO Trial II
6.4 Discussion
6.4.1 Obstruction increases number of bladder macrophages
6.4.2 Clodronate effectively mitigates obstruction-related macrophage increment
6.4.3 Cytokines correlate with bladder function
6.4.4 Clodronate treatment preserves function in the obstructed bladder

7 General Discussion and Future Directions
7.1 Animal model of nerve-sparing mid-urethral obstruction
7.2 Macrophages in obstruction-induced bladder remodeling
7.2.1 Cytokine involvement in remodeling by macrophages
7.2.2 TIMP and MMP involvement in remodeling of macrophages
7.2.3 Other roles of macrophages in tissue remodeling
7.3 Future work
7.3.1 Potential of the Nerve-sparing model
7.3.2 Future work with macrophage depletion models
7.3.3 Potential cytokine work
7.3.4 Understanding of macrophage to myofibroblast transdifferentiation
7.4 Conclusions

References

Copyright Acknowledgements
# List of Tables

## Chapter 3

| Table 3.1       | Overview of various pBOO methods used in mice and rats |

## Chapter 5

| Table 5.1       | Overview of methods assessing mouse bladder physiology |
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BOO</td>
<td>Bladder outlet obstruction</td>
</tr>
<tr>
<td>BOOI</td>
<td>Bladder outlet obstruction index</td>
</tr>
<tr>
<td>BPE</td>
<td>Benign prostatic enlargement</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BSMC</td>
<td>Bladder smooth muscle cell</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif ligand 2</td>
</tr>
<tr>
<td>CIC</td>
<td>Clean intermittent catheterization</td>
</tr>
<tr>
<td>CL</td>
<td>Clodronate liposomes</td>
</tr>
<tr>
<td>CyTOF®</td>
<td>Cytometry by time-of-flight</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DU</td>
<td>Detrusor underactivity</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Gd</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>HS</td>
<td>Hinman Syndrome</td>
</tr>
<tr>
<td>IC</td>
<td>Interstitial cell</td>
</tr>
<tr>
<td>ICC</td>
<td>Interstitial cells of Cajal</td>
</tr>
</tbody>
</table>
IFN-γ  Interferon gamma
IL  Interleukin
iNOS  Inducible Nitric Oxide synthetase
IP  Intraperitoneal
ko  Knock-out
LUTO  Lower urinary tract obstruction
LUTS  Lower urinary tract symptoms
MCP-1  Monocyte chemoattractant protein 1
MDSC  Myeloid derived suppressor cells
MFB  Myofibroblast
MIF  Macrophage migratory inhibitory factor
MMP  Matrix metalloproteinase
NeMO  Nerve-Sparing mid-urethral Obstruction
NGB  Neurogenic bladder
NS  Normal saline
OAB  Overactive bladder
OCT  Optimal cutting temperature
pBOO  Partial bladder outlet obstruction
PCR  Polymerase chain reaction
PFA  Paraformaldehyde
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUV</td>
<td>Posterior urethral valve</td>
</tr>
<tr>
<td>PVR</td>
<td>Post void residual</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNaseq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>VSOP</td>
<td>Voided stain on paper</td>
</tr>
<tr>
<td>VUR</td>
<td>Vesico-ureteral reflux</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
## List of Figures

### Chapter 3

| Figure 3.1 | Dissection in Nerve-sparing Mid-urethral Obstruction (NeMO) is less extensive than in Proximal Urethral obstruction |
| Figure 3.2 | Definition of measured and calculated micturition parameters describing bladder physiology |
| Figure 3.3 | After 2 weeks, NeMO led to changes similar to obstruction-induced human bladder pathology |
| Figure 3.4 | Increase in absolute bladder mass is moderate 2 weeks after PU sham and NeMO while excessive after PU |
| Figure 3.5 | PU sham, as well as, obstruction procedures, alter spontaneous contraction pattern |
| Figure 3.6 | PU sham procedure increased contractile strength of bladder strips |
| Figure 3.7 | Obstruction technique mediates nerve fibers morphometry coordinately with mean micturition fraction in sham animals |
| Figure 3.8 | Structural and functional changes in NeMO sham vs up to 12 weeks of obstruction |
| Figure 3.9 | Correlations of NeMO-induced bladder mass with bladder function |

### Chapter 4

| Figure 4.1 | NeMO effectively induces increase in bladder mass after 2 weeks |
| Figure 4.2 | Marked increase in residual urine 2 weeks after NeMO |
| Figure 4.3 | Mortality increased with degree of partial obstruction |
| Figure 4.4 | 26-gauge and 27-gauge placeholder during NeMO result in effective increase in relative bladder mass |
| Figure 4.5 | Maintained bladder emptying 2 weeks after NeMO |

**Chapter 5**

| Figure 5.1 | Equipment for weight-based micturition assessment in mice |
| Figure 5.2 | Data recording and discrimination of urine-induced increase in recorded weight |
| Figure 5.3 | Partial bladder outlet obstruction (pBOO) and treatment with S6K-inhibitor |

**Chapter 6**

| Figure 6.1 | Immunofluorescence staining of detrusor area of rat bladders 2 weeks after pBOO |
| Figure 6.2 | Correlation of number of macrophages with bladder mass and residual urine volume |
| Figure 6.3 | Number of macrophages in pooled bladder and spleen samples of controls 2 weeks after pBOO |
| Figure 6.4 | Cytokine levels are affected by CL-treatment and correlate with bladder function |
| Figure 6.5 | Obstruction increased bladder mass (Trial I) |
| Figure 6.6 | CL-treatment affects bladder physiology |
| Figure 6.7 | Obstruction increased bladder mass (Trial II) |
1 Literature Review

1.1 The Urinary Bladder and its Function

The urinary bladder is a hollow smooth muscle with two main functions, to collect and store urine at low pressure and to release urine efficiently and completely. The bladder is highly distensible, which allows it to adapt to high volume during the filling phase. A coordinated relaxation of its external sphincter along with a contraction of the detrusor muscle leads to almost complete emptying in a normal functioning bladder. All the layers of the bladder wall – the urothelium with its lamina propria, the submucosa, the detrusor, and the adventitia that is covered with serosa towards the peritoneal cavity – play a role in distension and contraction of the bladder; alterations in any of these compartments can cause bladder dysfunction. Smooth muscle hypertrophy or excess in extracellular matrix (ECM) for instance can lead to reduced bladder compliance, as well as, incomplete emptying.

Structurally, the bladder is divided into the superior part, which is referred to as the dome, while the inferior half is referred to as the bladder base. The wall of the bladder dome is more distensible and thinner than the bladder base. Urine from both kidneys drains into the bladder via the two ureters and their ostia and is then eliminated from the bladder through the urethra. The area between ostia and the internal urethral meatus is called the trigone.

The bladder wall is composed of the following layers: The mucosa consists of lamina propria and urothelium. The latter lines the lumen of the bladder, providing a distensible barrier protecting underlying tissue from urine. Large transitional epithelial cells form the surface layer of the urothelium; their capability to flatten for more surface area allow luminal expansion during bladder filling. The submucosa consists of blood vessels, nerve fibers, connective tissue, and smooth muscle, it provides the tissue with oxygen and nutrients. The strongest layer of the bladder wall is the detrusor, which is organized into three layers, an inner
and outer longitudinal layer, and a middle circular layer\(^1\). The latter forms a circular preprostatic sphincter around the bladder neck, contributing to continence at this level.

Cycles of urine accumulation and expulsion are controlled by complex neural circuits. These involve afferent and efferent nerve endings in the urothelium, the contractile smooth muscle cells, and in the ECM; pelvic, hypogastric, and pudendal nerve link the bladder to the spinal cord, the brainstem, and the cerebral cortex. With increased luminal volume during the filling phase, intravesical pressure rises only minimally\(^2\). The detrusor’s smooth muscle bundles are required to remain elongated for up to several hours so that low pressure can be maintained during the filling phase. In the normal bladder, the urothelium’s and the detrusor’s mechanoreceptors cause increased afferent activity when the threshold volume is reached. The micturition reflex involves decreased sympathetic efferent activity and decreased activity of the pudendal nerve innervating the external sphincter, leading to relaxation of both urethral sphincters, which is followed by a coordinated contraction of the detrusor muscle, allowing efficient bladder emptying\(^3\).

### 1.2 Partial Bladder Outlet Obstruction

#### 1.2.1 Clinical Consequences of partial Bladder Outlet Obstruction

Partial bladder outlet obstruction (pBOO) in humans is defined by the International Continence Society (ICS) as “the generic term for obstruction during voiding. It is a reduced urine flow rate and/or presence of a raised post void residual (PVR) and an increased detrusor pressure”\(^4\). More technically, pBOO is defined as an increase in bladder outlet obstruction index (BOOI), which in turn is derived from the slope of the line separating *equivocal* from *obstructed* in the Abrams-Griffiths nomogram, which plots maximum urinary flow against detrusor pressure at the time of maximum flow\(^5\). Notably, these criteria have been established in a male population, while they are not fully applicable in women, given their specific differences in mobility of the proximal urethra and the bladder neck, as well as, activity of the pelvic floor\(^6\). While some suggested defining pBOO in women based on radiographic evidence of urethral occlusion, others proposed that any detrusor peak pressure exceeding 60 cm H\(_2\)O along with a maximum flow rate of less than 15 ml/s and funneling of the bladder neck with the urethral sphincter relaxed should be considered BOO in female patients\(^7,8\). Some researchers also suggest
factoring in the abdominal pressure during a pressure-flow study, as abdominal pressure affects intravesical pressure\(^9\). In other words, bladder outlet obstruction is present when during voiding normal urinary flow requires elevated intravesical pressure or when normal intravesical pressure results in a diminished urinary flow. PBOO can affect humans of both genders at virtually any age. In a male fetus, for instance, posterior urethral valves (PUV) – affecting about 1 in 5000 life born boys – can lead to bladder outlet obstruction and often to consequent congenital kidney damage of variable degree. Notably, PUVs are one of the most common underlying causes of pediatric kidney transplantation\(^{10,11}\). In women, urethral stricture is the most common cause of pBOO, although there are other origins of intrinsic urethral obstruction, as well as, diverse reasons of extrinsic urethral compression by pelvic organ prolapse, for instance\(^6\).

Benign prostatic enlargement (BPE) is arguably the most common cause of pBOO and it is also the most common etiology of lower urinary tract symptoms in men (LUTS). Patients with BPE and associated LUTS present themselves at the doctor’s office with complaints such as urinary frequency, urgency, difficulty initiating urination, nocturia, interruption of stream, decreased force of stream, and sense of incomplete bladder emptying\(^12\). BPE affects 70% of men in the US 60-69 years of age and 80% of men 70 years of age or older\(^13\); at the same time, LUTS affect 35% of men 60-69 years, 56% of men 50-79 years, and 70% of men 80-89 years of age, and prevalence was increasing over the past 2 decades\(^{14,15}\). While it is possible to have LUTS without BPE and vice versa, still most men with BPE have LUTS and most men with LUTS have BPE\(^12\). BPE with its associated diagnosis and treatment is also an important public health issue. In 2000, BPE caused 1.1 billion dollars in direct health care costs, accounted for over 4.4 million office visits, 117,000 visits to emergency departments, 105,000 hospitalizations, as well as, about 30 million hours in lost productivity in the US. Also in 2000, direct costs of treatment for overactive bladder (OAB) in men were 1.8 billion dollars, with another 3.9 billion dollars of indirect costs. Estimated annual costs of BPE-treatment in the US total 3.9 billion dollars\(^{13,16,17}\). Although the prevalence of female bladder outlet obstruction (BOO) is lower than in men, it is estimated to range from 2.7% to 23% and results from causes listed below (1.2.2.2)\(^{18}\). Despite release of pBOO by partial prostatectomy, almost 25% of BPE patients have persistent poor bladder function\(^10\), while up to 50% of boys with PUV have persistent bladder dysfunction after surgical treatment\(^{10,20,21}\). Not only bladder function but also bladder structure can be
persistently altered after surgical release; evidence suggests that bladder wall thickness is persistently increased above norm values after BPE treatment\textsuperscript{22,23}.

1.2.2 Anatomical Bladder Outlet Obstruction

1.2.2.1 Congenital Bladder Outlet Obstruction

The presence of PUV is one of the most common causes of congenital lower urinary tract obstruction, which affects about 2.2 in 10,000 births, followed by Prune Belly Syndrome and rarer conditions such as urethral atresia, anterior urethral valves or diverticulum, prolapsing ureterocele, syringocele, megalourethra, megacystis-microcolon-hypoperistalsis syndrome, obstruction by hydrocolpos or tumor such as a sacro-coccygeal teratoma\textsuperscript{24,25}.

1.2.2.2 Acquired Bladder Outlet Obstruction

Post-surgical or post-traumatic scar formation, as well as, radiation-induced fibrosis, can lead to urethral strictures or extended narrowing in both genders. Especially in women, extrinsic urethral compression can result from pelvic organ prolapse or can be iatrogenic after mesh or sling procedures applied in anti-incontinence surgeries. Urologic malignancies and PBE, on the other hand, are more common in men.

1.2.3 Neurogenic and Functional Bladder Outlet Obstruction

Neurogenic forms of BOO range from neuronal dysfunction inherent or close to the bladder such as detrusor – external sphincter dyssynergy and non-relaxing urethral obstruction to central neurologic disorders such as congenital spinal dysraphism, acquired paraplegia, or cerebral defects or diseases, such as Parkinson’s disease or Multiple sclerosis\textsuperscript{18,26,27}. Diabetic cystopathy also often combines features of neurogenic damage and BOO\textsuperscript{28}. Functional BOO, on the other hand, is a non-neurogenic, non-anatomic obstruction with disruption of the normal micturition process such as dysfunctional voiding. In a neurologically normal patient, it is caused by “an intermittent and/or fluctuating flow rate due to involuntary intermittent contractions of the periurethral striated or levator muscles during voiding”\textsuperscript{4}. Other forms of functional BOO include primary bladder neck obstruction or Fowler’s syndrome in women\textsuperscript{18}. 
The Hinman Syndrome (HS) is a particular form of bladder dysfunction with functional BOO that typically occurs early in childhood; the etiology of HS is still poorly understood and can even lead to kidney failure\textsuperscript{29}.

### 1.2.4 Pathophysiology of the Obstructed Bladder

Bladder outlet obstruction leads to detrusor hypertrophy, increased intravesical pressure during voiding counteracting increased outflow resistance, thus maintaining normal urinary flow rates during voiding. The structural and functional response is adaptive at first but can become maladaptive leading to a decompensated bladder over time; both phases are insidious and only become apparent when damage to the bladder has been done. With increasing intravesical pressure built up by the hypertrophic detrusor muscle, otherwise competent ureteral ostia preventing vesico-ureteral reflux (VUR) can become incompetent. Normal voiding pressures in adult men and women range from 50 to 80 cm H\textsubscript{2}O and from 40 to 65 cm H\textsubscript{2}O, respectively\textsuperscript{30}. Based on intravesical pressure, children with neurogenic bladder sphincter dysfunction can be categorized into high- and low-risk groups for secondary kidney damage from a neurogenic bladder. In children, whose detrusor filling pressure exceeds 40 cm H\textsubscript{2}O, glomerular filtration rate decreases along with impaired pyelocaliceal and ureteral drainage, resulting in vesicoureteral reflux and obstructive hydronephrosis\textsuperscript{31}. Even without VUR or dilation of the upper urinary tract, high bladder pressure can impede drainage of urine into the bladder; any continuous or intermittent intravesical pressure exceeding 40 cm H\textsubscript{2}O places children at risk for urinary tract infections, upper urinary tract dysfunction and ultimately renal failure\textsuperscript{31}. These findings support that sterile VUR at low pressure – i.e. less than 40cm H\textsubscript{2}O – does not lead to kidney damage; however, it predisposes for ascending urinary tract infections (UTI) and associated post-infection renal scarring. High degree VUR however – such as often found in patients with PUV – is associated with congenital dysmorphism of the kidneys. Ongoing outlet obstruction causes progressive detrusor hypertrophy, which in turn results in reduced bladder capacity and compliance. Furthermore, chronic distension reduces perfusion and thus tissue hypoxia/ischemia\textsuperscript{32,33}. Functionally, the obstructed detrusor can develop overactivity, underactivity, or a combination of both. The ICS defines detrusor overactivity as “a urodynamic observation characterized by involuntary detrusor contractions during the filling phase which may be spontaneous or provoked\textsuperscript{34}; these involuntary detrusor contractions can occur at different stages of bladder filling and may or may not lead to urinary incontinence.
Detrusor **underactivity** (DU) on the other hand is defined as “a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or a failure to achieve complete bladder emptying within a normal time span”\(^{34}\). An extreme form of detrusor underactivity is the acontractile detrusor. A useful quantitative measure for DU is the post void residual (PVR), which is the amount of urine left in the bladder at the end of micturition.

### 1.2.5 Tissue and Cellular Response to Obstructive Stimuli

The obstruction-induced increase in intravesical pressure directly correlates with increased bladder wall tension, which on a cellular level translates into increased stretch and tension stimuli placed on individual bladder smooth muscle cells (BSMC), as well as, to reduced microvascular tissue perfusion and subsequent tissue hypoxia\(^ {33}\). The various cell types within the urinary bladder respond in different ways to stimuli correlated with BOO. BSMC for instance, respond to stretch, hypoxia, and to denatured extracellular matrix (ECM) with increased proliferation, hypertrophy, and a further increase of extracellular matrix proteins\(^ {35-37}\). Hypoxia, as well as, distension of BSMC, can induce matrix metalloproteinase (MMP) activation\(^ {38}\), and proliferation\(^ {39,40}\). Under stretch, BSMC, as well as, vascular SMC, undergo dedifferentiation\(^ {38,41,42}\), phenotypic signs of dedifferentiation are a loss of smooth muscle actin, as well as, an increase in expression of ECM components such as Elastin, Fibronectin, or different types of Collagen\(^ {43,44}\). In chronic obstruction leading to bladder decompensation, BSMC are then gradually replaced with ECM, leading to bladder fibrosis along with a loss in contractility\(^ {45,46}\).

The **urothelium** senses various mechanical, thermal, and chemical stimuli and responds by releasing chemical factors such as adenosine triphosphate (ATP)\(^ {47,48}\). ATP, in turn, acts on P2 purinergic receptors on afferent nerve fibers in the submucosa, signaling bladder filling\(^ {49,50}\). ATP also stimulates release of acetylcholine and cytokines from the urothelium\(^ {51,52}\). BOO also increases urothelial ATP-release, which may contribute to non-voiding contractions and OAB symptoms in individuals with BOO\(^ {53}\); inducible nitric-oxide synthase (iNOS) is also known to be increased in BOO, possibly improving tissue oxygenation\(^ {54,55}\). Increase in urothelial proliferation is mediated by various growth factors such as epidermal growth factor\(^ {56,57}\), activators of the estrogen receptor\(^ {58,59}\), transforming growth factor-α (TGF-α)\(^ {60}\), transforming growth factor-β (TGF-β)\(^ {61}\), fibroblast growth factors (FGF)-1\(^ {60}\), FGF-2\(^ {62}\), and FGF-10\(^ {63}\).
originating from the lamina propria, and insulin growth factors 1 and 2. Interstitial cells (IC) – whose intestinal counterparts, the Interstitial Cells of Cajal (ICC), act as pacemaker for smooth muscle contraction – also seem to be involved in obstructive bladder pathophysiology. In the bladder, IC functionally connect urothelial cells, BSMC, and autonomic nerve endings. Evidence suggests them to be involved in the development of various forms of urinary tract dysfunction. One of the functional connections of the urothelium and the sensory nerve endings via IC seems to act through their purinergic receptor P2X3, which was shown to be increasingly expressed with ongoing pBOO, potentially contributing to perception of pain associated with obstruction or LUTS. In a rabbit model of pBOO, fibroblasts predominantly located in the serosal layer can become activated myofibroblasts (MFB) and even transform into immature SMC under the influence of various growth factors, of which TGF-β seems to play a central role. Besides developing contractile features, MFB also are more proliferative and more actively produce ECM proteins. During pBOO, fibroblasts, as well as, BSMC, contribute to a pathologic increase in collagen content of the bladder wall, ultimately leading to a poorly compliant fibrotic bladder. The role of immune cells will be discussed in paragraph 1.4 “Macrophages in bladder remodeling.”

### 1.3 Treatment of partial Bladder Outlet Obstruction

#### 1.3.1 Pharmacologic and Surgical Options

The primary aim of pBOO treatment by decreasing urinary outflow resistance is preservation of renal function. Treatment of BOO depends on the underlying cause. Again, anatomic BOO in the form of BPE is the most common form of obstruction. First line therapy in men with moderate symptoms from BPE are alpha-blockers and 5α-reductase inhibitors, with the first being prescribed in the US about 3-times more often for these patients. If medical treatment is not sufficient, treatment of BPE progresses to a partial resection of the prostate. Further surgical forms of therapy for anatomic BOO include treatment of urethral strictures by dilatation, transurethral incision or urethral reconstruction, surgical correction of an obstructing mesh or incision of a sling after anti-incontinence surgery, or therapy for pelvic organ prolapse in women. Management/care of neurogenic BOO includes medication such as baclofen or injection of botulinum toxin A, sacral neuromodulation, clean intermittent catheterization (CIC) with or without prior creation of a catheterizeable channel. Strategies addressing functional
BOO includes medication such as α-blocker or baclofen, pelvic floor physical therapy, sacral neuromodulation, sphincter injection of botulinum toxin A, CIC, or even surgical intervention\textsuperscript{18,72,73}.

For the fetus, unilateral upper urinary tract obstruction has generally a good prognosis, lower urinary tract obstruction (LUTO) on the other hand often results in severe postnatal morbidity or even in mortality due to renal damage and pulmonary hypoplasia. These severe consequences of LUTO have incited prenatal treatment attempts such as fetal vesico-amniotic shunting, which may limit the severity of congenital kidney damage and oligo hydramnios-associated pulmonary dysplasia in select cases, while benefit on bladder function is even less certain\textsuperscript{74,75}.

1.3.2 Treatment of LUTS

Despite adequate physical release of BOO such as surgical treatment of BPE by partial resection of the prostate, 25% to 31% of patients report postoperative LUTS, while other patients develop bladder overactivity after prostatectomy\textsuperscript{32,76,77}. In fact, there is only a weak correlation between pre- and post-prostatectomy obstructive symptoms and voiding function, which suggests that additional factors are supporting dysfunction after obstruction release\textsuperscript{78}. Bladder dysfunction also persists despite successful surgical treatment of PUV\textsuperscript{20,79}. In addition to persistence of symptoms after release of pBOO that are directly related to severity and duration of obstruction\textsuperscript{80}, only partially reversible structural changes are increased thickness of the bladder wall\textsuperscript{81}, and in rat models increased collagen expression\textsuperscript{82,83}, poor tissue compliance, enlargement of muscle bundle volumes\textsuperscript{84}, and hypercontractility\textsuperscript{85}. Evidence suggests that poor reversibility of obstruction-induced bladder dysfunction and structural alterations are at least in part due to epigenetic regulation\textsuperscript{86,87}. Therapy for persistent detrusor overactivity or storage LUTS in general after release of obstruction is typically done with antimuscarinics or β-3 adrenergic agonists\textsuperscript{76,88}. Patients who fail to respond can also be treated with intravesical vanilloid, which also seems to increase bladder capacity in patients with detrusor overactivity and to reduce bladder pain in interstitial cystitis\textsuperscript{89,90}. Additional agents such as α1-adrenoreceptor blockers and phosphodiesterase type 5 inhibitors have also proven effective in the treatment of OAB\textsuperscript{91,92}. Detrusor underactivity, which often seems to improve with the release of obstruction\textsuperscript{93}, remains challenging to be treated medically and still
sometimes needs to be addressed with intermittent catheterization. Pharmacological options include α-adrenoreceptors antagonists, muscarinic receptor agonists such as carbachol or bethanechol and acetylcholinesterase-inhibitors (e.g. distigmine). However, the effect of available treatment options in DU patients is limited due to their moderate efficacy and to their common side-effects. Furthermore, an age-related decrease of acetylcholinesterase-positive nerves, as well as, of muscarinic receptors (M3-type) reduces the density of pharmacological targets and thus diminishes efficacy of such drugs.

### 1.4 Macrophages and their Subtypes

Macrophages are a type of white blood cells. They are capable of recognizing, engulf, and digest substances foreign to the body, such as cellular debris, cancer cells, microbes, and anything else not featuring proteins specific to the host body’s cells and ECM. This process is also called phagocytosis. Macrophages are present in virtually all tissues, patrolling for possible pathogens. By phagocytosing microorganism, macrophages play a pivotal role innate immunity. Additionally, by presenting phagocytosed surface proteins to B-lymphocytes and T-cells, they can initiate a specific immune response, also called adaptive immunity. Circulating monocytes are bone-marrow derived phagocytic cells able to infiltrate various tissues; tissue resident macrophages, on the other hand, are considered to be yolk-sac derived and have slightly different surface markers. Depending on their location, tissue resident macrophages are called Kupffer cells, microglia, histiocytes, Langerhans cells, dendritic cells, or alveolar macrophages. Macrophages are extremely plastic in their phenotype, which is largely influenced by their tissue environment. Therefore, the landscape of potential macrophage phenotypes is much more complex than the traditional separation into pro-inflammatory M1-macrophages and anti-inflammatory and pro-fibrotic M2-macrophages. Especially tissue-resident macrophages are important regulators of organ structure homeostasis, contributing to development and resolution of fibrotic changes.

### 1.5 Tissue Fibrosis and the role of Macrophages in Remodeling

In most organs, fibroblasts are the predominant source of ECM, their activation towards the pro-fibrotic, proliferative and migratory phenotype called myofibroblasts (MFB) is commonly the result of changes in the fibroblast environment. These changes tend to fall in one or more of
four major categories: Cell stress, activation of the Transforming Growth Factor-β (TGF-β) pathway, immune cell activation, and pathologic extracellular matrix (ECM)\textsuperscript{101,102}. Concurrence of these activating stimuli is common for most fibrotic processes, with TGF-β arguably being the most potent pro-fibrotic cytokine\textsuperscript{103,104}. Lysophosphatidic acid (LPA), Endothelin-1 (ET-1), Platelet-Derived Growth Factor (PDGF)\textsuperscript{105}, Connective Tissue Growth Factor, Integrins\textsuperscript{104}, and Interleukins (IL) such as IL-4 and IL-13 are just some of many other factors activating fibroblast to MFB\textsuperscript{106}. While again fibroblasts or MFB are the major sources of ECM in most organs, cardiomyocytes and BSCM also contribute to the excess of matrix proteins in the respective organ fibrosis\textsuperscript{107,108}. Considering MFB and SMC as leading effectors of organ fibrosis, macrophages are believed to be the master regulators in developing fibrosis. Their central role in tissue remodeling and development of organ fibrosis has been shown for hepatic\textsuperscript{109}, cardiac\textsuperscript{110}, renal\textsuperscript{111}, intestinal\textsuperscript{112}, skin\textsuperscript{113}, and pulmonary fibrosis\textsuperscript{106}, but as yet poorly described in the bladder. Depletion of macrophages has proven potential to protect from organ fibrosis to various noxious stimuli. Macrophages can turn fibroblasts into proliferative, migratory and matrix-producing MFB via TGF-β\textsuperscript{106,114}, lysophosphatidic acid (LPA)\textsuperscript{115}, Endothelin-1 (ET-1)\textsuperscript{116}, as well as, through other cytokines such as IL-4, IL-13\textsuperscript{117}, or platelet-derived growth factor (PDGF)\textsuperscript{105}. Thus, macrophages can indirectly induce production of excess ECM observed in fibrosis. Furthermore, macrophages may secrete direct pro-fibrotic agents such as matrix metalloproteinase (MMP) 7 and 12\textsuperscript{118,119}, and by the release of tissue inhibitors of matrix proteinases (TIMPs)\textsuperscript{120}. Lastly, macrophages have the potential to produce ECM proteins themselves\textsuperscript{121,122} or to transdifferentiate under CD4+ cell control into ECM-producing fibroblasts at sites of tissue repair, which seems to be an important mechanism in lung fibrosis and also contribute to renal fibrosis\textsuperscript{123-127}. Interestingly, macrophages are not only master regulators in the development of fibrosis, they have also proven to mediate reversal of fibrosis\textsuperscript{128,129}. Macrophages appear to have the capability of inducing a restorative tissue environment, which is marked by absence of activating signals to the MFB, by a shift in the MMP-TIMP balance in favor of the MMPs and by the presence of a particular restorative macrophage phenotype promoting fibrosis resolution\textsuperscript{100,130}. This restorative macrophage type can even be promoted in vivo by administration of liposomes. This phagocytic stimulus induces an in situ phenotypic switch of macrophages already present in the tissue. The switch towards the restorative phenotype not
only withdraws pro-fibrotic stimuli from MFB but it also starts a process of active fibrosis resolution by phagocytosis of ECM proteins and production of anti-fibrotic MMPs\textsuperscript{131,132}. Taken together, controlling macrophage phenotype potentially offers a physiologic anti-fibrotic strategy, that could eventually also find its application in treatment of bladders that were structurally damaged from long-standing BOO.

1.6 Immune cells in Bladder Remodeling

1.6.1 Circulating Immune Cells in Bladder Outlet Obstruction

A recent study showed that the number of circulating inhibitory regulatory T-cells (Treg) is increased in rats during pBOO\textsuperscript{133}, along with increased bladder levels of IL-6 and IL-17, a pro-inflammatory cytokine typically produced by Th17, a subset of T-helper cells. However, it remained unclear whether the increase in bladder tissue-levels of IL-17 and IL-6 directly correlated with an increase in Th17 or other pro-inflammatory cells in the bladder. Similarly, circulating levels of myeloid-derived suppressor cells (MDCS), IFN-\(\gamma\), IL-10, and Aldosterone was found to be increased during pBOO in rats\textsuperscript{134}. Since bladder tissue was again not directly examined, other than recording of bladder mass, findings from circulating blood may just be reflective of a general stress response, while an effect of altered MDCS, IFN-\(\gamma\), and IL-10 levels on the bladder remain elusive. Indeed, a systemic stress response in addition to bladder oxidative tissue damage during pBOO in rabbits has been described by the same author\textsuperscript{135}. In brief, the systemic response to BOO seems to involve the immune system; however, these findings still need to be correlated with local involvement in and effects on bladder structure and function.

1.6.2 Macrophages in Bladder Outlet Obstruction

While the pivotal regulatory function of macrophages in tissue remodeling in general and the local inflammatory component in early stages of BOO has been studied\textsuperscript{70,136}, very little has been discovered about the specific role of macrophages in obstruction-induced bladder remodeling. PBOO in female rats increased the number of macrophages in the detrusor at 1 week and 4 weeks after the procedure compared to sham operated animals, which corresponded with an increase in expression of inflammatory cytokines such as monocyte chemotactic
protein 1 (MCP1; synonymous with: C-C motif chemokine ligand 2, CCL2), Interleukin-6 (IL-6), IL-17, or RANTES\textsuperscript{137}. One study examined the effect of deficiency in macrophage migratory inhibitory factor (MIF) in a pBOO model in female mice\textsuperscript{138}. MIF knock-out (ko) mice showed less submucosal and detrusor fibrosis, as well as, preserved muscle mass 3 weeks after pBOO compared to wild-type (WT) mice. The same study detected a pro-apoptotic effect of MIF on BSCM in culture. However, the specific contribution of macrophages to the observed differences between WT and MIF-ko mice seen \textit{in vivo} still remained unexplored.

1.7 Limitations in Modelling pBOO in Animals

Animal models are an essential component in bladder research. Models of pBOO have been devised for species\textsuperscript{139}, such as rat\textsuperscript{140}, rabbit\textsuperscript{141}, pig\textsuperscript{142}, and mouse\textsuperscript{143, 144}. Choice of species, gender, and method of obstruction is influenced by several factors. If the experimental plan involves \textit{in vivo} transgenic manipulation, which has become an increasingly important aspect of pBOO research\textsuperscript{145, 146}, then mice are virtually the only species routinely available for this purpose. Also, analysis tools such as RNaseq are more easily analyzed in species with the most accurately annotated genomes such as mice. However, in BOO research, mice are the smallest and thus most challenging species to reliably and reproducibly induce the surgical procedure of pBOO and to perform functional (eg. cystometric) evaluation or other physiologic measurements. In this regard, the planned \textit{endpoints} of a study predominantly affect the type of study animal\textsuperscript{147}. Histologic assessment of the bladder can be performed in any animal used. However, if bladder function is studied by cystometry, requiring implantation of a pressure-measuring catheter, severely affects bladder structure. Therefore, cystometry limits validity of histologic work-up of the bladder, as well as, value of RNA-quantification performed in tissue that is undergoing active wound healing\textsuperscript{147}. For these reasons, the only moderately established non-invasive methods to assess bladder pathophysiology in mice require further development.

Most of the basic and translational research in pBOO still focuses on pathways generating the obstructive response during pBOO rather than the consequences of established obstruction and on the mechanisms to reverse them. Whereas the pathophysiology of long-term obstruction and the restorative process after release of obstruction are clinically more relevant to understand, since patients usually do not present themselves at the doctor’s office during the phase of effective BOO before onset of symptoms. Patients generally would seek medical advice when
symptoms are already established. The restorative processes after release of obstruction are still poorly understood. Therefore, animal models in BOO ideally feature a type of obstruction that can be removed, so that the post-release phase can be studied. Furthermore, cost for purchase and husbandry of study animals, as well as, cost of study drugs that are usually given in proportion to body weight need to be considered as well.

One of the most common pBOO models is partial obstruction of the very proximal urethra adjacent to the bladder neck, thus anatomically mimicking urethral constriction by BPE. The majority of studies applying this proximal pBOO approach use female rats, while the same principle is applicable in pigs, rabbits, guinea pigs, and mice. However, great variability in the degree of obstruction (i.e. unreliable reproducibility), as well as, animal mortality are only some of the concerns associated with this procedure. Since this proximal urethral obstruction technique requires an abdominal incision and dissection around the bladder neck, the proximal urethra and the bladder are subject to inflammation and wound healing process, even in sham operated animals (Chapter 3). The late repair stages are still evident at the time of bladder harvest – usually, several weeks after the initial procedure – they are evident as broad attachments of the bladder to the abdominal wall and scarring at the bladder neck and the proximal urethra. Since nerve fibers entering the bladder in the female rat traverse the area of dissection for proximal urethral obstruction, possible denervation injury in obstructed and in sham operated animals is an additional major concern of this technique, most likely significantly confounding results in studies employing this pBOO method especially in the so-called ‘surgical sham’ animals. Chapter 3 discusses our findings examining the limitations and novel alternative to the proximal obstruction method in more detail. The proximal urethral obstruction technique has also been applied in female mice, not surprisingly with significant variability in the degree of obstruction and high animal mortality, which is often not even reported. A similar technique has been adapted for male mice; although inflammation, wound healing, and denervation injury affecting the bladder, as well as, morbidity and variability remain protean concerns with this approach.

To reduce the confounding effect of endogenous estrogen on bladder remodeling induced by pBOO, some animal studies performed a bilateral ovariectomy at the time of surgical pBOO; the estrogen levels of ovariectomized rats were reduced by 53% compared to controls. Histologically, ovariectomy was associated with increased blood vessel density in the bladder
wall, even when estrogen was supplemented; the functional effect of ovariectomy in BOO was not studied. In a rabbit model it was detected that while ovariectomy seemed to reduce expression and transcription of myosin, both parameters were increased by estrogen supplementation\textsuperscript{156}; additionally, ovariectomy in female rats with pBOO seemed to reduce expression of gap junctions in the bladder along with reduced effectivity of gap junction blockers\textsuperscript{157}. In summary, endogenous estrogen seems to affect the bladder’s structural and functional response to outlet obstruction. However, it remains unclear if ovariectomy truly reduces hormone-induced confounding or whether the added morbidity of ovariectomy even introduces other unknown factors to biologic response to pBOO.

For the foregoing reasons, despite the fact that pBOO is more prevalent in adult men than women, most experimental BOO studies work with female rodents, although some authors clearly advocate the use of male study animals, as their urogenital anatomy and hormonal status may more closely model human obstruction\textsuperscript{154,156}. Obstruction models in male rodents either involve dissection between the prostate and the bladder, which is more extensive in nature than periurethral dissection in female rodents.

Our own work suggests that this dissection very probably elicits a more pronounced local inflammatory/wound-healing response and a denervation injury as discussed with the proximal urethral obstruction approach\textsuperscript{158}. More distal pBOO methods in male rats resulted in reflux into seminal vesicles along with prostatitis\textsuperscript{154,159}. The use of male rodents for experimental BOO, which reduces the confounding effect of endogenous estrogen, comes at the price of more extensive dissection and subsequent inflammation, which too is confounding and, moreover, leads to increased animal morbidity.

Other male mouse models utilize genetic modification to study BOO. For example, some models overexpress aromatase and develop obstructive functional bladder phenotype in the absence of prostatic enlargement and physical obstruction\textsuperscript{160}, while other mice require exogenous estrogen treatment for 4-6 months to develop prostatic enlargement with subsequent BOO\textsuperscript{161}. Given their long-term exposure to increased levels of sex hormones, it seems questionable whether these BOO models are superior in modeling human BOO compared to surgically increased urethral resistance. Moreover, both hormone induced models clearly fail the criteria of reversibility of obstruction, meaning that the important post-release
pathophysiology of the bladder itself—be it reparative or with ongoing irreversible bladder pathology despite de-obstruction—cannot be studied with these models.

In summary, pBOO models used in the past, while allowing an effective release of obstruction feature a high degree of variability, high animal morbidity, and even mortality. Also, as detrusor and bladder neck are affected by the inflammatory process of wound healing even in the *sham* animals, the traditional proximal urethral obstruction model needs refinement, to enable accurate evaluation of the role of immune cells in obstruction-induced bladder remodeling. Moreover, any adjusted or novel pBOO model would ideally be applicable in mice, to allow employment of genetic tools of modification and analysis. Finally, non-invasive methods to assess bladder pathophysiology in mice are not well established and thus require further development.
2  Hypothesis and Aims

The impact of pBOO on quality of life in wide parts of the population, as well as, the related immense health care cost warrant the great research effort focusing on pathophysiology and treatment of BOO-related diseases. Current animal models seem confounded by either surgically induced inflammation and possibly denervation injury or by over-exposure to sex hormones; in several models, BOO cannot be released, whereby the clinically highly relevant post-release pathophysiology deserves being investigated more than what was done in the past. An improved animal model along with a method to assess bladder function also needs to be applicable in mice, as this species offers the full range of transgenic modification and thus more specific research results.

While macrophages are notorious master regulators of remodeling in several types of tissue, including hollow organs such as the heart or the gastro-intestinal tract, the role of these immune cells has remained unexplored so far in the context of obstruction-induced bladder remodeling. Moreover, macrophage-dependent histopathologic remodeling was shown to correlate with organ function; whether a comparable correlation is applicable in bladder remodeling is unknown.

2.1  Hypothesis

Bladder remodeling during partial outlet obstruction is modulated by macrophages.
2.2 Aims

I. Development of a reversible pBOO Model less confounded by Wound Healing and Denervation.

II. Transfer novel pBOO Model to Mice.

III. Establish a non-invasive method to assess murine bladder function.

IV. Describe the Effect of Macrophages in Obstruction-Induced Bladder Remodeling.

Given our goal of studying the role of macrophages in partial outlet obstruction, we first needed to develop a model that did not induce an inflammatory response from wound healing caused by dissection around the bladder neck; we also planned to histologically and functionally examine the effect of a possible denervation injury caused by surgical access to the proximal urethra.

Once an improved animal model was established, our second aim was to transfer the model to mice. This step would allow a manifold reduction in cost of the expensive study-drug that we intended to use in aim IV.

In a third step, we had to establish a reliable method to non-invasively assess bladder function in mice. Previously described assessment methods were either invasive, expensive, not applicable for long-term measurement, or did not provide satisfactory accuracy given the small voiding volumes in mice.

The fourth and main goal was to describe the functional and structural effect of macrophages in the mouse bladder after partial outlet obstruction.
3 Novel Model for Partial Bladder Outlet Obstruction in Mice

This chapter is modified from the following:

Martin Sidler, Karen Aitken, Janet Jiang, Dominika Bijos, Jacques Belik, Darius Bägli

Finding NeMO – Nerve-sparing Midurethral Obstruction: A Pathophysiologically Accurate Model of Rodent Partial Bladder Outlet Obstruction

Urology, 2017.
3.1 Introduction

Partial bladder outlet obstruction (pBOO), as well as, its associated sequelae in men with benign prostate hyperplasia (BPH), is a widespread health care problem. Rodents are most frequently used to model and investigate pathogenesis and therapy of obstruction-associated structural bladder alterations such as hypertrophy, hyperplasia, and fibrosis and their associated functional perturbations.

Traditionally, placing a suture tie around the proximal urethra creates pBOO in female rodents such as rats, mice, rabbits or guinea pigs. However, this obstruction model is notorious for its variability in the degree of obstruction. Furthermore, the mortality rate is usually 15% or more, even when a very standardized technical degree of obstruction is applied. Moreover, the obstructed animals as well as, importantly, the respective sham animals are both at a minimum subjected to dissection around the bladder neck. Furthermore, one can assume that this manipulation at least partially disrupts bladder innervation, and resultant bladder-neck bladder-body cross-talk either by denervation, neuropraxis, or inflammation. The healing process of the abdominal incision goes along with inflammation and adhesion to the bladder to the abdominal wall, both aspects further invite disturbances to bladder function and are not typically part of human pBOO. The female rat is most often chosen for its apparent lack of urethral complexity vs the male. While obstructing the proximal urethra has been historically intuitive given analogies to BPH, this is reasoning is flawed for obvious gender-specific reasons, creating undue technical challenges. Approaching the urethra in female rat at the more distal mid-urethra avoids the manifold downsides of the traditional technique. Indeed, due to the inherent biological noise, numerous investigators have even abandoned sham operations for convenience in the proximal urethral obstruction (PU) model in favor of completely unoperated controls. However, sham surgery is a scientifically sound, historically validated and necessary principle to uphold.

We hypothesized that placing a partial urethral obstruction distal to the pubic symphysis would be both effective in inducing typical obstruction-related changes such as bladder hypertrophy while at the same time preserving bladder neck innervation and normal bladder function in critical sham operated animals. We present evidence that this novel model of pBOO, termed
NeMO (Nerve-sparing Mid-urethral Obstruction), is a significant advance upon the existing approach with the potential of moving the field of bladder functional biology forward.

3.2 Material and Methods

3.2.1 Experimental animal groups

*Group 1:* For the purpose of comparing traditional PU, to NeMO, and to unoperated controls, 38 female Sprague-Dawley rats (227-278g) were randomly divided into two groups to undergo either a procedure for PU or NeMO respectively. In each group, a sham procedure was done in seven animals whereas twelve received a pBOO. A group of five rats was left as unoperated.

*Group 2:* For the purpose of longitudinal comparisons, 24 rats with the same characteristics were used to study the functional and structural changes secondary to NeMO over a period of 6-12 weeks after the procedure; for each time point, 4 rats underwent obstruction while 2 rats underwent NeMO sham surgery. 4 more rats served as unoperated controls and we harvested their bladders together with the 12-week animals. The rats were housed under standard conditions. The institutional animal care committee approved the experimental protocol.

3.2.2 Surgical Procedures

3.2.2.1 Proximal Urethral Obstruction (PU)

The surgical technique for PU and PU sham was performed as described previously. In brief: After lower abdominal incision and minimal dissection around the bladder-urethral junction, we passed a 4-0 silk suture behind the proximal urethra. A 0.9mm metal rod was placed alongside and the ligature was snugly tied around both, followed by removal of the rod. The wound was closed in layers with absorbable suture (Figure 3.1, A-E). Sham procedures were performed identically except for the placement of the ligature.
Figure 3.1. Dissection in Nerve-sparing Midurethral Obstruction (NeMO) is less extensive than in Proximal Urethral obstruction (PU) and spares the bladder neck. Comparison of surgical technique of traditional proximal urethral obstruction (PU; A-E) technique and nerve-sparing mid-urethral obstruction (NeMO; F-J). Asterisk (*) placed at urethral meatus, arrow (➡) indicates the urethra, arrowhead (▶) marks the ureters. Steps for PU: A) Lower midline abdominal incision. B) Exteriorized bladder with dissection around the proximal urethra. C) Blunt passage of suture behind urethra. D) Tying 4-0 silk suture with a 0.9mm rod temporarily placed alongside the urethra. E) Wound after closure in

3.2.2.2 Nerve-sparing mid-urethral obstruction

NeMO or NeMO sham procedures were done as follows. In anesthetized rats, we exposed the mid part of the urethra and temporarily stented the urethra with a 20G angiocath in order to facilitate its delineation intraoperatively. The genital (clitoral) neurovascular bundle running on top of the urethra was carefully detached in order to exclude it from the ligature. After minimal dissection between vagina and urethra, a 4-0 silk suture was passed behind the urethra. The catheter was removed and the 0.9mm metal rod temporarily placed para-urethrally while tying the suture. The wound is closed in two layers (Figure 3.1, F-J). The procedure for sham animals was identical except for placing the silk tie. We observed that the majority of rats had a normally filled bladder prior to the surgical procedure.

3.2.2.3 Reversibility of Obstruction — Urethral Suture Removal

For both techniques, the partially obstructing silk tie was easy to remove in anesthetised rats without injuring the urethra; this was confirmed in all animals at the time of bladder harvest, whereupon the animals were sacrificed by exsanguination.

3.2.3 Bladder functional parameters

The animals’ micturition pattern was recorded for 10 hours during the light cycle at 2 weeks after the operation in Group 1, and at 6, 8, 10, and 12 weeks for Group 2. The rats were placed in metabolic cages funneling urine into a continuously weighed cup. An increase of more than 0.1g of collected urine per 2 minutes was considered a micturition. For the purpose of analysis, small voids were defined as less than 25% of maximum voided volume in control animals (i.e. <0.25g). The number of small voids divided by the total number of voids resulted in the small void ratio, which served as a surrogate parameter for an over active voiding pattern. At the time of bladder harvest residual urine, bladder mass and animal weight were recorded. Other
parameters characterizing micturition patterns were maximum bladder capacity, which equals the sum of the largest void plus residual volume. Voiding efficiency was determined by maximum micturition fraction, defined as maximum voided volume divided by maximum bladder capacity, and mean micturition fraction, defined as the average voided volume divided by maximum bladder capacity (Figure 3.2).
Figure 3.2. Definition of measured and calculated micturition parameters describing bladder physiology. A) Maximum micturition volume was the largest void measured during the 10 hours of micturition recording. Residual volume was measured by direct needle aspiration before bladder harvest. Micturition volume (MV) was any recorded increase in collected urine. B) Maximum bladder capacity
(MBC) was determined by adding maximum micturition volume to aspirated residual volume. Micturition fraction was the ratio of a single voided volume and maximum bladder capacity. Copyrights received.

3.2.4 Contractility assay

In anesthetised rats, bladders were harvested, weighed, cut in four longitudinal strips, and immediately placed in ice-cold Krebs solution (118.4 mM NaCl, 7.7 mM KCl, 1.9 mM CaCl$_2$$\cdot$7H$_2$O, 1.15 mM MgSO$_4$, 1.15 mM KH$_2$PO$_4$, 4.2 mM dextrose, 4.2 mM NaHCO$_3$). Isometric contractions of bladder wall strips were measured in a 25-ml tissue bath filled with Krebs solution at 37°C and bubbled with 95% O$_2$–5% CO$_2$, with strips connected to a low-compliance force transducer (Radnoti, Monrovia, CA). Contraction force was continuously recorded for further analysis (LabChart 8; ADInstruments, Colorado Spring, CO). Muscle strips were tensed to 1.0–1.5 g and let equilibrate for 30 minutes. A 2-minute exposure to 100 nM Carbachol was done to prove strip viability before washing. At the end of the following 30-minutes resting phase, we selected a 10-minute period in which we determined the maximum power frequency (MPF) – describing the frequency carrying the greatest physical power, the maximum amplitude, and the mean contractile force, using the analysis tools in LabChart. For comparison, we normalized the maximum amplitude, as well as, the mean contractile force by the strips average cross-sectional area (calculated as weight over length). We also measured the contractile force with increasing doses of Carbachol, starting at 10 nM and using a maximum dose of 30 µM; exposure times to Carbachol was 2 minutes with repeated washes and 5 minutes of rest before application of the next higher dose.

3.2.5 Histology

3.2.5.1 Immunofluorescence for Nerve fibers

Coronal sections involving the proximal urethra, bladder neck, and bladder base were stained with β$_3$-Tubulin antibody. Cell nuclei were stained with Hoechst 33342. We quantitated nerve fiber density and nerve fiber area in the lamina propria of the bladder neck sections using the image processing program Volocity (Version 6.3; PerkinElmer Inc.).
3.2.5.2 ECM Quantification

Picrosirius red staining was used on mid-equatorial cross-sections from bladders of the second group (6–12 weeks obstruction) to determine structural parameters as outlined in Figure 6. Automated image analysis was performed using Pannoramic Viewer (3DHISTECH, Budapest, Hungary) with the HistoQuant module.

3.2.6 Statistics

Statistical analysis was performed using IBM SPSS Statistics 23 (SPSS Inc., Chicago, IL). Detection of statistically significant differences among all groups was done by ANOVA. Direct comparison between 2 groups was done using two-tailed T-Test, or Welch T-Test where equality of variances was not given, respectively. p-values <0.001 were considered highly significant. Comparison of variances among groups was done using a two-tailed F-test. Numerical results were summarized graphically using boxplots, the box encompassing the interquartile range (IQR) from the first quartile (Q1) to the third quartile (Q3), the bold transverse bar representing the median. The whiskers mark maximum and minimum values. Outliers represented by a circle-symbol (○) are more than a 1.5-fold IQR away from Q1 or Q3, respectively; extreme values are represented by an asterisk (*), indicating they are more than a 3-fold IQR away from Q1 or Q3, respectively. 2-tailed Pearson’s correlation was calculated for most correlations; 2-tailed Spearman’s correlation was used to correlate data pairs.

3.3 Results

3.3.1 Obstructed Animals

3.3.1.1 Safety, efficacy, consistency, and shorter operating time of NeMO.

After two weeks, PU, as well as, NeMO caused a significant increase in relative bladder mass (details see Figure 2). Absolute bladder mass after NeMO was also significantly increased (Supplemental Figure 3). Three out of twelve PU animals died secondary to bladder rupture, while no complications occurred among the animals undergoing NeMO. Additionally, the variance in absolute and relative bladder mass of the NeMO group was significantly less than
of the PU group. The mean operating time for NeMO (9.9 minutes) was significantly shorter by 1.7 minutes than for PU (data not shown).

3.3.1.2 NeMO incites functional Changes similar to obstruction-induced Human Bladder Pathology.

Compared to control, the average residual volume during obstruction rose more than 8-fold and greater than 3-fold for the PU and the NeMO groups, respectively. NeMO also tended to increase the ratio of small voids compared to NeMO sham (p=0.067). While NeMO did not lead to short term changes of maximum bladder capacity, the PU group showed marked dilative changes with more than twice the maximum bladder capacity compared to the controls (Figure 3.3).
Figure 3.3. After 2 weeks, NeMO led to changes similar to obstruction-induced human bladder pathology. PU induced an excessive increase in bladder mass and marked bladder dilatation. “○” and “*” mark outliers and extreme values, respectively. A) Changes in bladder to body mass ratio 2 weeks after NeMO or PU procedures, respectively, compared to un-operated controls. NeMO effectively led to increases in bladder to body mass ratio of 119% (vs controls) and a 71% increase (vs NeMO sham). p<0.05; †. PU sham procedure slightly increased the bladder to body mass ratio by 86% compared to
controls, p=0.07; ‡, and also tended to increase the ratio by 46% when compared to NeMO sham, p=0.09. PU led to a 64% greater relative bladder mass than NeMO, p<0.05, and to >2.5-fold increase in relative bladder mass compared to controls, p<0.001; §. The variability in bladder to body mass ratio was significantly greater by 2-fold in PU than in NeMO (standard deviation 0.38 vs 0.19; p=0.018). B) The volume of residual urine was increased by NeMO compared to NeMO sham by a mean of 0.37ml (p=0.013; †) and by 3-fold compared to controls. PU shams had significantly more residual urine than NeMO sham by >5-fold (or 0.12ml; ‡). Obstruction residual urine in PU was almost 2.5-fold greater compared to NeMO (p=0.03; §). C) PU resulted in an average bladder capacity of 2.7ml, vs 1.3ml for NeMO (p=0.03; †), which was similar to the other groups. D) PU shams had a decrease in mean micturition fraction of over 30% vs controls (p=0.045) and a decrease of over 33% vs NeMO shams (p<0.001). E) PU sham rats tended to have an ~3-fold ratio of small voids compared to controls (p=0.07; †). Obstruction NeMO tended to increase the ratio of small voids compared to NeMO sham (p=0.067; ‡).

3.3.2 Sham Animals

3.3.2.1 PU Sham Procedure increases Bladder Mass more than a NeMO Sham Intervention

Compared to control, the sham procedure for PU resulted in a significant increase in both absolute and relative bladder mass by 45% and 85%, respectively (Figure 3.3; Figure 3.4); absolute bladder mass, as well as, relative bladder mass of NeMO sham, on the other hand, was not different from controls. Also, the variance of bladder mass of the NeMO sham group was significantly lower than the variance among PU shams.
3.3.2.2 PU sham procedure increases residual urine, leads to inefficient voiding, and displays signs of bladder overactivity.

Rats that underwent PU sham procedure had an average residual urine volume of 150µl, which was significantly, almost 5-fold, higher than NeMO shams. The fact that the majority of unoperated control rats did not void during induction of anaesthesia before bladder harvest led to the greater average amount of residual urine in control rats compared to the NeMO sham group (Figure 2). Additionally, the mean micturition fraction in PU sham-operated rats was significantly reduced by almost 33% compared to unoperated controls, and reduced by >33%
compared to NeMO shams (Figure 2), attesting to the reliability and accuracy of the NeMO shams. The maximum micturition fraction of PU shams also tended to be lower than in NeMO shams (p=0.067). PU sham operated rats also tended to a three-fold higher ratio of small voids than NeMO shams (p=0.07; Figure 3.3).

3.3.2.3 Obstruction, as well as, PU-sham procedures increase contraction amplitude and susceptibility to cholinergic stimulation in isolated bladder strips.

We isolated 4 longitudinal muscle strips from two-weeks sham and obstructed bladders, comparing how the NeMO and PU approach affect intrinsic aspects of spontaneous bladder contraction (e.g. Amplitude and Frequency). Visual comparison of spontaneous contraction curves shows an obvious increase in amplitude in obstructed and in PU sham groups (Figure 3.5). Obstruction procedure seems to decrease spontaneous contraction frequency in both, PU and NeMO approaches. Quantitative comparison of spontaneous contraction recording among our groups confirmed the visual findings (Figure 3.6). Again, PU sham procedure also led to an increase in amplitude of spontaneous contractions. When normalizing the raw amplitude by the strip cross sectional area, the NeMO sham strips were similar to control strips while NeMO, PU sham, as well as, PU had a significantly greater amplitude of spontaneous contractions. NeMO, as well as, PU led to a significant decrease in maximum power frequency to less than half compared to their respective shams. Of note, in proximally operated animals (PU and PU sham), the higher maximum power frequency correlated significantly with increased ratio of small voids. However, no such correlation was seen in NeMO and NeMO sham rats or in control animals (data not shown). Dose response curves of both, sham operated and obstructed animals showed a significant shift towards higher susceptibility to Carbachol stimulation after proximal approach compared to NeMO.
Figure 3.5. PU sham, as well as, obstruction procedures, alter spontaneous contraction pattern while NeMO sham strips are similar to controls. A) – E) Representative recordings of spontaneous contractions of bladder strips for each group. Copyrights received.
Figure 3.6. PU sham procedure increased the contractile strength of bladder strips; spontaneous activity of bladder strips correlated with overactive voiding behavior in PU but not in NeMO animals. A) For both, NeMO and PU, strips from obstructed bladders had a significantly greater contractile force by over 2-fold than their respective shams (†) and strips from control bladders (p=0.04 and p=0.042, respectively). Raw amplitude in PU shams tended to be over 50% greater than NeMO shams (p=0.07; ‡). B) The average cross section-adjusted amplitude in PU shams (†), as well as, in NeMO(‡)
were significantly higher than in NeMO shams by almost two-fold. PU animals were solely significantly different from NeMO shams(§). C) NeMO(†), as well as, PU(‡) had a significantly lower frequency in spontaneous contraction of less than half, compared to their respective shams. D) In both PU and PU shams, the ratio of small voids positively correlated with the maximum power frequency of isolated bladder strips (p=0.029). There was no such correlation in NeMO and in unoperated control rats. E&F) Dose-response curves for the contraction elicited by Carbachol in bladder strips from PU sham and NeMO sham operated animals, showing significantly higher susceptibility of PU sham strips at 300nM and 10µM. Comparing PU and NeMO obstruction, the dose-response curve shift was less pronounced, yet still significant at 3µM Carbachol. Copyrights received.

3.3.3 Structural Responses to Dissection

3.3.3.1 Denervation injury results from dissection around the proximal urethra, but not the mid-urethra.

We quantitated the number of nerve fibers in the lamina propria of coronal sections of the bladder neck as number of nerve fibers per cell nucleus – or nerve density. Normalizing the number of nerve fibers to the number of nuclei accounted for tissue edema or other alterations of the extracellular matrix. We found that in the bladder neck area of PU shams nerve density was significantly reduced by 60% compared to NeMO shams. Indeed, the latter maintained a nerve-density similar to unoperated controls (Figure 3.7). The average nerve fiber size in PU shams was only 1/3 compared to NeMO shams (p=0.06). Nerve fiber size of all proximally operated animals (PU and PU shams) was significantly smaller compared to all NeMO and control animals (p=0.03). The decrease in nerve density in PU shams tended to correlate with inefficient voiding as seen on the decrease in mean micturition fraction (p=0.06).
Figure 3.7. Obstruction technique mediates nerve fibers morphometry coordinately with mean micturition fraction in sham animals. A) Tissue sections of the bladder neck stained for nerve fibers with anti-β3-tubulin and nuclear stain with Hoechst 33342 show loss of nerve fibers in PU sham. B) PU sham animals had a significantly lower density of nerve fibers of less than 40% compared to NeMO shams.
and of less than one third compared to controls (p=0.05; †). C) The average size of nerve fibers in PU shams was less than one third compared to NeMO sham (borderline significant, p=0.06; ‡). Average nerve fiber size for PU, as well as, PU-sham was 50% smaller compared to NeMO, NeMO-sham, and controls (p=0.03; §) D) The lower number of nerve fibers in PU sham tended to correlate with lower average micturition fraction, while NeMO sham animals had both preserved nerve density and micturition fraction (p=0.06). Copyrights received.

3.3.4 Mid-term characteristics of NeMO

NeMO induces a persistent increase in bladder mass, progressive detrusor fibrosis, and lamina propria thickening. Relative bladder mass was significantly increased by NeMO more than 2-fold compared to NeMO shams (Figure 3.8); sham bladder mass was similar to controls. While the difference in relative extracellular matrix (ECM) area in the detrusor was not significant between NeMO sham and NeMO animals, the latter showed a significant and increase in relative ECM-area over time (Figures 3.2 & 5), consistent with that seen in physiologically relevant obstruction. A similar increase in thickness of the lamina propria was noted over time (p=0.015). The difference between NeMO sham and NeMO in endomysial fibrosis (percentage of ECM-area within smooth muscle bundles) was not significant and the degree of endomysial fibrosis did not seem to change over time. NeMO-induced increase in relative bladder mass was positively correlated with the amount of residual urine, maximum bladder capacity, and was negatively correlated with micturition fraction (Figure 3.9).

NeMO exhibits a stable maximum bladder capacity and increase in residual urine volumes. There were no significant differences among control, sham, and obstructed animals in maximum bladder capacity (Figure 3.8). Residual urine tended to be increased 3-fold by NeMO when compared to shams from 6 to 12 weeks after the procedure (p=0.07).
Figure 3.8. Structural and functional changes in NeMO sham vs up to 12 weeks of obstruction. A) Obstructed animals show a >2-fold increase in bladder mass compared to shams (p=0.023; †), bladder mass of sham animals was similar to controls. B) Percentage of ECM-area in the detrusor tended to be 20% lower in obstructed bladders compared to shams (p=0.07; †). Obstructed bladders showed a
significant increase in detrusor fibrosis over time (p=0.05; §) C) In obstructed bladders, duration of obstruction was a significant factor associated with an increase in lamina propria thickness (p=0.015; †). D) Muscle-bundle ECM area was not significantly different among groups, nor was there a significant change over time. E) There were no significant differences among groups in maximum bladder capacity. F) Obstruction tended to lead to a 3-fold increase in residual urine compared to sham animals (p=0.07; †). Copyrights received.
Figure 3.9. Correlations of NeMO-induced bladder mass with bladder function. A+B) Highly significant correlation of the obstruction-induced increase in bladder mass with the amount of residual urine and maximum bladder capacity (p<0.001). C) The obstruction-induced increase in relative bladder
mass is negatively correlated with voiding efficiency, measured by maximum micturition fraction (p<0.001). Copyrights received.

3.4 Discussion

By modeling pBOO using a PU approach, the prevailing literature is plagued with concerns such as the considerable mortality of operated animals, dissection-associated disruption of innervation around the bladder neck, induction of a wound healing response, and great variability in the degree of obstruction. We observe NeMO (mid-urethral approach) be a preferred, reproducible, and reversible obstruction technique that reduces or eliminates drawbacks of the traditional PU model. Besides sparing bladder neck innervation, periurethral dissection and placement of ligature in NeMO also occur at a level distal to the striated sphincter\textsuperscript{166,167}, which plays an important role in bladder emptying in the rat and thus is important to preserve during dissection.

3.4.1 NeMO safely models functional and structural changes of moderate human bladder outlet obstruction.

As predicted, NeMO reliably induced an increase in the relative bladder mass and the absolute bladder mass, as well as, in the residual urine volume. As 2 weeks of pBOO is relatively short, maximum bladder capacity was still unchanged. Responding to obstruction with an increase in mass before developing dilative bladder changes is in fact similar to the bladder pathophysiology in the context of prototypical human pBOO by BPH, where wall thickening and increased bladder mass precede dilative changes. Conversely, PU caused a marked increase in maximum bladder capacity even after only 2 weeks. Such early dilation lies in stark contrast to the pathophysiology of pBOO in humans and might also be in part due to denervation injury around the bladder neck\textsuperscript{168}. Furthermore, NeMO is both faster than a PU approach and devoid of complications or even deaths in obstructed animals. Indeed, bladder rupture in the deceased PU animals consistently occurred at the apical dome; we believe that tissue trauma resulting from necessary traction on the bladder during dissection was most likely a significant factor
leading to bladder rupture and associated mortality in obstructed rats. Additionally, the variance in the increase in bladder mass in response to obstruction was significantly lower with NeMO than with PU obstruction. The higher residual urine volumes in PU vs NeMO animals is likely due to the denervation injury associated with PU procedure, possibly from impairment of detrusor-sphincter crosstalk in proximally operated animals resulting in inefficient voiding, as indicated by their reduced mean and maximum micturition fraction.

3.4.2 NeMO shams lack the confounding features of PU shams.

Besides verifying the favorable outcome with the NeMO technique, we detected critically important differences in bladder function and structure between the sham-operated groups, especially revealed by comparison to an unoperated control group. The PU-sham procedure was associated with an undesirable increase in bladder mass, as well as, an overactive micturition pattern. Two mechanisms might contribute to this observation. First, bladder denervation in rats is known to alter baseline phasic contractility. Second, the inflammatory response induced by wound healing around the bladder neck area after dissection may also cause bladder overactivity. Indeed, the less pronounced though more reliable and reproducible increase in organ mass after NeMO in obstructed animals is likely more physiological and closer to the gradually developing pathology seen in BPH than induced by PU.

3.4.3 Detrusor strip contractility

Further evidence that PU sham procedure has undesired effects on bladder function was further provided by the increase in the amplitude of spontaneous contractions of bladder strips. The observation that the spontaneous inherent contractile frequency in PU bladder strips was directly correlated with bladder overactivity in vivo suggests that bladders in the PU groups lack external neuronal control, as no such correlation was seen in the NeMO or control groups. Additionally, the observed shift in dose response curve after PU procedure is consistent with the observation in experimentally transected bladders, further indicating that dissection around the proximal urethra is associated with undesired microstructural and functional changes.
3.4.4 Denervation

Nerve disruption was further evidenced by a clear loss in nerve density, as well as, a decrease in nerve fiber size in the bladder neck area in the PU sham animals. The nerve loss correlated with inefficient voiding highlighting the potential for confounding structural and functional disruptions in PU shams. A disturbance in bladder outlet innervation may be a potential source of inefficient voiding. This would cause the detrusor to contract against a non-relaxed sphincter, similar to the dysfunctional bladder outlet obstruction in neurogenic bladders (NGB). Ironically, the traditional PU sham procedure may indeed serve as a robust animal model for non-spinal cord injury NGB or for isolating a local neuropathic component not previously recognized to increase outflow resistance. Pelvic gangliectomy, leading to loss of nerve fibers in the bladder’s mid-equatorial and more cranial areas would be another option to create a partially denervated bladder, though more surgically involved than NeMO. Finally, inflammatory responses occurring around the nerve-rich bladder neck area and in the adjacent pre-vesical abdominal wall may also contribute to altered bladder function, since inflammatory changes, in general, are involved in bladder overactivity. Dissection around the bladder neck in PU urethral procedures may also impair the bladder’s blood supply and thus contribute to the inflammation and dysfunction seen in PU shams; however, we did not evaluate our samples for ischemia-specific changes.

3.4.5 Time course

Longer term obstruction caused a significant increase in bladder mass and residual urine with NeMO, including time-dependent increases in detrusor ECM (extracellular matrix) content and thickness of the lamina propria, indicating a trend towards obstruction-induced bladder fibrosis. These findings are consistent with long-term pBOO in human disease. Stable maximum bladder capacity with absent dilative changes is also more similar to long-term pBOO in humans than the massive dilation observed after 12 weeks of PU pBOO. As we did not observe any complete obstruction in our NeMO model, we propose that the use of a thinner rod along the urethra may be safely attempted if the intention is to model higher outflow resistance.
3.4.6 Relating NeMO to other pBOO models

Table 3.1 summarizes published rodent models of pBOO, some of which suggest an approach other than the female PU. Most of the models involve an abdominal approach, with bladder neck and proximal urethra dissection. Injecting a bulking agent along the urethra seems similar to NeMO, yet it may be difficult to quantitatively and predictably standardize the degree of obstruction. Such standardization is a critical holy grail in obstruction models if studies are to harness high throughput biological techniques where data mining and analysis will be crucially dependent on the underlying assumptions of the disease model. Moreover, the expense, durability, and reversibility remain unaddressed in rodent pBOO models.173

PU in male rats via a perineal approach led to dilation of seminal vesicles likely from refluxing urine.159 In contrast, obstruction along the penile urethra in male rats has also suggested inducing a mild degree of obstruction more representative of most human obstructive bladder pathologies.174 However, this animal model involves extensive dissection to separate penile urethra from corpus cavernosum and may lead to reflux into the seminal vesicles such as described by others, potentially offering a pop-off mechanism given the increased pressure in the urethra proximal to the obstruction. NeMO shares the limited applicability to female rodents with most of the commonly used pBOO animal models, although using male animals when modeling bladder pathophysiology in the context of obstructing prostate hyperplasia would seem ideal.

Finally, the ease of releasing/reversing the pBOO after NeMO is an additional important feature of our technique (data not shown). Most studies on pBOO remodeling focus on adaptation during obstruction. However, the phase after release of obstruction is the more important, more common, and the most relevant clinical state facing patients with ongoing symptoms and dysfunction. The analysis of persistent bladder pathobiology and dysfunction following reversal of NeMO is currently under analysis and will be reported separately. In addition, the ease of the model will enable its use in mice. At present, most murine models of pBOO are fraught with high variability and mortality that are rarely reported in publications. NeMO provides low variability and low mortality, which could enable it to be used in mice, including potential use in transgenic animals.
3.5 Conclusion

NeMO in rats is highly effective in inducing the desired pBOO-associated changes such as an increase in bladder mass and residual urine. It is consistent and produces virtually no associated animal morbidity and mortality. NeMO also recreates the bladder fibrosis associated with pBOO. Critically, the reduced variability, as well as, preserved innervation and function in sham animals, compared to PU animals, strongly suggests NeMO be adopted as the new standard animal model when investigating pBOO in vivo in rodents. The functional and structural response to NeMO is much closer to the pathophysiology of human pBOO than the exaggerated response obtained with PU. Given the technical and clinical advantages of NeMO will enable the full spectrum of experimental approaches to research bladder remodeling, allowing for more specific strategies to understand and treat obstructive bladder diseases including persistent post-release pathophysiology.

Table 3.1. Overview of various pBOO methods used in mice and rats.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Mortality, Reference</th>
<th>Laparotomy</th>
<th>Reversibility</th>
<th>Durability</th>
<th>Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal partial obstruction using a suture tie</td>
<td>3/20 (15%)\textsuperscript{148,149}</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Female rats</td>
</tr>
<tr>
<td></td>
<td>4/24 (16.7%)\textsuperscript{149}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jeweler’s ring at proximal urethra</td>
<td>4/40 (10%)\textsuperscript{175}</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Female rats</td>
</tr>
<tr>
<td>Proximal partial obstruction (suture tie) with intravaginal knot</td>
<td>2/12 (16.7%)\textsuperscript{176}</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Female rats</td>
</tr>
<tr>
<td>Condition</td>
<td>Rate (Obs.)</td>
<td>Response</td>
<td>NeMO</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>-------------</td>
<td>----------</td>
<td>------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Peri-urethral injection of Hyaluronic Acid</td>
<td>0 (0%)</td>
<td>No</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Female rats</td>
</tr>
<tr>
<td>Partial obstruction proximal to the prostate</td>
<td>n/a</td>
<td>Yes</td>
<td>Unknown</td>
<td>Yes</td>
<td>Male mice</td>
</tr>
<tr>
<td>Partial obstruction of the penile urethra using a tie</td>
<td>9/100 (9%)</td>
<td>No</td>
<td>Unknown</td>
<td>Yes</td>
<td>Male rats</td>
</tr>
<tr>
<td>NeMO</td>
<td>0/36</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Female rats</td>
</tr>
</tbody>
</table>
4 Application of Nerve-Sparing mid-urethral Obstruction (NeMO) in Female Mice

This chapter is modified from the following:

Martin Sidler, Karen Aitken, Janet Jiang, Darius Bägli

Nerve-sparing Mid-urethral Obstruction (NeMO) in Female Small Rodents


A visualized version of this study can be viewed at the following:

www.jove.com
4.1 Introduction

Partial bladder outlet obstruction (pBOO) has a high prevalence and can result in severe bladder dysfunction\(^{177}\); the spectrum ranges from congenital malformations such as posterior urethral valves or hypospadias, over acquired urethral strictures to benign prostatic hyperplasia. The latter affecting more than 30\% of men sixty years and older\(^{178}\). Great patient burden and immense health care costs associated with pBOO warrant the considerable research effort put into studying bladder remodeling in response to increased outflow resistance\(^{179}\). From 2006 until 2015, over 220 PubMed indexed articles were published concerning the effect of pBOO on the urinary bladder. Although animal models for pBOO have been devised in several species\(^{139}\), such as the rat\(^{140}\), the rabbit\(^{141}\), pig\(^{142}\), and mouse\(^{143,144}\), arguably the most commonly used animal model, however, are female rats undergoing partial obstruction of the proximal urethra; access to the animals’ abdomen, exteriorizing of the bladder, and dissection around the bladder neck are inevitable with this traditional proximal urethral obstruction technique. Variability in the degree of obstruction and animal mortality are only some of the concerns associated with this procedure\(^{148,149}\); we showed that in sham operated animals, dissection around the proximal urethra leads to physiologic changes that correlate with loss of nerve fibers at the bladder neck\(^{180}\). This finding indicates, that the most commonly used pBOO animal model, which involves accessing the proximal urethra in female rodents, leads to a denervation injury with associated structural and functional changes of the bladder, affecting sham and obstructed animals. Therefore, an alternate approach avoiding denervation injury was needed.

Our lab developed and evaluated a Nerve-sparing Mid-urethral Obstruction (NeMO) approach, effective in inducing expected obstruction-associated changes in the bladder such as an increase in organ mass and residual urine, whilst sham-operated animals were indistinguishable from unoperated control animals. Also, the striated urethral sphincter remained untouched as it lies proximal to the level of dissection. Furthermore, variability in the obstruction-induced increase in bladder mass was significantly lower than in traditional proximal urethral obstruction and animal mortality was zero. We also successfully applied NeMO in female mice with a less than 10\% mortality in obstructed animals, while all pBOO models for mice described to date were associated with a mortality around 50\%. Studying bladder remodeling in the context of pBOO
in mice will benefit from applicability of the whole spectrum of transgenic modifications. Dissecting around the mid-urethra in female rodents does not induce the undesirable and confounding structural or functional changes in the urinary bladder observed in the traditional proximal obstruction model. Nevertheless, inducing a partial obstruction at the mid-urethral level still induces bladder hypertrophy and increased residual urine, as expected from an animal model for pBOO. Importantly, performing NeMO in mice opens investigation of bladder remodeling in pBOO to transgenic methods, which are virtually unavailable in larger rodents.

4.2 Material and Methods

4.2.1 NeMO in female rats

12 female Sprague-Dawley rats of around 200g underwent pBOO, while 7 rats underwent sham procedure. Bladders were harvested after 2 weeks and assessed for residual urine volume and bladder mass.

4.2.1.1 Surgical procedure

a. Preparation

i. Place a female Sprague-Dawley rat weighing around 200 g under 3% isoflurane anesthesia, weigh the animal, and shave and disinfect the prepubic area using an iodine solution.

ii. On a heating pad, tape hind limbs and tail down and catheterize the bladder with a 20-gauge angiocatheter, which is also gently taped down to prevent sliding.

b. Dissection

i. Use a scalpel to perform an 8 mm longitudinal skin incision from the palpable pubic symphysis towards the urethral meatus. Lift off the skin on either side to facilitate later closure.

ii. Dissect bluntly to identify the stented urethra by longitudinal spreading with fine scissors. Place fine self-retaining skin hooks to enhance exposure of the
urethra. Lift off the neuro-vascular bundle running ventrally on the urethra by analogous blunt dissection.

iii. Perform some gentle longitudinal spreading using curved scissors dorsal to the urethra to develop the plane between urethra and vagina. Then, bluntly pass a 4-0 silk suture behind the urethra and prepare the double throw of a surgeon’s knot.

c. Partial urethral obstruction

i. Remove the angiocatheter, place a 0.9 mm metal rod parallel to the urethra, and then tighten the knot around the urethra and metal rod, so that the latter still slides out easily thereafter.

ii. Secure the knot with 3 more throws using gentle tension before removing the rod. Cut the suture ends about 3 mm long, or 4 mm if a later release of obstruction is part of the experiment.

d. Wound closure

i. Approximate the paraurethral glands using a buried single stitch of absorbable braided suture 4-0.

ii. Close the skin with a buried horizontal mattress stitch using the same absorbable suture.

e. After care

i. Inject buprenorphine 0.1 mg/kg subcutaneously before placing the rat in a recovery cage.

ii. Offer some soft recovery diet for the first 24 h to the single housed rat. After this period, house animals again in the same pairs as before the procedure provided they are doing well and belong to the same treatment group.

iii. Check the rats daily for bladder size and general well-being; record their weight at least weekly.
4.2.2 NeMO in female mice

21 female C57/Bl6 mice weighing around 18g underwent pBOO, while 18 underwent sham procedure. To determine the appropriate size of placeholder to be used alongside the urethra during placement of the partially obstructing tie, we used 3 different gauges (G) of injection cannulas, 25 G, 26 G, and 27 G. After 2 weeks, animals were weighed, bladders were harvested and assessed for residual urine volume and bladder mass.

4.2.2.1 Surgical procedure

a. Preparation

i. Place a female C57bl/6 mouse weighing around 18 g and proceed as detailed in step 1.1.1.

ii. On a heating pad, tape hind limbs and tail down and catheterize the bladder with a 24-gauge angiocatheter, which is also gently taped down to prevent sliding.

b. Dissection

i. Use a scalpel to perform a 6 mm longitudinal skin incision from the palpable pubic symphysis towards the urethral meatus.

ii. Dissect bluntly to identify the stented urethra by longitudinal spreading with fine scissors. Lift off the neuro-vascular bundle running ventrally on the urethra by analogous blunt dissection.

iii. Perform some gentle longitudinal spreading using curved scissors dorsal to the urethra to develop the plane between urethra and vagina. Then, bluntly pass a 5-0 non-absorbable braided suture behind the urethra and prepare the double throw of a surgeon’s knot.

c. Partial urethral obstruction

i. Connect the angiocatheter with a 1 mL syringe and inject 0.1 mL of normal saline into the bladder.

ii. Place a 26-gauge cannula parallel to the urethra. Gently tighten the knot around urethra and cannula while pulling back the angiocath.
iii. Secure the knot with 3 more throws using gentle tension before removing the cannula.

iv. Test for urine appearing at the meatus upon gentle pressure on the animal’s bladder; loosen the tie if unable to express urine. Otherwise, cut the suture ends about 3 mm long.

d. Wound closure

i. Close the skin with a buried horizontal mattress stitch using a 5-0 braided absorbable suture.

e. After care

i. Inject 0.1 mg/kg buprenorphine subcutaneously before placing the mouse in a recovery cage.

ii. Offer some soft recovery diet for the first 48 h to the single housed mouse. After this period, house animals again in the same groups as before the procedure provided they are doing well and belong to the same treatment group.

Note: Mice with a palpable distended bladder 24 h after the procedure that do not void when handled are over-obstructed and will die within the next 1-2 days unless taken back under anesthesia for removal of the obstructing ligature. Check the mice daily for bladder size and general well-being; record their weight at least weekly.

4.3 Results

4.3.1 Nerve-sparing Mid-urethral Obstruction (NeMO) in female rats

Mortality. We performed a mid-urethral obstruction on over 40 female rats so far and had no mortalities.

Bladder mass. Average relative bladder mass (bladder-to-body-mass-ratio) in sham operated animals was 0.33% two weeks after the procedure, while obstructed rats had a mean relative bladder mass of 0.60% which translates into an 85% increase (p=0.004; Figure 4.1).
**Residual urine.** Sham operated rats had virtually no residual urine that we could aspirate by direct bladder puncture at the time of organ harvest, mid-urethral obstruction animals, in contrast, had a significantly greater mean amount of residual urine of 0.42 mL (p=0.01; Figure 4.2).

**Figure 4.1:** NeMO effectively induces an increase in bladder mass after 2 weeks. NeMO led to an 85% increase in bladder-to-body-mass-ratio within 2 weeks (p=0.004; §).

**Figure 4.2:** Marked increase in residual urine 2 weeks after NeMO. While sham operated rats had virtually no residual urine that could be needle-aspirated at the time of bladder harvest, mid-urethral obstruction animals had almost 0.4 mL of residual urine at average (p=0.013; §). Copyright received.
4.3.2 Nerve-sparing Mid-urethral Obstruction (NeMO) in female mice

**Mortality.** Overall, 18 female mice underwent a sham NeMO procedure, and one of them died. In order to calibrate the necessary degree of obstruction, we evaluated 3 different diameters of cannulas (25-gauge, 26-gauge, and 27-gauge) placed along the urethra when tying the suture. None of the 5 mice died when we used a 25-gauge cannula as a placeholder along the urethra (Figure 3). On the other hand, mortality significantly increased with the smaller size placeholders. 1/8 or 5/8 mice died when using a 26-gauge or 27-gauge cannula, respectively (p=0.039, Fisher’s exact test).

![Figure 4.3: Mortality increased with the degree of partial obstruction. A) One out of 18 mice died after sham NeMO procedure. B) When using a 25-gauge cannula as a placeholder along the urethra, all 5 operated mice survived, while 1/8 or 5/8 mice died when using a 26-gauge or 27-gauge cannula, respectively (p=0.039, Fisher’s exact test). Copyright received.

**Bladder mass.** NeMO in female mice using a 25-gauge cannula did not effectively increase bladder to body mass ratio (Figure 4.4). The use of a 26-gauge cannula resulted in an over 2-fold increase in relative bladder mass (p=0.04). When we applied a 27-gauge placeholder an over 60% increase in relative bladder mass occurred after 2 weeks (p=0.004).
Figure 4.4: 26-gauge and 27-gauge placeholder during NeMO result in an effective increase in relative bladder mass after 2 weeks. NeMO in female mice using a 25-gauge cannula did not result in a significant increase in relative bladder mass ($\S$); when an only 26-gauge cannula was used, an over 2-fold increase in relative bladder mass occurred ($p=0.04; \dagger$); obstructions with a 27-gauge placeholder resulted in an over 60% increase in relative bladder mass ($p=0.004; \ddagger$). Copyright received.

**Residual urine.** Similar to rats, female mice tend to void at induction of anesthesia. When harvesting the bladders, we occluded the bladder neck with forceps and weighed the bladder with urine and after draining the urine, this allowed for precise calculation of residual urine present at bladder harvest. PBOO placing a 25-gauge or a 26-gauge cannula along the urethra did not result in a change in residual urine within 2 weeks (Figure 4.5). Notably, only very few animals voided during induction of anesthesia, which is in contrast to rats that virtually all void when induced. In the 3 mice surviving NeMO using a 27-gauge cannula, mean residual urine volume was increased by 3-fold compared to the respective shams. However, the difference did not achieve statistical significance, most likely due to the low $n$. 

![Bladder mass as % of body mass](image)
4.4 Discussion

Animal mortality is one of the major concerns of the traditional model of pBOO at the proximal urethra, at 15% or more in many reports\cite{148,149,176}. NeMO appears to have minimal mortality when applied in female rats. Proximal urethral obstruction in mice is technically more challenging than in rats and thus even more prone to complications. Using a 26-gauge cannula as a placeholder for our mid-urethral approach, we had only minimal animal mortality. In an ongoing study where we applied NeMO in female mice using a 26-gauge cannula only 2 out of 18 mice were over-obstructed (data not shown). Using a smaller placeholder such as a 27-gauge cannula over half of the obstructed mice died due to over-obstruction with urinary retention or bladder rupture. Therefore, we consider a 26-gauge placeholder to be a reasonable caliber for female mice of about 18 g.

Increase in bladder mass is one of the hallmarks of pBOO in human disease. We identified NeMO to effectively induce a significant increase in bladder mass after only 2 weeks in rats, as well as, in mice. The average increase in bladder mass after pBOO at the proximal urethra is over 2.5-fold after 2 weeks and hence much higher than what we observed after NeMO\cite{180}. Such an excessive increase in organ mass, however, does not reflect the majority of infravesical obstructive uropathies which researchers aim to model when applying pBOO; in this regard, NeMO offers a less invasive and well-tolerated pBOO model with a more
moderate remodeling-response that is much closer to human pathology than the traditional proximal pBOO. The more pronounced increase in bladder mass in proximal pBOO is most likely related to a denervation injury resulting from dissection around the bladder neck; denervation then may jeopardize detrusor-sphincter crosstalk and cause a functional outlet obstruction even in sham operated animals\textsuperscript{180}.

**Residual urine** was also significantly increased in female rats undergoing NeMO after only 2 weeks. In mice on the other hand, only using the 27-gauge cannula as a placeholder tended to increase the amount of residual urine in mice. However, while rats virtually all void when placed under gaseous anesthesia, we did not observe this in mice where only very few voided when induced. This non-voiding behavior of mice probably masks the true amount of residual urine and necessitates another stimulus to induce micturition in future studies.

While the **degree of obstruction** using a 0.9 mm rod along the urethra in female rats seems to be appropriate, combining minimal animal mortality with a clear increase in bladder mass and residual urine, calibrating the degree of obstruction for NeMO in female mice is more challenging. On one hand, using a 25-gauge cannula did not lead to a detectable increase in bladder mass, using the 27-gauge cannula, on the other hand, resulted in over-obstruction in more than half of the mice undergoing pBOO. Only the 26-gauge cannula had a reasonable response in terms of organ growth along with a low animal mortality.

Since pBOO is clearly more prevalent in men than in women, using male animals would be preferable when modeling human pBOO. Our nerve sparing mid-urethral obstruction (NeMO) technique obviously does not address this shortcoming of accepting animals’ hormone status influencing the inflammatory response that initiates tissue remodeling. The low variability and low animal mortality of NeMO in mice, however, will make investigation of bladder remodeling amenable to the full spectrum of transgenic methods, which are very limited in larger rodents.

In summary, NeMO for female rats and female mice is easy to do, has a low animal mortality, and avoids the denervation injury to the bladder resulting from dissection around the bladder neck.
5 Assessment of Murine Bladder Function

This chapter is modified from the following:

Martin Sidler, Karen Aitken, Darius Bägli

Assessment of murine bladder function.

Submitted to: Bladder, 2017
5.1 Introduction

Animal models are an essential part of research in bladder physiology and pathophysiology. Drug-effects, structural and functional changes resulting from experimental procedures, or phenotypic changes based on genetic manipulations can be assessed with regards to bladder physiology in rodents. However, the physiologic parameters measured in rodent bladders have only limited translational value to human bladder pathology. To avoid confusion, the terms used to describe animal bladder physiology should be clearly defined in experimental studies and terms describing human bladder physiology should be avoided\(^{181}\). Despite these translational limitations, rodents have been used extensively in research of LUTS\(^{182,183}\). A research project’s hypothesis, methods involved, and the planned endpoints determine the choice of species to be used in a study. Using mice as experimental animals has the powerful advantage of the full availability of transgenic manipulation and generally lower cost than larger animals. While histologic workup and molecular analysis of bladder tissue are routinely done in all species used, there are three main techniques within a spectrum of options to assess murine bladder function.

1) **Continuous urine weight recording (CUWR)** as an assessment of bladder physiology has been used in different studies with some variation in the technical set-ups\(^{184,185}\). However, the basic principle of collecting and weighing urine directly under a mouse cage remains. Also, measurement of post-void residual urine (PVR) at the time of bladder harvest is commonly done in studies using CUWR. Although the method is simple and requires little specialized equipment, it was not widely applied in the past. CUWR is a common method to record micturition pattern in rats, whereby urine is funneled in a measuring cup while solid material such as feces or chow dropped by the study animal gets collected separately. However, the same equipment is unsuitable to quantify micturitions in smaller rodents such as mice, as their much smaller voided volume would adhere to the funnel and thus not reach the measuring cup\(^{186,187}\).

2) **VOIDED STAIN ON PAPER (VSOP)**, alternatively named “voided stain assay” (VSA), takes advantage of filter paper getting discolored by contact with liquids (i.e. urine). The first
description of this technique also used automated paper propulsion, which allowed for recordings of up to 24 hrs duration\textsuperscript{188}. The more recent applications of VSOP mostly used static paper underneath a mouse cage, which provides data about voided volumes and number of micturitions per time in a semiquantitative way\textsuperscript{189-191}. For this technique, the duration of measurement is limited to a few hours, unless the paper is changed regularly. The VSOP technique was also further developed based on continuously transported filter paper as described earlier\textsuperscript{188}, with the addition of automated scanning and quantification of urine spots on laminated paper – this technique was named automated VSOP (aVSOP)\textsuperscript{146}.

3) **Invasive cystometry** or the cystometrogram (CMG) probably yield the broadest spectrum of functional parameters. One of the major limitations of CMG is that bladder histology and RNA expression patterns may not remain undisturbed after this invasive type of functional assessment\textsuperscript{147,192}. Also, cystometric studies in mice are much more challenging to obtain than in larger animals such as rabbits or guinea pigs. However, the latter is obviously more expensive to purchase and to hold and anesthesia is more challenging than rats or mice. Additionally, they require more of potentially expensive study drugs based on their greater body mass. As an extension of cystometry, video-urodynamics have recently been described as well, combining CMG with bladder ultrasonography\textsuperscript{193}; image-derived measurement of bladder volume seems to be the added advantage of that technique.

Here, we present our experience with novel tracing analyses derived by continuous urine weight recording (CUWR) to determine bladder physiologic parameters in mice. We also tested our set-up of CUWR and applied it in a pilot study, evaluating the functional effect of treatment with an mTOR pathway inhibitor\textsuperscript{38,44}, (S6K-inhibitor PF-4708671) in female mice with partial bladder outlet obstruction (pBOO). We also provide a review of other described options to assess murine bladder physiology and compare it to CUWR, a method that is non-invasive efficient, low cost, not labor intensive, and that can be done over extended periods of time.
5.2 Material and Methods

5.2.1 Cages and Recording Equipment

We adapted metabolic cages available in our animal facility that were designed for rats (Figure 5.1 B); in the original configuration, voided urine was funneled into a measuring cup, while feces was collected in a separate container. Despite hydrophobic coating of the funnel, some drops of urine from each void would stick to the funnel, making this method unsuitable for micturition recording in mice. To circumvent this problem, we removed the funnel and let all excrements drop on a paper-covered plate residing on a weight scale underneath the cage. We evaluated Polytetrafluoroethylene (PTFE) mesh, as well as, different sizes of stainless steel mesh treated with hydrophobic spray to keep feces from dropping onto the scale while letting urine pass. We directly compared recordings with and without a mesh above the scale and consequently removed the mesh and adopted a new approach. We developed a novel tracing analysis algorithm further described below. The algorithm can discriminate whether a weight-increase was caused by solid or fluid material based on the acute peak of the former and evaporation of the latter (Figure 5.2 A and B). Essentially, analyses included tracing shape, frequency per unit time, and the amount of urine weight gain for each event as detected by the scale. The weight scales (Scout® Pro, Ohaus® Corporation, Parsippany, NJ, USA) had a resolution of 10mg and were connected via USB hub to a lap top computer running a recording software (Logger Pro®, Vernier Software & Technology®, Beaverton, OR, USA). The scales were placed 15cm underneath the cages so that mice’s tail would not touch the scale and thus lead to artifacts. To describe the effect of a mesh placed underneath a metabolic cage, normal saline (NS) ranging from 20 µL to 250 µL was pipetted onto 2 paper-covered weight scales, with one of them having a wire mesh 5 cm above it and weight was recorded once per minute; weight increases recorded by the scale with mesh were for the most part lower than the increase recorded by the scale without mesh (Figure 5.1 F); the weights as detected by the analysis algorithm are slightly lower than the actual amounts dropped onto the scales, which is a result of averaging recordings immediately before and after a weight increase (Figure 5.1 G). For the in vivo testing, we recorded micturition for 10 hours during the animals’ sleep cycle after letting them adapt in the metabolic cage for one hour.
Figure 5.1. Equipment for weight-based micturition assessment in mice. A) Metabolic cages initially designed for rats were adjusted so that all excrements could be collected and weighed directly without loss of urine sticking to a screen interposed between the animal and the scale. B) Original set-
up of metabolic cages to assess micturition parameters in rats with urine funneled into a cup residing on a scale. C) Urine and fecal pellets were allowed to drop directly onto the paper-lined plate on the weight scale, where fluid components evaporate. D) Several channels were recorded simultaneously connecting the scales to a laptop running LoggerPro® software via a USB hub. E) Example of urine drop with droplets remaining on the mesh. F) Traces of weight-recording from one scale with and another scale without mesh above, with identical amounts of Normal Saline (NS), pipetted on them; volumes were repeated 3 times. G) Detected amounts of Normal Saline dropped on a scale with mesh were consistently lower than amounts detected by a scale with mesh above. Copyright pending.
Figure 5.2. Data recording and discrimination of urine-induced increase in recorded weight. A) Micturitions are recorded as steep increase, followed by a slightly curved decrease caused by evaporation of urine. B) If a solid particle such as a chow pellet happens to drop on the scale, the weight
increase is sustainable. C) Example of recorded trace with analysis results. D) Sample traces from all 4
groups of animals; sham operated animals showed very few small voids, whereas, obstructed animals –
drug treated or vehicle – demonstrated an increase in small voids, indicative of hyper-activity, similar
to overactivity in obstructed patients. Copyright pending.

5.2.2 Data Analysis Algorithm

The recorded data was transferred to a spread sheet (Excel®, Microsoft corporation, USA) and
we devised a simple macro that would detect weight increase, under the condition that it was
followed by a decrease (Figure 5.2 C and Appendix I). This prerequisite would allow
distinction between fluid/evaporative and solid material dropped onto the scale (Figure 5.2 A
and B). The calculation also averaged cell values to account for minor fluctuations in
recordings caused by airflow. Additionally, we programmed a software macro to avoid double
counting of a single micturition; based on the column featuring the raw weight recordings, the
macro detected micturitions in a first step, while it would edit the actual micturition-induced
weight increase in a second step. Parameters setting the various thresholds referenced by the
macro are listed as “Master parameters” and would be identical throughout the project.
Resulting variables were mean micturition volume, maximum micturition volume, total
number of voids, number of small voids (i.e. less than 50 µL), and total urine volume over 10
hours of recording; the ratio of small voids was calculated as the number of small voids
divided by total number of voids and served as a surrogate for hyperactive bladder.

5.2.3 Surgical Procedure and Drug Treatment

We evaluated our method of assessing murine bladder physiology by continuous urine weight
recording in a pilot study, examining the effect of S6K-inhibition on mice with partial bladder
outlet obstruction (pBOO). To this end, 28 female C57/Bl6 mice weighing 17-20 g were
randomly divided into two groups, 16 underwent pBOO as previously described, while 12
mice underwent the respective sham procedure (JoVE reference194), two of the first group
were inadvertently completely obstructed and consequently had to be euthanized. Half of each
group received daily intraperitoneal injections with the S6K-Inhibitor PF-4708671 at 75mg/kg
body mass, while the other half received vehicle195. Bladders were harvested after 2 weeks,
and bladder mass was recorded. Mice were housed in groups under standard conditions and had free access to food and water. All experiments with animals undergoing micturition assessment were approved by the institution's animal care committee.

5.2.4 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 24 (SPSS Inc., Chicago, IL). Direct comparison between 2 groups was done using two-tailed T-Test, or Welch T-Test where equality of variances was not given, respectively. p-values <0.001 were considered highly significant. Numerical results were summarized graphically using boxplots, the box encompassing the interquartile range (IQR) from the first quartile (Q1) to the third quartile (Q3), the bold transverse bar representing the median. The whiskers mark maximum and minimum values. Outliers represented by a circle-symbol (○) are more than a 1.5-fold IQR away from Q1 or Q3, respectively.

5.3 Results

5.3.1 Micturition recording and analysis method

We found a wire mesh with 11x11 by square inch grid (opening size of 2 mm; wire diameter 0.45 mm) to reliably retain mouse feces, larger mesh size would allow feces to pass through while smaller sizes would increase the amount of urine sticking to the mesh despite treatment with hydrophobic coating such as silicone spray (Figure 1E). In a direct comparison of identical amounts of NS pipetted onto a scale without and on another scale with wire mesh above, the scale with the mesh failed to pick up small voids of 20 μL and almost consistently led to lower amounts of recorded weight-increase (Figure 5.1 F and G).

Also, when recording for several hours, contamination of the mesh by dried urine, as well as, the increasing amount of feces on the mesh would potentially cause progressively more urine to be absorbed over time. We also evaluated a Polytetrafluoroethylene (PTFE) mesh with a similar size as the wire mesh; however, due to its highly hydrophobic capacity, the urine drops would mostly not even pass the mesh but stay on top of it, we, therefore, did not use PTFE mesh in further trials. Since any mesh seemed to reduce the amount of urine detected, we decided to leave the mesh aside and recorded all material falling underneath the cage, i.e.
urine, feces, occasional pellets of chow, when a mouse could move one out of the food tray (Figure 5.2 A and B). We also weighed feces pellets which were around 20 and 30 mg. Since they had only a slow evaporation-related mass decrease, feces led to changes in the recording that were not followed by the same decrease in recorded weight as voided urine. We also noted that airflow reduction in the measurement room visibly reduced the extent of random fluctuations in recorded weight. The iterative changes to a simple macro allowed the recorded weight-traces to be analyzed by an optimized algorithm, which reliably detected micturitions while weight increases caused by solid material dropped onto the scale were not classified as micturition. The smallest voided volume we were able to detect weighed 20 – 30 mg, airflow-induced fluctuations in recorded weight, as well as, maximum resolution of the scales used did not allow to detect smaller voids.

5.3.2 Functional Changes associated with pBOO, as well as, Treatment with S6K-Inhibitor are detected using Novel Analysis Algorithm

The ratio of small was almost double in obstructed and drug treated mice compared to obstructed and vehicle treated mice (p=0.023); similarly, the ratio of small voids was increased by 2-fold in all obstructed mice compared to all sham operated mice (p=0.011; Figure 5.3 A). Two weeks after pBOO or sham procedure, respectively, we found that obstruction tended to lead to a 2-fold increase in relative bladder mass (p=0.082; Figure 3E) and absolute bladder mass (p=0.066; Figure 5.3 F). In sham animals, treatment with S6K-inhibitor induced an increase in relative bladder mass by almost 60% (p=0.014; Figure 5.3 E), as well as, an increase in absolute bladder mass of over 50% compared to sham operated and vehicle treated animals (p=0.016; Figure 5.3 F). The changes in bladder mass among the obstructed groups were not statistically significant. Total number of micturitions, total voided volume, and mean micturition volume over 10 hours of recording were similar among all four groups (for details see Figures 5.3 C and D).
Figure 5.3. Partial bladder outlet obstruction (pBOO) and treatment with S6K-inhibitor affects bladder mass and leads to a hyperactive voiding pattern. A) The ratio of small voids as a surrogate of bladder hyperactivity was almost double in obstructed and drug treated mice compared to obstructed and vehicle treated mice (*; p=0.023); this ratio was also increased by 2-fold in all obstructed animals pooled compared to all sham operated animals (§; p=0.011). B-D) Mean micturition volume, total

E-F) Relative bladder mass (%)

G-H) Bladder mass (mg)
number of micturitions, and total voided volume over 10 hours of recording was similar among groups. **E)** Relative bladder mass in sham operated mice was increased by treatment with S6K-inhibitor by almost 60% (*; p=0.014) compared to sham operated and vehicle treated animals, while the almost 2-fold increase in relative bladder mass induced by obstruction was not statistically significant (§; p=0.082). **F)** Obstruction tended to lead to a 2-fold increase in absolute bladder mass (§; p=0.066) in vehicle treated mice, while treatment with S6K-Inhibitor resulted in an over 50% increase in bladder mass (p=0.016). Copyright pending.

5.3.3 Literature review

From 2007 to 2016, 78 PubMed-indexed studies were published using **mouse cystometry**. We also found 25 studies utilising **VSOP** or **VSA** to assess murine bladder function, while **aVSOP** was applied in 5 studies during the same period, respectively. Only 3 studies used the **weight-based** micturition recording during the 10-year period examined. The earliest description of the aVSOP method dates back to the late 1970s and was presented in a modified set-up in 2002 and 2008146,188-190, mouse cystometry was first described in the late 1980s196, while the addition of concomitant bladder ultrasound was only recently suggested193; the first report involving weight-based micturition recording was published in 2001184.

5.4 Discussion

Animal models – and rodent models in particular – are an essential part in studying bladder physiology and pathophysiology183. While models for benign bladder diseases that required a surgical intervention such as pBOO were traditionally for the most part done in rats, there seems to be a recent shift towards the use of mice also in this field of research. The full availability of genetic modifications in mice arguably being the leading reason for this trend also highlight the need for accurate functional methods that are non-invasive so as not to disturb underlying the underlying genomic and epigenomic responses while maintaining reliable phenobiology of this field. Morphologically, the mouse bladder also may be more like human bladder since both contain intramural ganglia, as opposed to the rat bladder, which is typically ganglia-free197. However, assessment of murine bladder function is technically far
more challenging given the small bladder size, delicate tissue architecture, and low micturition volumes. Together, this rationale argues for new non-invasive methodologies to assess murine bladder function.

The observation of increased bladder mass in the groups treated with S6K-inhibitor was unexpected, given that inhibition of S6K is known to lead to decreased cell size and low body mass\(^{198,199}\). While we confirmed the S6K-inhibitory effect of PF-4708671 on cardiac tissue in vivo in a small pilot experiment earlier, the mechanism by which treatment led to increased bladder mass will need to be explored.

5.4.1 Equipment and data analysis for CUWR

Our lab previously established a novel, reliable pBOO-model in female mice with a much-reduced signal-to-noise ratio in sham operated animals with very low animal morbidity and mortality\(^{194}\). Subsequently, we had to determine a method to assess murine bladder function. The three principle options were cystometry, a-VSOP, and continuous urine weight recording (CUWR). We considered the latter to be the least labor intensive, the least expensive, and obviously also a non-invasive method. Additionally, CUWR lends itself for micturition assessment over extended periods of time with minimal additional effort and expenses. Based on equipment originally designed to record rat micturition behaviour, we modified the method to render it suitable for mice. The challenges in refining CUWR in mice included how to either prevent solid waste elimination from confounding the recording strategy while maximizing the capture of liquid (urine) elimination patterns.

Our CUWR method with the corresponding analysis allowed capture and detection of mouse micturitions weighing as little as 20 – 30 µg, which is a significantly higher capture resolution than reported in other studies using CUWR, where voided volumes did not seem to be much less than 50 µg\(^{146,184,185}\). In one of the studies, this difference might be due to the wire mesh placed underneath the cage to retain feces, but which also retarded capture of some of the voided urine thus causing small voids to remain undetected despite the use of very precise weight scales\(^{184}\). In another study employing CUWR, detected micturitions also were greater than 100 µg for the most part\(^{185}\); it is unclear whether this finding could be related to the strain of mice used (not indicated), which notoriously affects functional bladder parameters\(^{200}\). Indeed, it is important to note that micturitions in mice of both genders and at various animal
ages are often less than 50 μL\textsuperscript{201}. In addition, our method of CUWR to assess murine bladder function allows for precise detection of an array of parameters including maximum voided volume, micturition frequency, absolute number of small voids, as well as, the ratio of small voids, which can serve as a surrogate of hyperactive voiding behavior. CUWR is not labor intensive, allows for parallel recording of multiple animals, and can be done repetitively and over extended periods of time.

We chose to evaluate our CUWR method in a pilot study applying a recently established pBOO model in female mice\textsuperscript{194}; additionally, we sought to determine the functional effect of mTOR inhibition during pBOO in mice using an inhibitor of S6K, a downstream signaling partner of the mTOR pathway. In previous studies, our lab showed that S6K-activity was increased in the partially obstructed bladder, which was associated with a loss of smooth muscle cell differentiation\textsuperscript{38}. While mechanistic evaluation of the involved pathway itself is beyond the scope of this report, our functional results showed the anticipated increase in bladder mass secondary to obstruction. Also, we found that obstruction leads to an increased ratio of small voids, which serves as a surrogate for hyperactive voiding pattern; in this regard, the pBOO model used leads to functional changes similar to pBOO in men with BPH\textsuperscript{88}. Interestingly, our CUWR strategy was able to detect a further increase in bladder hyperactivity after treatment with S6K-inhibitor. Our results also show that pBOO, as well as, S6K-inhibition affect bladder mass and lead to an altered voiding patterns, detected in both cases vs. control animals using our CUWR method.

In studies involving bladder physiology, post-void residual urine (PVR) can be assessed using ultrasound\textsuperscript{193}; however, it is more commonly determined via direct volume measurement at the time of bladder harvest. While in rats, this parameter is can be determined via needle-aspiration of urine, the friable and much smaller mouse bladder does not allow for the same technique to be used reliably. The most accurate measurement of murine residual urine during bladder harvest can be done by temporarily clamping the bladder at its neck, weighing the urine-containing bladder on a scale, so that the empty bladder mass can then be subtracted to derive the weight of residual urine. While female rats virtually all void during induction of anesthesia, this behaviour is much less consistent in mice. Mouse handling around induction of anesthesia clearly affects whether the animals empty their bladder at that time or not\textsuperscript{193}; we therefore also recommend that micturition during induction should be observed and recorded.
5.4.2 Overview of options to assess murine bladder function

In general, CUWR yields directly measured parameters such as maximum micturition volume, mean micturition volume, micturition interval/micturition frequency, number of voids, and number of small voids (Table 5.1). Additionally, provided the animals void during induction of anesthesia, PVR can be determined as well. Furthermore, PVR plus maximum voided volume may be considered as maximum bladder capacity. As a surrogate of voiding efficiency, mean micturition fraction is the ratio of average voided volume divided by maximum bladder capacity. The ratio of small to total number of voids, by definition more frequent than normal voiding, is a marker of a hyperactive voiding pattern (NeMO-reference, when available). The ease of recording and data analysis makes CUWR a non-invasive, efficient and little labor intensive method. Also, besides modified cages, only weight scales and data recording software is necessary to apply CUWR, which makes this method arguably the most affordable of all options discussed here. On the other hand, a limitation of CUWR is obviously that no intravesical pressure data can be recorded and non-voiding contractions go unnoticed.

Voided stain on paper (VSOP) is another non-invasive method to assess murine bladder function (Table 5.1). Parameters determined by VSOP are similar to CUWR, however micturition frequency may be less accurate, given the limited duration of measurement of 2-4 hours\(^{190,191}\). Since stained paper needs to be recovered and scanned with resulting images analysed to extract micturition data, VSOP is likely more labor intensive than CUWR and only little specialized equipment is necessary. Limitations are the restricted duration of measurement, and the possible overlap of stains from repeated micturitions in the same area, which becomes more likely as time of measurement increases. In an attempt to reduce artifacts, some researchers also did not provide water to mice, which further limited the possible duration of VSOP-measurement\(^{191}\), as well as, may affect bladder function both from under filling, and increased urine concentration. As a variant of VSOP, a VSOP-method with automated paper propulsion was described in 1978\(^{188}\). A further refined method with automated image processing was described in 2012 and named automated VSOP (aVSOP)\(^{146}\). Parameters determined by aVSOP are again similar to CUWR and virtually the same as in VSOP; however, measurement can be done over extended periods of time and the resolution regarding time of micturition-event is improved compared to VSOP. With the automated
scanning and image processing, aVSOP might be less labor intensive than VSOP, while the equipment necessary, as well as, quality and quantity of laminated filter paper used almost certainly increase the cost for aVSOP, especially if a protocol requires long-term or repeated measurements. Although accuracy in recording time of a single micturition is improved in aVSOP, overlap of urine stains is still of concern and especially small voids might go unnoticed when urine drips on paper in an area of a previous larger void, which limits accuracy of this method.

**Cystometry** theoretically offers the broadest spectrum of physiologic parameters of all assessment options (Table 1)\(^ {147}\), whereby direct measurement of MV still requires urine collection in a cup or on filter paper\(^ {147,186}\). Other authors also used manual collection of urine from bottom of the cage\(^ {202}\). While the criteria described for CMG in animals appear similar to those registered in human urodynamic studies, terminology should reflect the distinct differences; *detrusor overactivity* for example is defined by ICS as “involuntary detrusor contractions during the filling phase which can be spontaneous or provoked”, or *cystometric bladder capacity* is defined by ICS as “the bladder volume at the end of the filling cystometrogram, when ‘permission to void’ is usually given”\(^ {34}\). Both criteria involve active cooperation of the subject studied, which is obviously not possible in animals. Instead of detrusor overactivity the term bladder hyperactivity should be used when non-voiding contractions are recorded or when increased micturition frequency is observed\(^ {147}\)

Additionally, urodynamic testing is defined as “an interactive diagnostic study of the lower urinary tract composed of a number of tests that can be used to obtain functional information about bladder filling, urine storage and emptying”\(^ {203}\); considering this definition, the term urodynamic testing may also deserve to be limited to studies in humans.

Rats are probably the most widely used species for cystometry, but it is also applicable in rabbits or guinea pigs\(^ {204,205}\). Cystometry in mice on the other hand, is technically more difficult given the small size of their bladder\(^ {144,206}\). Most commonly, suprapubic catheter insertion is performed for mouse cystometry, the rarely used transurethral technique in anesthetized mice only yields a limited choice of functional parameters\(^ {196}\). A recent description of how the entry point of transvesically inserted catheters affects cystometric results also deserves consideration when planning an experiment or comparing results\(^ {193}\). The small catheters used for mouse cystometry are prone to twisting and occlusion, they can also
lead to irritation of the small bladder which obviously translates into artifactually induced bladder activity\textsuperscript{147}. Restraining mice for cystometry reduces the risk of mechanical dysfunction of the catheter; however, the technique increases sympathetic activity and thus increases bladder capacity, again introducing unpredictable and spurious functional measurements\textsuperscript{192}. Cystometry can also be done in anesthetized animals, but results have to be interpreted with caution, as virtually any anesthetic confounds bladder functional responses\textsuperscript{207}. Catheter implantation and execution of the measurement protocol makes rodent cystometry extremely labor intensive and leads to loss of animals. Considering the equipment necessary, cystometry is also the most expensive method to assess murine bladder physiology.

Despite its popularity, cystometry in rodents also has significant limitations. Histologic work-up of rat bladders 2 days after catheter implantation showed granulation, severe edema and hemorrhage, while 7 days after the procedure, these changes were still in a repairing stage\textsuperscript{192}. Because of these changes, histologic analysis of bladders after CMG is of limited value.

Despite the fact that rodent bladders are still in a repair stage one week after catheter implantation, most cystometries are still done within the first 3 days thereafter\textsuperscript{181,186}. An indwelling catheter not only affects bladder histology and function\textsuperscript{193}, it almost certainly also affects gene expression patterns in the bladder\textsuperscript{208}, especially over the first days after catheter implantation – the active wound healing process after laparotomy and catheter insertion makes results from gene expression analysis difficult if not impossible to interpret.

Furthermore, time of recording also seems to be critical in cystometry. During the first days after catheter implantation, bladder activity and pressures seem to be increased along with low micturition volume; these parameters stabilize by day 6 after catheter implantation\textsuperscript{207}. The same study also found that after one week, 15\% of catheters lost function for reasons such as occlusion dislodgement, biting, stone formation, or bleeding; mean post implantation survival was only 22 days; therefore, CMG with implanted catheters does not seem to be a suitable method for longitudinal observations with repeated measurements. It was also noticed that CMG-results are less consistent across laboratories than non-invasive method such as VSOP\textsuperscript{191}. Besides the variability resulting from slightly different application of a certain measurement technique, mouse strain, sex, age, and husbandry practices, as well as, time point in the animals’ circadian rhythm clearly affect physiologic results as well, which mandates that this kind of information is provided in experimental reports\textsuperscript{200,209,210}. 

5.5 Conclusion

Among the methods to assess murine bladder function, continuous urine weight recording is critically non-invasive and seems to be the most accurate, efficient, arguably the least expensive, and most suitable option for long-term recording. It can be applied with little specialised equipment, sometimes even with just minor modifications of animal cages available in most animal facilities. With our novel approach, using a software algorithm to discern solid versus liquid material dropping on the weight scale, no urine gets caught in the mesh, allowing for small micturitions to be recorded reliably. Detection of small voided volumes is essential in detection of hyperactive voiding patterns such as induced by pBOO. VSOP on the other hand is usually limited to short-term measurements and has limited precision in time-resolution of events; aVSOP mitigates these shortcomings, while more specialised and expensive equipment becomes necessary. Invasive measurement methods such as cystometry or video-urodynamic studies offer the advantage of yielding a broad range of parameters, including intravesical pressure data; these methods are obviously a lot costlier and labor intensive than non-invasive methods, the yielded results are also less consistent between laboratories than non-invasive measurements and thus more challenging to compare. Disturbed histology and limited value of gene expression analysis of bladder tissue after cystometry are significant drawbacks of this method. Choice of the assessment method for murine bladder function is influenced by the anticipated effect of an experimental intervention and the consequential endpoints a study aims to monitor. Since animal strain, gender, age, and husbandry practices, as well as, measurement technique affect bladder function and measured physiologic parameters, these aspects should be precisely defined in scientific reports to facilitate interpretation of results.

Table 5.1. Overview of methods assessing mouse bladder physiology

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameters measured</th>
<th>Equipment necessary</th>
<th>Cost</th>
<th>Invasive</th>
<th>Reported duration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystometry</td>
<td>Basal pressure, maximum pressure, threshold pressure, intermicturition</td>
<td>Bladder catheter, 3-way valve, pressure transducer, infusion</td>
<td>$$$</td>
<td>Yes</td>
<td>1-2 hrs</td>
<td>147,186,187,191,196, 211,212</td>
</tr>
<tr>
<td>Video-Urodyamics</td>
<td>As cystometry. Additionally, ultrasonographic measurement of bladder capacity and PVR</td>
<td>As cystometry. Additionally: High resolution ultrasound probe.</td>
<td>$$$$</td>
<td>Yes</td>
<td>1-2 hrs</td>
<td>193,213,214</td>
</tr>
<tr>
<td>VSOP</td>
<td>Micturition volume, amount of urine per hour, micturition frequency, PVR at time of harvest.</td>
<td>Filter paper. Image scanning and analysis equipment/software.</td>
<td>$</td>
<td>No</td>
<td>2-4 hrs</td>
<td>190,191,212</td>
</tr>
<tr>
<td>aVSOP</td>
<td>As VSOP, enhanced time-resolution.</td>
<td>As VSOP. Special metabolic cage with automated transport of filter paper and automated image-scanning.</td>
<td>$$</td>
<td>No</td>
<td>Up to 8 days</td>
<td>146,201,213</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>-----</td>
<td>-----</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>CUWR</td>
<td>Micturition volume, ratio of small voids, micturition frequency. PVR at time of harvest. Calculated bladder capacity and micturition fraction.</td>
<td>Modified animal cages. Digital weight scales with USB-connection. Data recording software.</td>
<td>$</td>
<td>No</td>
<td>Up to 7 days</td>
<td>184,185</td>
</tr>
</tbody>
</table>
6 Role of Macrophages in Obstruction-Induced Bladder Remodeling

6.1 Introduction

Partial bladder outlet obstruction (pBOO) has a high prevalence and can affect people at any age and both genders. The most common form of pBOO results from benign prostatic enlargement (BPE), affecting 70% of men in the US at 60-69 years of age and 80% of men of 70 years or older\textsuperscript{13}. To a significant extent, obstruction-induced bladder tissue remodeling and mass increase can be attributed to the activation and proliferation of bladder smooth muscle cells (BSMC). Three distinct stimuli leading to BSMC activation have been identified and mechanistically described\textsuperscript{38}, mechanical strain, hypoxia, and denatured extracellular matrix\textsuperscript{37,87}. The obstruction-induced remodeling process in the urinary bladder follows a sequence of inflammation, compensatory smooth muscle hypertrophy, and ultimately and tissue fibrosis, which is irreversible and marks the functionally decompensated state\textsuperscript{70,215,216}. This latter state is marked on one hand by storage dysfunction such as decreased bladder compliance, detrusor overactivity, and urinary incontinence; on the other hand, voiding dysfunction such as incomplete voiding, and detrusor underactivity (often leading to recurrent urinary tract infections) are also frequent complaints after long-term pBOO.

However, while macrophages are known to be involved in remodeling of other organs, such as the heart\textsuperscript{110}, intestine\textsuperscript{112}, skin\textsuperscript{113}, lungs\textsuperscript{106}, kidneys\textsuperscript{111}, and liver\textsuperscript{109}, their specific role in bladder remodeling has not been explored so far. One study examined the effect of deficiency in macrophage migratory inhibitory factor (MIF) in a pBOO model in female mice\textsuperscript{138}. MIF knock-out (ko) mice showed less submucosal and detrusor fibrosis, as well as, preserved muscle mass 3 weeks after pBOO when compared to wild-type (WT) mice. The same study detected a pro-apoptotic effect of MIF on BSCM in culture. However, the specific contribution of macrophages to the observed differences between WT and MIF-ko mice seen \textit{in vivo} remained unexplored. Other researchers found that pBOO in female rats led to an increased number of macrophages in the detrusor at 1 and 4 weeks after the procedure when compared to sham operated animals. The increase in macrophages corresponded with an
increase in expression of various inflammatory cytokines\textsuperscript{137}. Particularly, IL-1β was found to mediate an increase in bladder mass, bladder capacity, and micturition pressure in female mice with pBOO\textsuperscript{217}. Elevated levels of IL-6, IL-17, and tumor growth factor-β (TGF-β) in urine were also found during pBOO in a rat model\textsuperscript{133}.

To study immune cells and cytokines, we used a pBOO model that allows to dissect and partially obstruct the urethra at a safe distance to the bladder\textsuperscript{194}, thus avoiding the dissection-induced wound healing response at the bladder neck along with infiltration of inflammatory cells.

6.2 Material and Methods

6.2.1 Preliminary assessment of macrophage density in rat bladder after pBOO

6.2.1.1 Surgical procedure

9 female Sprague-Dawley rats of 220-250 g underwent pBOO. In the anesthetized animals, we exposed the mid part of the urethra caudal to the pubic symphysis and temporarily stented the urethra with a 20G angiocath to facilitate its delineation. The genital (clitoral) neurovascular bundle running on top of the urethra was carefully detached to exclude it from the ligature. After minimal dissection between vagina and urethra, a 4-0 braided non-absorbable suture was passed behind the urethra. The catheter was removed and a 0.9mm metal rod was temporarily placed alongside the urethra while tying the suture, which was done with variable degrees of tension, to achieve some variation in degree of obstruction. The wound was closed in two layers. The procedure for sham animals was identical except for placing the tie. After 2 weeks, animals were anesthetized, residual urine was measured by direct aspiration from the bladder, before bladders were harvested, weighed, and embedded in optimal cutting temperature (OCT) compound. Rats were euthanized by exsanguination.

6.2.1.2 Immunofluorescence for rat bladder tissue

Midequatorial bladder cryosections of 7µm were fixed in 4% paraformaldehyde (PFA), then permeabilized with a 0.2% Triton-X solution in PBS for 10 min at room temperature (RT) and
then blocked in 10% serum, 0.3% BSA, 0.3M Glycine solution in PBS for 1 hour before incubation with primary antibodies: Mouse anti rat CD68 (1:200; Abcam, Cambridge, MA, USA) to identify macrophages; rabbit anti Myosin heavy chain (1:200; Sigma, St. Louis, MO, USA). Secondary antibodies were donkey anti rabbit AlexaFluor® 647-conjugated and goat anti mouse Cy™3-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used at a 1:400 dilution in 1% BSA PBS solution and incubated for 1 hr at RT. Nuclei were counterstained with Hoechst 33342. Images were automatically analyzed using the image processing program Volocity (Version 6.3; PerkinElmer Inc., Woodbridge, ON, Canada). Number of macrophages per high power field was averaged from 3-4 pictures taken from the detrusor area of each sample.

6.2.2 Depletion of bladder macrophages using Clodronate Liposomes®

6.2.2.1 Surgical procedure and drug treatment

To quantify macrophage number in whole mouse bladders, 10 female C57Bl/6 mice of 18-21 g body mass underwent pBOO at the mid-urethral level. Detailed description and a link to videography material demonstrating the technique are available in chapter 4. Briefly, in anesthetized mice, we exposed the mid part of the urethra caudal to the pubic symphysis and temporarily stented the urethra with a 24-gauge angiocath to facilitate its delineation. After dissection between vagina and urethra, a 5-0 braided non-absorbable suture was passed behind the urethra. The catheter was removed and a 26-gauge hypodermic needle was temporarily placed alongside the urethra while tying the suture. The wound was closed using braided absorbable suture. 5 of the obstructed mice received intraperitoneal (IP) injections with Clodronate Liposomes (CL; ClodronateLiposomes, Amsterdam, Netherlands) at 50 mg per kg body mass 3 times per week, the other 5 obstructed mice received Normal Saline (NS; 0.9% NaCl) injections, 5 mice served as unoperated controls. After 2 weeks, animals were anesthetized for bladder harvest. Mice were euthanized by exsanguination.

6.2.2.2 Quantification of macrophages by CyTOF® mass cytometry

The 5 bladders of each group and 2 spleens – serving as positive controls – of control mice were pooled, morcelized, and digested into a single cell suspension using collagenase. Single cell suspensions were quantitated on a CyTOF2® (cytometry by time of flight) mass
cytometer (Fluidigm, San Francisco, CA, USA). The following antibodies were used: Rat anti mouse CD45 to detect myeloid cells, rat anti mouse CD3 to label T-cells, rat anti mouse CD11b to label macrophages (all Fluidigm), rat anti CD19 to label B-cells, and rat anti mouse CD4 to label T-helper cells (Biolegend). Data analysis was done on Cytobank (Cytobank, Santa Clara, CA, USA).

6.2.3 Cytokine assay

6.2.3.1 Surgical procedure

As part of a dose-finding experiment requested by the institutions animal care committee, 16 female C57Bl/6 mice of 18-21 g body mass underwent pBOO at the mid-urethral level as described under 6.2.2.1. 6 mice received intraperitoneal (IP) injections with Clodronate Liposomes (CL; ClodronateLiposomes, Amsterdam, Netherlands) at 50 mg per kg body mass 3 times per week, 6 mice received half of the dose, 4 mice received Normal Saline (NS; 0.9% NaCl) injections. After 2 weeks, animals underwent micturition recording for 10 hrs as described in Chapter 5. Thereafter, mice were anesthetized, residual urine was determined by the difference in bladder weight containing urine and bladder weight without urine. The harvested bladders were homogenized using a Dounce homogenizer. Protein content in supernatant was quantitated. Four NS-injected mice, 2 mice that had received 50mg of CL, and 2 mice that had received half the dose, we screened and quantitated cytokines and chemokines using an array kit (Proteome Profiler Mouse Cytokine Array Kit, R&D Systems, McKinley Place, MN, USA) according to the manufacturers protocol; resulting films were scanned and pixel intensity was quantitated using Image Studio™ Lite (Li-Cor Biosciences, Lincoln, NE, USA). Mice were euthanized by exsanguination.

6.2.4 Partial bladder outlet obstruction and macrophage depletion trial I

21 female C57/Bl6 mice that were retired breeders of 24 to 29 g body mass underwent pBOO at the mid-urethral level as described under 6.2.2.1. 14 received CL IP injections 3 times per week at a dose of 50 mg Clodronate per kg body mass; 7 mice received NS injections instead of CL. 14 mice underwent sham procedure, of which 9 received CL injections also at 50 mg/kg 3 times per week. After 2 weeks, animals underwent micturition recording as described
in detail in chapter 5. Bladder mass, volume of residual urine, and body mass was determined at the time of bladder harvest.

6.2.5 Partial bladder outlet obstruction and macrophage depletion trial II

41 female C57/Bi6 mice of 18 to 21 g body mass underwent pBOO at the mid-urethral level as described under 6.2.2.1, 26 mice underwent sham procedure; 25 of the pBOO mice received CL IP injections 3 times per week at a dose of 50 mg Clodronate per kg body mass, while 16 mice received NS injections instead, 15 of the sham operated mice received CL treatment. After 2 weeks, animals underwent micturition recording as described in detail in Chapter 5. Bladder mass, volume of residual urine, and body mass was determined at the time of bladder harvest. The bladders of half of each group were randomly assigned either for histologic work-up or for RNA quantification; for histology, bladders were embedded in OCT compound while bladders for the latter purpose were stored in RNAlater for 24 hours at 4°C before storage at -80°C.

6.2.6 Recording and analysis of bladder function

Bladder function was assessed non-invasively by continuous recording of voided urine. Mice were placed in metabolic cages during the light-cycle with free access to food and water. Recording was initiated after allowing for 1 hour of acclimatization and carried out for 10 hours. Recorded weight-traces were analyzed using a software-algorithm that detected weight increase and discriminated fluid from solid material based on weight decrease by evaporation of urine. Parameters determined were maximum voided volume, mean voided volume, and ratio of small voids, which serves as a surrogate of bladder hyperactivity. For a detailed description of the method please refer to Chapter 5.

6.2.7 Histologic evaluation

Midequatorial bladder cryosections of 7µm were fixed in 4% paraformaldehyde (PFA), then permeabilized with a 0.2% Triton-X solution in PBS for 10 min at room temperature (RT) and then blocked in 10% serum, 0.3% BSA, 0.3M Glycine solution in PBS for 1 hour before incubation with primary antibodies: Rat anti mouse CD68 (1:200; Abcam, Cambridge, MA, USA) to identify macrophages; rabbit anti mouse Myosin heavy chain (1:200; Sigma, St.
Louis, MO, USA). Secondary antibodies were donkey anti rabbit AlexaFluor® 647-conjugated and goat anti rat AlexaFluor® 594-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used at a 1:400 dilution in 1% BSA PBS solution and incubated for 1 hr at RT. Nuclei were counterstained with Hoechst 33342. Images were automatically analyzed using the image processing program Volocity (Version 6.3; PerkinElmer Inc., Woodbridge, ON, Canada).

6.2.8 Statistics

Independent sample T-Test (two-tailed) was performed to directly compare two independent groups. P-values less than 0.05 were considered significant, p-values less than 0.001 were considered highly significant. Pearson correlation was applied to correlate physiologic data with structural findings, PCR data, and cytokine assay data. Numerical results were summarized graphically using boxplots, the box encompassing the interquartile range (IQR) from the first quartile (Q1) to the third quartile (Q3), the bold transverse bar representing the median. The whiskers mark maximum and minimum values. Outliers represented by a circle-symbol (○) are more than a 1.5-fold IQR away from Q1 or Q3, respectively; extreme values are represented by an asterisk (*), indicating they are more than a 3-fold IQR away from Q1 or Q3, respectively. Statistical analysis was performed using IBM SPSS Statistics 24 (SPSS Inc., Chicago, IL USA). Heat maps were created using R statistical software.

6.3 Results

6.3.1 Macrophage density correlates with residual urine and bladder mass in rat bladder after pBOO

After 2 weeks of pBOO, bladder mass ranged from 146 mg to 744 mg, residual urine ranged from 0.01 mL to 8.5 mL, average number of macrophages per high power field (macrophage
density) was between 0.2 and 15.8 (Figure 6.1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.1.png}
\caption{Immunofluorescence staining of detrusor area of rat bladders 2 weeks after pBOO. A) Low number of macrophages in detrusor of bladders with low mass after pBOO. B) Higher number of macrophages in detrusor of bladders with greater mass.}
\end{figure}

Macrophage density in the rat bladder detrusor significantly correlated with absolute bladder mass (p=0.011), as well as, with the volume of residual urine (p=0.003; Figure 6.2.).
Figure 6.2 Correlation of number of macrophages with bladder mass and residual urine volume. 
A) Number of macrophages per high power field correlated with bladder mass (p=0.011) and B) volume of residual urine (p=0.003).

6.3.2 Bladder macrophage depletion and quantification in mouse

While in a pooled sample of 5 control bladders, only 3.5% of live cells were macrophages, this ratio increased to 17% after 2 weeks of pBOO; CL-treatment of obstructed animals reduced bladder macrophages to 7% of live cells (Figure 6.3). The spleen sample served as positive control.
Figure 6.3 Number of macrophages in pooled bladder and spleen samples of controls 2 weeks after pBOO. A) Of the 129’000 live cells that were counted from the spleen sample, almost 12’500 (almost 10%) were CD11b+ macrophages. B) From pooled control bladders, 3.5% of live cells were CD11b+ macrophages, C) while this percentage was 17% in a pooled sample from obstructed bladders. D) The ratio of CD11b+ macrophages in bladders from obstructed and CL-treated animals was only 7% of live cells.
6.3.3 Dose Finding and Cytokine Screen

Four out of 6 obstructed mice receiving 25 mg/kg CL died over the course of 2 weeks, at least one of them from unintended complete BOO. Only 2 out of 6 mice receiving CL at 50 mg/kg died. For cytokine analysis, the 4 NS-injected mice were compared to 2 mice from each CL dose group. Cytokine levels were comparable between mice receiving 25 mg/kg versus 50 mg/kg of CL. While CL-treatment significantly reduced the measured amount of IL-1-alpha by almost 4-fold, levels of IL-2 were increased by over 4-fold; also, I-309 tended to be increased by 3–fold (Figure 6.4). We correlated the level of cytokines in the supernatant with the physiologic parameters and found that amounts of IL-23 and T Lymphocyte-Secreted Protein I-309 (I-309) were negatively correlated with the ratio of small voids, stromal cell-derived factor 1 (SDF-1) tended to be negatively correlated with the ratio of small voids; levels of TIMP-1 were negatively correlated with maximum voided volume, and tended to be negatively correlated with mean voided volume.
Figure 6.4 Cytokine levels are affected by CL-treatment and correlate with bladder function. A) Sample cytokine array film, screening for 40 cytokines. B) CL-treatment significantly reduced the measured amount of IL-1-alpha by almost 4-fold (p=0.010), while IL-2 was over 4-fold increased (p=0.025). I-309 tended to be increased by 3-fold (p=0.078). C) Amounts of IL-23 and I-309 were negatively correlated with the ratio of small voids (p=0.05 and p=0.028, respectively), SDF-1 tended to be negatively correlated with the ratio of small voids (p=0.075); levels of TIMP-1 were
negatively correlated with maximum voided volume (p=0.05), and tended to be negatively correlated with mean voided volume (p=0.08).

6.3.4 Macrophage Depletion during pBOO Trial I

6.3.4.1 Mortality

Among the CL-treated animals, 7 of 9 sham operated mice and 9 of the 14 obstructed mice died within the planned 2-weeks course. Two more obstructed mice had to be removed from the study because of complete BOO, one CL-treated and one mouse receiving NS-injections.

6.3.4.2 Physiologic Results

Maximum voided volume, mean voided volume, residual volume, and the ratio of small voids was not significantly different between the 4 treatment groups.

![Obstruction lead to an increase in relative bladder mass](image)

**Figure 6.5 Obstruction increased bladder mass.** Obstruction increased relative bladder mass by over 2-fold in mice receiving NS-injections (\(*\); p=0.04).
6.3.5 Macrophage Depletion during pBOO Trial II

6.3.5.1 Physiologic Results

6.3.5.1.1 Animal Mortality

None of the sham operated and NS-injected mice died, while 2 out of 15 sham/CL-treated and 2 out of 16 obstructed and NS-injected mice had to be euthanized for complete BOO. 10 of the 25 obstructed and CL-treated mice died or had to be euthanized because of poor body condition, 3 more had to be euthanized for complete BOO over the course of 2 weeks.

6.3.5.1.2 Obstruction-induced functional Changes are Mitigated by Clodronate Treatment

Among sham operated mice, CL-treatment did not significantly affect any of the measured physiologic parameters. Mean voided volume was decreased by obstruction in NS-injected mice to almost 1/3 compared to unobstructed mice (p=0.001; Figure 6.6 A); this decrease was similar in CL-treated mice, in which obstructed animals had a mean voided volume of less than half compared to sham operated mice (p=0.021). Maximum voided volume is decreased by obstruction alone by over 1/3 compared to sham (p=0.008); among CL-treated mice, maximum voided volume was less than half in the obstructed group (p=0.001; Figure 6.6 B). Obstruction alone increased the ratio of small voids by more than 2-fold (p=0.028), while CL-treatment reduced this ratio back to sham-levels (p=0.009, compared to Obstruction + NS; Figure 6.6 C). In NS-injected mice, mean residual volume was increased after obstruction by over 3-fold compared to sham animals (p=0.024); CL-treatment tended to reduce this increase by over 50% (p=0.058; Figure 6.6 D).
Figure 6.6 CL-treatment affects bladder physiology. A) In NS-injected mice, obstruction decreased mean voided volume to almost 1/3 compared to unobstructed mice (*; p=0.001); the effect was similar in CL-treated mice with obstructed mice having a mean voided volume of less than half compared to sham operated mice (**; p=0.021) B) Obstruction alone led to a decrease in maximum voided volume by over 1/3 compared to sham s(*; p=0.008); among CL-treated mice, maximum voided volume was less than half in the obstructed group (**; p=0.001). C) Obstruction alone increased the ratio of small voids by more than 2-fold (*; p=0.028); CL-treatment reduced this ratio to sham-levels (**; p=0.009 compared to Obstruction + NS) D) Obstruction alone increased average residual volume by over 3-fold (*; p=0.024); CL-treatment tends to reduce this increase by over 50% (**; p=0.058).
6.3.5.1.3 Clodronate Treatment did not affect Bladder Mass

Bladder to body mass ratio, as well as, absolute bladder mass was increased by obstruction alone by more than 2-fold (p=0.003 and 0.002, respectively; Figure 6.7. A & B) compared to sham animals receiving NS-injections. Among CL-treated animals, bladder mass and a bladder to body mass ratio that was almost 1/3 increased after obstruction compared to sham-operated mice (p=0.016 and 0.030, respectively). Among obstructed animals, CL-treatment did not have a significant effect on bladder mass or bladder to body mass ratio.

![Figure 6.7 Obstruction increased bladder mass. A & B](image)

Figure 6.7 Obstruction increased bladder mass. A & B) In NS-injected mice, obstruction led to an over 2-fold increase of bladder to body mass ratio, as well as, absolute bladder mass (*; p=0.003 and 0.002, respectively). Among CL-treated animals, obstructed bladders had a bladder mass and a bladder to body mass ratio that was almost 1/3 increased compared to sham-operated mice (**; p=0.016 and 0.030, respectively). Among obstructed animals, CL-treatment did not have a significant effect on bladder mass or bladder to body mass ratio.

6.4 Discussion

BOO has a high prevalence especially in aging men and is a common cause of lower urinary tract symptoms (LUTS). If obstruction is left untreated, the remodeling process ultimately leads to irreversible tissue damage and bladder fibrosis, which is accompanied by severe bladder dysfunction. BOO and bladder dysfunction can also lead to kidney failure.
Development of fibrosis has been shown to be mediated by macrophages in a broad variety of tissues. However, very little is known so far about the role of macrophages in bladder remodeling. We examined the effect of macrophage depletion in a pBOO model in female mice.

6.4.1 Obstruction increases the number of bladder macrophages

Our initial findings were that pBOO in female rats effectively increased the number of macrophages in the detrusor area; furthermore, the number of macrophages was positively correlated with the increase in bladder mass. This indicated that macrophages responded to the obstructive stimulus with either proliferation of tissue resident macrophages or monocyte recruitment. The urinary bladder and the heart not only share essential functional and structural features such as repeating cycles of contraction/expulsion and relaxation/storage, they also respond to pressure overload or hormonal stimuli with a similar response of hypertrophy, increase in extracellular matrix deposition, and ultimately fibrosis\textsuperscript{218-220}. Studying the role of cardiac macrophages it was found, that tissue remodeling in the heart induced by angiotensin-II infusion was marked by both, local proliferation and recruitment of phagocytes from circulating monocytes\textsuperscript{221}. The expansion of the cardiac macrophage population was present before hypertrophy and fibrosis occurred, suggesting that inflammatory changes precede hypertrophic tissue remodeling. It was also observed, that matrix metalloproteinase-9 (MMP-9) overexpression in macrophages exacerbated hypertension-induced cardiac hypertrophy, along with increased tissue inflammation and fibrosis\textsuperscript{222}, highlighting their specific role in smooth muscle organ remodeling.

6.4.2 Clodronate effectively mitigates obstruction-related macrophage increment

6.4.2.1 Possible animal models for macrophage depletion

Various animal models were considered that allow reduction of tissue macrophages. Gadolinium (Gd) chloride, for instance, was described over 20 years ago as means to induce macrophage apoptosis in vitro and in vivo\textsuperscript{223-226}. However, Gd chloride can form insoluble Gd hydroxide colloid and lead to emboli particularly in lung and kidney\textsuperscript{227,228}. Moreover, Gd
chloride blocks stretch-induced responses of arterial and bladder SMC\textsuperscript{229,230}, which clearly precludes the use of Gd for our experiments studying stretch-induced bladder remodeling.

Another option would be the use of a transgenic model. One of them has a drug-inducible gene that leads to Fas-mediated apoptosis of macrophages\textsuperscript{231}. However, mice of this strain (also known as MaFia mice) are expensive; moreover, induction, as well as, maintenance of macrophage depletion requires repeated intravenous injections under general anesthesia\textsuperscript{232}. For these reasons, we did not use these mice for our experiments. Another transgenic model allows for macrophage depletion with diphtheria toxin injection\textsuperscript{233}. Because an increase in inflammatory cytokines in kidneys and other organs was observed with the use of diphtheria toxin\textsuperscript{234}, we did not consider this depletion method for our purpose. Arguably the most commonly used macrophage depletion model is the use of Clodronate liposomes (CL)\textsuperscript{235,236}, which deliver the active compound selectively to phagocytic cells. Clodronate seems to act in macrophages through depletion of both the intracellular pool of ATP, as well as, iron\textsuperscript{237}. Effective macrophage depletion in gastrointestinal tissue with CL as also been reported\textsuperscript{238}. We chose this method for its ease of intraperitoneal administration, the little amount of effective drug with no significant side effects on smooth muscle cells, and the proven effectiveness for abdominal organs\textsuperscript{239-241}.

### 6.4.2.2 Effectivity of macrophage depletion by intraperitoneal CL injection

To quantify macrophages in control and obstructed bladders by an additional method, and to confirm effectivity of the macrophage depletion method using CL, we analyzed pooled samples of suspended bladders using a mass-cytometry approach. We applied pBOO in female mice at a safe distance to the bladder, thus avoiding macrophage infiltration as part of the wound healing process; after 2 weeks, the number of macrophages in the obstructed bladders was almost five-fold compared to controls. The macrophage depletion method with intraperitoneal injection of CL also proved to be effective, as it led to an almost 60% reduction of macrophages detected in suspended bladders, which is considerably more than the 30% reduction of macrophages seen with CL injections in a urinary tract infection model\textsuperscript{242}. 
6.4.3 Cytokines correlate with bladder function

We found that quantity of only a few cytokines was significantly different comparing CL treated to NS injected mice; several other signaling factors also appeared to be affected, while the number of tested samples was probably too small to reach statistical significance, given the variability within groups. However, we detected important correlations of cytokine quantity with functional changes that are typically obstruction-related such as the ratio of small voids, which serves as a surrogate for hyperactive voiding, or the mean and maximum voided volumes. Given the small sample number, these findings need to be considered pilot data and deserve confirmation by a larger dataset. However, published evidence shows, that the obstruction-induced increase in macrophages corresponds with an increase in expression of inflammatory cytokines such as monocyte chemotactic protein 1 (MCP1; synonymous with: C-C motif chemokine ligand 2, CCL2), Interleukin-6 (IL-6), IL-17, or RANTES\(^{137}\). Elevated urinary levels of IL-6, IL-17, and tumor growth factor-β (TGF-β) were also found to be increased during pBOO in a rat model\(^ {133}\). IL-1β, in particular, was found to mediate an increase in bladder mass, bladder capacity, and micturition pressure in female mice with pBOO\(^ {217}\). IL-1β also increases release of IL-6, IL-8, CCL2, and CCL5 from cultured BSMC\(^ {243,244}\). Interestingly macrophages are a possible source of IL-1β, whereby the inflammasome NLRP3 (NACHT, LRR and PYD domains-containing protein 3) mediates its release\(^ {245}\). Furthermore, use of an inhibitor of the NLRP3-inflammasome suppressed the obstruction-related inflammatory process and mitigated bladder hypertrophy, as well as, associated alterations in bladder function\(^ {246}\). Activating factors of the NLRP3 inflammasome are bacterial toxins and (adenosine trisphosphate) ATP\(^ {247}\), but also oxidative stress in general\(^ {248}\), which is typically increased in the obstructed bladder. Additionally, evidence suggests that tissue damage can also trigger activation of NLRP3\(^ {249,250}\).

6.4.4 Clodronate treatment preserves function in the obstructed bladder

In the NS-injected groups within only 2 weeks, pBOO led to the typical obstruction-related changes such as increase in bladder mass, rise in residual urine volume, decrease of mean voided volume, and increase in the ratio of small voids, which is reflective of a hyperactive micturition pattern. Interestingly, the latter two functional alterations were significantly mitigated by CL-treatment. However, bladder mass was not affected by CL.
To what extent number and phenotype of bladder macrophages correlates with these observations cannot be determined yet, since quantification of macrophages in bladder tissue has not yet been completed.

6.4.4.1 Animal mortality under Clodronate liposome treatment

When retired breeders were used, we noted a high rate of animal mortality associated with CL-treatment. The older mice also seemed more prone to complete inability to void after intended partial BOO. When using younger mice, CL-associated mortality was lower than reported by others. We observed that mice had reduced fluid and food intake for about 24 hours after CL-injections. Macroscopically, the intestine of some mice that died or had to be euthanized after CL-treatment showed intramural gas in the cecum. However, we were unable to determine whether these were post-mortem changes related to Clodronate or not.

6.4.4.2 Clodronate may contribute directly to preserved bladder function

Clodronate is a first-generation bisphosphonate, inhibiting osteoclast activation and inducing osteoclast apoptosis, thus inhibiting bone resorption. In animal studies, Clodronate has a half-life of about 15 minutes. Clinical indications for bisphosphonate use are tumor-induced hypercalcemia, treatment of osteoporosis, and Paget’s disease. In its liposome-encapsulated form, it is preferentially taken up by phagocytic cells, whereby Clodronate is metabolized intracellularly to a compound modifying ATP into a non-functioning molecule, which ultimately induced apoptosis of the phagocytic cell.

In clinical use as osteoporosis treatment, bisphosphonates were particularly studied regarding their cardiac side-effects. Controversy still exists about a potential cardioprotective effect of bisphosphonates; while some trials found a reduced risk of myocardial infarction associated with the use of bisphosphonates, review studies were unable to confirm this effect. Similarly, the correlation between atrial fibrillation and bisphosphonate is unclear.

Alendronate was shown to diminish the contractile response of human coronary artery tissue by affecting ATP-sensitive potassium channels. However, different bisphosphonates may have differential effects on cardiac function.

The in vivo effect on nociception of bisphosphonates was also studied in mice using different pain stimuli and central, as well as, peripheral route of application. While Clodronate seemed
to have central, as well as, peripheral analgesic effect, intestinal transit time was not prolonged, indicating that the effect was not mediated by opioid receptors; however, the used dose was only 5 mg/kg body mass, which is only 10% of the single dose commonly used for macrophage depletion\(^{255}\). Clodronate could potentially reduce bladder-associated pain via its mild antagonistic capacity against the P2X3 subunit of pain-mediating purinoceptors in the bladder\(^{261}\). Another bisphosphonate, Alendronate, has proven to reduce the tone of the rat lower esophagus sphincter by inhibiting cholinergic nervous activity\(^{262}\). Therefore, Clodronate might affect afferent, as well as, efferent neuronal function.

Several authors also studied the direct effect of bisphosphonates on SMCs \textit{in vitro}. Alendronate, for instance, was shown to decrease osteogenic transdifferentiation and ECM mineralization of vascular SMC (VSMC) exposed to osteogenic medium\(^{263}\); inhibited calcification in cultured SMC by bisphosphonates was also found by others\(^{264}\).

In summary, there seems to be an effect of bisphosphonates on smooth muscle organs and SMC \textit{in vitro}. While it still deserves confirmation, it seems unlikely that Clodronate could preserve function in the obstructed bladder as observed in our study, given the only 3 intraperitoneal applications per week along with the very short half-life of Clodronate.
7 General Discussion and Future Directions

7.1 Animal model of nerve-sparing mid-urethral obstruction

Animal models of pBOO are an essential component of research in this common benign urologic condition. We developed a nerve-sparing mid-urethral obstruction (NeMO) model that avoids the severely confounding effects of dissection around the bladder neck, spares the animal from a laparotomy, and has a low animal mortality. NeMO is applicable in female rats, as well as, in female mice. For mice, our model seems to be the first one that can be carried out with low variability in degree of obstruction and low animal mortality; applying pBOO in mice allows for the full use of transgenic manipulations, such as knock-out or overexpression of certain genes, thus enabling very specific research questions, addressing pathophysiology, mechanisms and studying potential therapeutic targets. The exclusive use of female animals in the present study to investigate a pathology that is most prevalent in men will still invite further validation in male animals. However, voiding dysfunction more broadly is also highly prevalent in the female human population. Moreover, avoiding the confounding effect of surgical induction of a wound healing response and a denervation injury to the bladder most likely outweighs the confounding effect of exposure to female sex hormones.

In the future, we plan to study the necessary duration for irreversible structural and functional bladder damage to occur in female mice after NeMO. As the pBOO can be easily released, we also intend to study the pathophysiology of the post-release phase. The latter is, in fact, the clinically more relevant phase than the period of onset of obstruction-induced changes and symptoms since patients typically present themselves when obstructive symptoms are already established. Many patients remain symptomatic despite the release of obstruction. In this regard, understanding the post-release pathophysiology and ultimately finding therapeutic strategies to enhance post-release recovery has the potential to benefit many patients.
7.2 Macrophages in obstruction-induced bladder remodeling

The work presented here shows, that macrophages are involved in obstruction-induced bladder pathophysiology, with hyperactive voiding ranking as the most significant association. As hyperactive voiding can be linked to ineffective contractile function, neuronal stimulation and direct stimulation of the SMC through various pathways, there are a number of possible mechanisms for the effect of the macrophage depletion. We quantitatively showed that intraperitoneal Clodronate liposome (CL) injection effectively reduces bladder macrophages and affects cytokine quantity detectable in the bladder. Interestingly, various cytokines also significantly correlated with obstruction-related bladder dysfunction, such as hyperactive voiding or mean voided volume (see section 7.2.1 for cytokines). However, the question remains as to whether the major effectors of obstruction-induced macrophages are cytokines, or the other functionalities of macrophages (see following sections).

7.2.1 Cytokine involvement in remodeling by macrophages

The cytokines that were most altered by macrophage depletion in the bladder included CCL1, IL1-α and IL2. The chemokine CCL1 is not usually produced by macrophages, however but is produced by activated T cells to call in monocytes, NK cells, and other cell types. It is possible that CCL1 levels increase due to overproduction by activated T cells, to compensate for the reduction of the macrophage depletion. IL-2 is produced by numerous leukocytes including lymphocytes. Its increase during Clodronate treatment is similar to the increase of CCL1, and may also reflect a compensatory mechanism of the T cells to regulate the immune responses during macrophage depletion. In vascular SMC, it can modulate proliferative responses and prostacyclin synthesis in response to angiotensin II (AT II), through heparin-binding epidermal growth factor-like growth factor (HB-EGF) and Ca++. This is significant since, AT II, HB-EGF and Ca++ are known to be important regulators of prostacyclin synthesis in stretched bladder SMC (Nguyen et al, 2001, Park et al, 1999). However, in the depleted animals, IL-2 is still increased, which opens the possibility that IL-2 may increase particular relaxant prostacyclin.

IL1-α is one of the cytokines that are typically produced by activated macrophages and epithelial cells. We found that it was reduced during the Clodronate treatment. It is pro-inflammatory and synergizes with TNF-α, which itself is known to have a role in bladder
dysfunction. The wide-ranging roles of IL1 alpha in the regulation of many cellular processes in many cell types make it an interesting therapeutic target. In particular, it can affect fibrosis, e.g. through stimulation of collagenase section. In addition, it influences prostaglandin E-2 (PGE-2) release and cyclooxygenase-2 (COX-2) synthesis. The effects of IL1alpha can induce smooth muscle cells to produce heme-oxygenase, as well as, nitric oxide synthase. In addition, vascular SMC proliferate rapidly in response to IL-α, though they maintain a differentiated morphology.

All of these pathways converge in a conflicting manner on contractile function, and likely play a role in the altered function of the bladder though future work will be required to dissect out the relative roles of each (see Section: Future Work).

7.2.2 TIMP and MMP involvement in remodeling of macrophages

Tissue inhibitors of MMPs (TIMPs) and MMPs have been studied individually in both the bladder pathology and macrophages, but in this work we have seen that TIMP-1 was associated with the maximum voided volumes during obstruction. TIMP-1 is known to regulate both extracellular MMPs, and its downregulation is associated with matrix degradation, decreased proliferation and cell growth (Hornebeck et al, 2004). A rise in MMP activity has been noted in bladder SMC in response to stretch, bladder distension, and obstruction. In addition, the increased MMP activity in conditioned media from distended bladders is associated with increased proliferation of BSMC\textsuperscript{35}. TIMP-1 activity, however, could counter this rise in MMP activity and potentially have a role in not only affecting the MMPs. It is therefore interesting that there appears in this relatively small number of samples that we find an increase in TIMP-1 consistent with the trend toward increased mean voided volumes and the increased maximum voided volumes.

7.2.3 Other roles of macrophages in tissue remodeling

Macrophages are scavengers for many by-products of tissue degradation, including reactive oxygen species, which may play a role in bladder pathology during obstruction.
7.3 Future work

7.3.1 Potential of the Nerve-sparing model

In the future, we plan to study the necessary duration for irreversible structural and functional bladder damage to occur in female mice after NeMO. As the pBOO can be easily released, we also intend to study the pathophysiology of the post-release phase. The latter is, in fact, the clinically more relevant phase than the period of onset of obstruction-induced changes and symptoms since patients typically present themselves when obstructive symptoms are already established. Many patients remain symptomatic despite the release of obstruction. In this regard, understanding the post-release pathophysiology and ultimately finding therapeutic strategies to enhance post-release recovery has the potential to benefit many patients.

7.3.2 Future work with macrophage depletion models

Future work with the biologic samples available after the experiments with pBOO and CL treatment will comprise histologic work up and gene expression analysis. Histologic features to be studied are smooth muscle cell (SMC) size and degree of loss of differentiation, quantitating myosin heavy in immunofluorescent (IF) staining, for instance. Also using IF, the number of macrophages present in the bladder wall will be quantitated, along with the amount of ECM, especially collagen. Ideally, type of macrophages will also be studied on the sectioned bladder tissue; epitopes such as CD163 or CD206 can serve as markers for M2-phenotype, whereas F4/80 positivity and low presentation of Ly6C can be indicative of tissue resident origin of macrophages as opposed to recruited macrophages from the circulating monocyte pool. While recruitment of bone marrow derived cells into the obstructed bladder has been shown and methods to monitor monocyte release from bone marrow have been described, it is unknown whether pBOO directly affects bone marrow activity.\(^{266-268}\).

Origin and recruitment mechanism of macrophages involved in bladder remodeling are important as they would lend themselves as potential targets in modifying the number and possibly phenotype of bladder macrophages. Finding that bone marrow derived, circulating monocytes give rise to the increased number of macrophages in the obstructed bladder would make it inviting to study, whether blockage of CCR2, CCR5, or CX3CR1 lead to altered
tissue remodeling – similar to the markedly reduced atheroma formation in blood vessels when these factors are blocked\textsuperscript{269-271}.

Gene expression analysis using qPCR will focus on genes coding for products such as extracellular matrix proteins (i.e. collagen, elastin, fibronectin, calponin), cytokines that proved to correlate with bladder function, and factors known to be involved in obstruction-induced bladder remodeling such as brain-derived neurotrophic factor (BDNF).

Discriminating whether the CL-treatment associated effects are more related to macrophage depletion or to direct effects of Clodronate will also need to be addressed. One option to study this would be to do the same experiment as described in \textbf{6.2.5}, with the modification that another macrophage depletion method is used (such as MaFIA mice or CD11b-DTR mice).

The role of macrophages in the post-release tissue remodeling would also be of interest. Knowing that macrophages are essential in recovery from fibrosis in other tissues, they might be involved in post-release bladder remodeling as well. Moreover, inducing an effective restorative macrophage phenotype after the release of pBOO might, in fact, lend itself to a potential therapeutic strategy to enhance recovery from obstruction-induced structural and functional changes. In a clinical setting, an agent modifying macrophage function could be directly deposited into the bladder wall at the time of transurethral resection of an enlarged prostate.

\textbf{7.3.3 Potential cytokine work}

An understanding of the specific cytokines that are involved in the pathology may be of interest as well. In other organs, cytokines have direct effects on myocyte and neuronal function, and therefore may be of interest in terms of both remodeling, as well as, contractile function. For instance, IL-6 and TNF-\(\alpha\) have known hypertrophic effects on cardiomyocytes. In our own work, IL1-\(\alpha\), IL2, and CCL1 may have a role in mediating not only the fibrotic effects of macrophages but also the frequency of voiding, as these cytokines are altered in concordance with frequency dysregulation. Examining whether these three cytokines directly affect contractility of strips would be of interest. Another test would include directly treating BSMC in vitro for an examination of altered Ca++ handling and contractile function (gel
contraction and response to agonists). Finally, knockouts of these cytokines or their antagonists could be used to examine their effects on bladder function.

However, other cytokines may be mediating some of the effects of the Clodronate depletion of macrophages, which could be examined through more RNAseq or SILAC proteomics of the bladder extracts during obstruction with and without depletion. To examine if these cytokines have translational potential, we might need to perform time courses to identify understand their alterations during both the compensatory and decompensatory phases of obstruction, as well as, healing phases (after de-obstruction).

### 7.3.4 Understanding of macrophage to myofibroblast transdifferentiation

In the vasculature, work by Gary Owens and others has shown that smooth muscle cells can have the potential to transdifferentiate into other cell types, including macrophages\(^{272,273}\). It might be of interest to examine by lineage tracing if the CD11b positive macrophages that increase in number during obstruction are transdifferentiated smooth muscle cells via an osteochondrogenic precursor-like cell. To test this, one could lineage mark smooth muscle cells (with an SMC-cre), then identify by immunostaining macrophage-like cells with galectin-3, CD11b, and other macrophage markers. If any macrophages do derive from myocytes, their sensitivity to Clodronate would likely still remain, though it would still have to be tested. While the role of any SMC-derived macrophages in pathophysiology would remain a difficult question to be tested, its theoretical importance could be significant if the SMC-derived macrophages form a large proportion of the macrophages seen during obstruction.

### 7.4 Conclusions

This work supports a role for the involvement of macrophages in obstructive bladder pathophysiology. The establishment of clear and useful models for precise genetic models has been a clear move forward for the field in terms of understanding precise components of anatomic vs. neurologic aspects of the disease. The association of cytokine alterations during macrophage depletion is consistent with a role for several cytokines during murine partial
obstruction. In addition, it also supports a role for macrophages in the enticement of hyperactivity during bladder obstruction.
References

19. Hoekstra, R. J., Van Melick, H. H. E., Kok, E. T. & Ruud Bosch, J. L. H. A 10-year follow-up after transurethral resection of the prostate, contact laser prostatectomy and


91. Chapple, C. R., Cardozo, L., Nitti, V. W., Siddiqui, E. & Michel, M. C. Mirabegron...


127. Wang, Y.-Y. et al. Macrophage-to-Myofibroblast Transition Contributes to


220. Zou, Y. et al. Mechanical stress activates angiotensin II type 1 receptor without the


237. van Rooijen, N. Extracellular and intracellular action of clodronate in osteolytic bone


271. Combadière, C., Potteaux, S., Rodero, M. & Simon, T. Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C hi and Ly6C lo monocytosis and almost


Copyright Acknowledgements

Figure 3.1 to 3.7 are adapted from “Finding NeMO - Nerve-sparing Midurethral Obstruction: A Pathophysiologically Accurate Model of Rodent Partial Bladder Outlet Obstruction”. *Urology* (2017). Reproduced with permission.

Figure 4.1 to 4.5 are adapted from 194. Reproduced with permission.

Figure 5.1 to 5.3 are adapted from “Assessment of murine bladder function” in *Bladder*.

Copyrights were received for the included manuscripts from *The Journal of Visualized Experiments* and *Urology*.

Copyrights pending from *Bladder*.